Purification and some Properties of Glutamine Synthetase from the Nitrogen-fixing Cyanobacteria *Anabaena cylindrica* and a *Nostoc* sp.

By MARIA JOSE A. M. SAMPAIO, PETER ROWELL
AND WILLIAM D. P. STEWART

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, Scotland

(Received 4 September 1978)

Glutamine synthetase has been purified to homogeneity from two N_2 -fixing cyanobacteria, *Anabaena cylindrica* and a species of *Nostoc* (the phycobiont of *Peltigera canina*). The activities of the *A. cylindrica* enzyme in the biosynthetic and transferase assays were, respectively, 9.4 and 32 μmol product formed min^{-1} (mg protein) $^{-1}$; the corresponding values for the *Nostoc* sp. enzyme were 6.5 and 20. Stabilization of the enzyme required Mg^{2+} , glutamate, EDTA and a thiol reagent to be present during purification.

The molecular weight of the *A. cylindrica* enzyme was 591 000 as estimated by sedimentation analysis, 660 000 by gel filtration and 565 000 by polyacrylamide gel electrophoresis; the *Nostoc* sp. enzyme gave values of 630 000 by gel filtration and 575 000 by electrophoresis. The molecular weights of the sub-units of each enzyme were approximately 49 000 to 50 000. Electron microscopy revealed that each molecule was composed of 12 sub-units arranged in two superimposed hexagonal rings. The maximum diameter of the rings was 13.6 nm and the distance between the centres of adjacent sub-units was 4.9 nm.

When dialysed in the absence of stabilizing ligands the *A. cylindrica* enzyme lost activity and the protein band characteristic of the native enzyme was replaced by three bands with approximate molecular weights of 510 000, 310 000 and 130 000. These sub-species re-associated and activity was restored by adding 2-mercaptoethanol and substrates. A similar reversible deactivation has been observed with glutamine synthetase from photosynthetic eukaryotes and yeast but no similar data have been reported for a N_2 -fixing prokaryote.

INTRODUCTION

Previously, we have provided evidence based on enzyme studies (Dharmawardene *et al.*, 1973; Rowell *et al.*, 1977), the use of analogues (Stewart & Rowell, 1975; Ladha *et al.*, 1978) and ^{15}N tracer studies (Stewart *et al.*, 1975) that glutamine synthetase is a major enzyme involved in NH_4^+ assimilation in N_2 -fixing cyanobacteria. This conclusion is also supported by other work (Wolk *et al.*, 1976; Meeks *et al.*, 1977; Ownby, 1977; Thomas *et al.*, 1977) and there is now substantial evidence that the glutamine synthetase/glutamate synthase pathway (see, for example, Brown *et al.*, 1974) is the primary route of NH_4^+ assimilation in N_2 -fixing cyanobacteria. This also appears to be the case in N_2 -fixing heterotrophic bacteria (Nagatani *et al.*, 1971) and in photosynthetic bacteria (Brown & Herbert, 1977a, b; Johansson & Gest, 1977).

The properties and regulation of the purified glutamine synthetase of N_2 -fixing cyanobacteria have not so far been reported in detail, but no evidence of regulation by an adenylation-deadenylation mechanism was obtained from studies using crude extracts and partially purified preparations (Dharmawardene *et al.*, 1973; Rowell *et al.*, 1977; Sawhney

& Nicholas, 1978). However, such a regulatory mechanism has been noted in most other N_2 -fixing prokaryotes so far studied, including various free-living heterotrophic bacteria (Tronick *et al.*, 1973), *Rhizobium* (Rao *et al.*, 1978) and photosynthetic bacteria (Johansson & Gest, 1977). As a prerequisite to detailed studies on the regulation of cyanobacterial glutamine synthetase, we report here a method for the purification of the enzyme from *Anabaena cylindrica*, and from a *Nostoc* species isolated from the lichen *Peltigera canina*, and present information on the structural characteristics of the purified enzymes as well as data on certain aspects of the regulatory properties of cyanobacterial glutamine synthetase. The data complement and extend those obtained by Stacey *et al.* (1977), who recently purified glutamine synthetase from a marine cyanobacterium, *Anabaena* strain CA.

METHODS

Organisms. *Anabaena cylindrica* Lemm. (CU 1403/2a) and the isolated *Nostoc* sp., phycobiont of the lichen *Peltigera canina* (obtained originally from Dr J. W. Millbank, Imperial College, London), were used in axenic culture.

Culturing of the cyanobacteria. *Anabaena cylindrica* and *Nostoc* sp. were grown in BG-11 medium (Stanier *et al.*, 1971) and in the medium of Allen & Arnon (1955), respectively, in continuous culture at 30 °C and 3000 lux with N_2 as the nitrogen source. Cyanobacteria used for purification of glutamine synthetase were grown in batch culture (50 to 100 l) at 30 °C and 3000 lux and harvested in the late-exponential growth phase.

Enzyme extraction and purification. Cultures were harvested by centrifugation, washed twice in 50 mM-Tris/HCl buffer, pH 7.5 (buffer A) and resuspended in buffer B (buffer A supplemented with 5 mM-MgCl₂, 10 mM-sodium glutamate, 5 mM-2-mercaptoethanol and 1 mM-EDTA). In all subsequent steps, buffer B was used unless otherwise stated. Cyanobacteria were disrupted by passage through a French pressure cell at 110 MPa. Debris was removed by centrifugation at 35000 g for 30 min, the supernatant was treated with (NH₄)₂SO₄ to 40 % saturation for 30 min, and then centrifuged at 35000 g for 30 min. The resulting supernatant was treated with (NH₄)₂SO₄ to 70 % saturation and, after 30 min, re-centrifuged at 35000 g for 30 min. The pellet was suspended in a minimal volume of buffer B and dialysed to remove (NH₄)₂SO₄. The resulting solution was chromatographed on DEAE-cellulose (30 × 3.2 cm column) and eluted with a gradient of 0 to 0.5 M-KCl in buffer B. Active fractions were bulked and concentrated to less than 3.0 ml by vacuum dialysis. The concentrated enzyme was then chromatographed on a 100 × 2.5 cm column of Sepharose 6B. Active fractions were bulked and concentrated by vacuum dialysis. Such preparations were occasionally homogeneous but it was usually necessary to further purify the enzyme by chromatography on a 10 × 1 cm column of aminohexane-Sepharose 4B, eluting the enzyme with a gradient of 0 to 0.8 M-sodium glutamate in buffer B. Active fractions were again concentrated by vacuum dialysis. All operations were carried out at 4 °C.

Glutamine synthetase assays (EC 6.3.1.2). (i) Biosynthetic activity, Mg²⁺-dependent. A modification of the method of Kingdon *et al.* (1968) was used in which the production of ADP was coupled to the oxidation of NADH. This assay, which allows continuous monitoring of activity, avoids problems which might arise from the more commonly used P_i release assay for an enzyme which shows slow, time-dependent, changes in activity. The reaction mixture contained, in addition to enzyme, in 3 ml: 150 μmol Tris/HCl buffer, pH 7.5; 3.0 μmol ATP; 200 μmol NH₄Cl; 60 μmol sodium glutamate; 150 μmol MgCl₂; 150 μmol KCl; 0.45 μmol NADH; 0.5 μmol phosphoenolpyruvate; 20 units lactate dehydrogenase; (EC 1.1.1.27); 8 units pyruvate kinase (EC 2.7.1.40). Unless otherwise stated, the reaction was initiated by adding glutamate and the rate of oxidation of NADH at 30 °C was measured at 340 nm. None of the reagents used affected the activity of the coupling enzymes.

(ii) γ-Glutamyl transferase activity, Mn²⁺-dependent. This was estimated by a modification of the method described by Pamiljans *et al.* (1962). The reaction mixture contained, in addition to enzyme, in 1 ml: 40 mM-Tris/HCl buffer, pH 7.0; 3 mM-MnCl₂; 20 mM-potassium arsenate; 0.4 mM-ADP (Na salt); 60 mM-hydroxylamine; 30 mM-glutamine. Incubations were at 30 °C for 10 min in the dark.

Protein determinations. The method of Lowry *et al.* (1951) was used, with bovine serum albumin as the standard. For purified enzyme preparations the spectrophotometric method of Layne (1957) was occasionally used (see Results).

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out according to Davis (1964) and, for molecular weight estimations, according to Hedrick & Smith (1968), on 5, 7.5, 11 and 15 % (w/v) gel columns using Tris/glycine buffer, pH 8.8, and a current of 3 mA per gel tube. The molecular weight

standards were fatty acid synthetase (520000), β -galactosidase (480000), ferritin (450000), bovine serum albumin dimer (136000) and monomer (68000). Gels were stained for protein with 0.2% (w/v) Coomassie Brilliant Blue in acetic acid/methanol/water (10:45:45, by vol.) for 5 to 15 h at room temperature, destained with acetic acid/methanol/water (10:25:65, by vol.) and scanned at 600 nm using a Gilford 2410-S gel scanner (Gilford Instruments, Teddington, Middx).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was carried out according to Weber & Osborn (1969) using 7.5% (w/v) gels and a current of 6.5 mA per gel tube. The molecular weight standards were bovine serum albumin dimer (136000) and monomer (68000), catalase (58000), γ -globulin-H-chain (50000), aldolase (40000), yeast alcohol dehydrogenase (37000), chymotrypsinogen (25700) and γ -globulin-L-chain (23500). Proteins in 1% (w/v) SDS plus 1% (w/v) 2-mercaptoethanol in phosphate buffer, pH 7.2, were denatured by incubation at 100 °C for 2 min. Bromophenol blue was used as the tracking dye and gels were stained as above.

Electron microscopy of purified glutamine synthetase. A carbon-coated grid (3.05 mm diam.) was touched on to a drop of purified glutamine synthetase solution for 30 s. The grid was then transferred to a drop of 2.6% (w/v) phosphotungstic acid for 30 s, dried and examined using an AEI model 801 electron microscope at 60 kV and a magnification of 63000.

Determination of molecular weight. Purified glutamine synthetase at an initial concentration of 0.3 mg ml⁻¹ in buffer B was centrifuged to equilibrium at 10 °C in a Beckman model E analytical ultracentrifuge (Yphantis, 1964). Short liquid columns (3 mm) in a cell with a double-sector centre-piece were used. The density of buffer B was 1.004 g ml⁻¹. The partial specific volume (v) of *A. cylindrica* glutamine synthetase was calculated from its amino acid composition according to Schachman (1957). The amino acid composition of the enzyme was determined after hydrolysis in 6 M-HCl for 24 h and 60 h at 110 °C *in vacuo*. Analyses were carried out according to Spackman *et al.* (1958) using a BioCal BC-100 amino acid analyser. Cysteine was determined after oxidation with performic acid (Moore, 1963) and tryptophan by the method of Spies & Chambers (1949).

The molecular weight of glutamine synthetase was also estimated by gel filtration on a 110×0.9 cm column of Sepharose 4B (void vol. 24 ml). The molecular weight standards were phosphorylase kinase (1300000), fatty acid synthetase (520000) and β -galactosidase (480000).

Chemicals. NADH, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, alcohol dehydrogenase and catalase were from Boehringer. 2-Mercaptoethanol, β -galactosidase, bovine serum albumin, aminohexane-Sepharose 4B, urease, blue dextran, ADP, ATP, hydroxylamine, L-glutamic acid, Sepharose 4B and Sepharose 6B were from Sigma. Phosphotungstic acid was from TAAB Laboratories, Reading, Berks. All other chemicals were from BDH.

RESULTS

Purification of glutamine synthetase

The activities and yields obtained at various stages of purification of the glutamine synthetase from N_2 -fixing *A. cylindrica* are presented in Table 1. The specific activities of the purified enzyme were 9.36 and 32.00 μ mol product formed min⁻¹ (mg protein)⁻¹ in the biosynthetic and transferase assays, respectively. The corresponding values for the purified *Nostoc* sp. enzyme were 6.53 and 20.00 μ mol min⁻¹ (mg protein)⁻¹. These values, based on protein concentrations determined by the method of Lowry *et al.* (1951), are somewhat higher than those obtained using protein concentrations estimated from the absorbance at 280 nm (Layne, 1957). The latter values are included in Table 1 to allow direct comparison with the results of Stacey *et al.* (1977) who purified glutamine synthetase from *Anabaena* CA. The purified enzymes showed higher biosynthetic activities than the *Anabaena* CA enzyme, the purifications obtained being 213-fold and 65-fold for the *A. cylindrica* and *Nostoc* sp. enzymes, respectively. Stabilization of the enzyme was achieved by using buffer B (containing Mg²⁺, glutamate, EDTA and 2-mercaptoethanol) at all stages of purification; the enzyme could then be stored at 4 °C for several days with little loss of activity. Biosynthetic activity was lost when Mg²⁺ was replaced by Mn²⁺. A slight increase in total activity during (NH₄)₂SO₄ fractionation was consistently observed and has previously been noted (Stacey *et al.*, 1977). The enzyme purified from each cyanobacterium showed only a single protein band on electrophoresis using 7.5 and 5% polyacrylamide gels (Fig. 1*a, b*; Fig. 2*a, b*).

Table 1. *Purification and activity of glutamine synthetase from A. cylindrica*

Activities were measured by the biosynthetic assay and are expressed as μmol product formed min^{-1} . Specific activities [μmol product formed min^{-1} (mg protein) $^{-1}$] are based on protein determinations by the method of Lowry *et al.* (1951), except those shown in parentheses which are based on protein determinations by the method of Layne (1957) (see text).

Sample	Total protein (mg)	Specific activity	Total activity	Yield (%)
Crude extract	4200	0.044	186.0	100
50 to 70 % $(\text{NH}_4)_2\text{SO}_4$ fraction	1560	0.137	213.5	115
DEAE-cellulose eluate	140	0.897	125.6	67
Sephacrose 6B eluate	9.1 (17.2)	6.69 (3.54)	60.9	32
Aminohexane-Sephacrose 4B eluate	4.0 (7.5)	9.36* (5.00)	37.5	20

* The transferase activity of this preparation was $32.0 \mu\text{mol}$ product formed min^{-1} (mg protein) $^{-1}$.

Fig. 1

Fig. 2

Fig. 1. Polyacrylamide gel electrophoresis of purified glutamine synthetase from *A. cylindrica*: (a) $30 \mu\text{g}$ protein on a 7.5 % gel; (b) $30 \mu\text{g}$ protein on a 5 % gel; (c) $30 \mu\text{g}$ protein on a sodium dodecyl sulphate gel. The origin is at the upper end of each gel.

Fig. 2. Polyacrylamide gel electrophoresis of purified glutamine synthetase from *Nostoc* sp.: (a) $10 \mu\text{g}$ protein on a 7.5 % gel; (b) $10 \mu\text{g}$ protein on a 5 % gel; (c) $20 \mu\text{g}$ protein on a sodium dodecyl sulphate gel. The origin is at the upper end of each gel.

Molecular weight

From the high-speed equilibrium analysis of the *A. cylindrica* enzyme (Fig. 3) and its partial specific volume, calculated from the amino acid composition to be 0.726 ml g^{-1} (Table 2), the molecular weight of the enzyme was calculated to be 591 000. Molecular weight values based on elution patterns of both enzymes from Sepharose 4B, relative to the elution of proteins of known molecular weight, were approximately 660 000 and 630 000 for the *A. cylindrica* and *Nostoc* sp. enzymes, respectively.

The molecular weight of the sub-units of glutamine synthetase from both organisms was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. A single band only was detected with each enzyme (Fig. 1c; Fig. 2c). By com-

Fig. 3. High-speed sedimentation equilibrium centrifugation of *A. cylindrica* glutamine synthetase in buffer B. The plot of $\log_{10}(\text{fringe displacement})$ against the square of the distance from the centre of rotation was obtained from interference patterns recorded after 18 h at 8000 rev. min⁻¹.

Table 2. *Amino acid composition of purified glutamine synthetase from A. cylindrica*

The values, except for half-cystine and tryptophan, were calculated from the amino acid compositions measured after 24 h and 60 h hydrolysis. Half-cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan was determined by the method of Spies & Chambers (1949).

Amino acid residue	Residues per minimum mol. wt of 49000	
	Calculated	Nearest integer
Aspartic acid	50.8	51
Threonine	25.7	26
Serine	24.7	25
Glutamic acid	46.3	46
Proline	26.9	27
Glycine	39.1	39
Alanine	28.4	28
Valine	14.7	15
Half-cystine	10.4	10
Methionine	13.6	14
Isoleucine	28.2	28
Leucine	29.7	30
Tyrosine	18.1	18
Phenylalanine	27.0	27
Tryptophan	2.3	2
Lysine	24.0	24
Histidine	7.6	8
Arginine	17.3	17
Total	—	435

parison with the electrophoretic mobility of proteins of known molecular weight, the approximate molecular weight of the sub-units of each enzyme was estimated to be 49000 to 50000.

Electron microscopic examination of glutamine synthetase

Electron microscopic examination of the purified enzymes from *A. cylindrica* and *Nostoc* sp. revealed that each molecule had 12 sub-units arranged symmetrically in two hexagonal layers (Fig. 4). Most of the molecules appeared face-on as hexagonal rings while molecules resting on an edge showed a tetrahedral arrangement. With the *A. cylindrica* enzyme, especially after dialysis against buffer A (Fig. 4a), many of the molecules appeared as broken rings; this was not the case with the *Nostoc* sp. enzyme. The dimensions of the *Nostoc* sp. enzyme were obtained from the superimposed images of two molecules (Fig. 4b, c), the maximum diameter of the hexagonal ring being approximately 13.6 nm. The

Fig. 4. Electron micrographs of preparations of purified glutamine synthetase from *A. cylindrica* and *Nostoc* sp.: (a) negatively stained preparation of the *A. cylindrica* enzyme (bar marker represents 100 nm); (b, c) the molecular organization of the *Nostoc* sp. enzyme as seen face-on (b) and end-on (c) when negatives of electron micrographs of two separate negatively stained molecules were superimposed and then printed. Prior to electron microscopy the purified enzyme was dialysed against buffer A, rather than buffer B, because the former gave a clearer background under the electron microscope.

width of each sub-unit and the distance between the centres of adjacent sub-units were approximately 4.9 nm. There were no apparent differences between the *A. cylindrica* and *Nostoc* sp. enzymes with respect to sub-unit arrangement and dimensions of the molecule.

Reversible deactivation of the purified glutamine synthetase from A. cylindrica

An active homogeneous preparation was invariably obtained when glutamine synthetase was purified from *A. cylindrica* in buffer B (see above). However, enzyme preparations of low activity which showed three bands on electrophoresis (Fig. 5a) and which could be partially reactivated were obtained in two ways. First, when buffer A was substituted for buffer B during purification, little enzyme activity was obtained but activity could subsequently be increased up to sixfold by adding 2-mercaptoethanol or dithiothreitol together with substrates during the biosynthetic assay. The rate of activation increased with increasing concentration of 2-mercaptoethanol (Fig. 6). Activity was similarly stimulated when such preparations were dialysed against buffer B prior to assay. Stimulation of activity in both cases was accompanied by a complete, or almost complete, disappearance of the three bands (Fig. 5a) and the appearance of a new band of slightly lower electrophoretic mobility (Fig. 5b). This latter band had the same electrophoretic mobility as the native enzyme

Fig. 5. Effect of treatment with 2-mercaptoethanol and substrates on the electrophoretic mobility of glutamine synthetase purified from *A. cylindrica*: (a) enzyme purified using Tris/HCl buffer, pH 7.5, in the absence of stabilizing ligands; (b) enzyme purified as above and then dialysed against 50 mM-Tris/HCl, pH 7.5, containing 20 mM-sodium glutamate, 1 mM-ATP and 40 mM-2-mercaptoethanol. Each gel was loaded with 20 μ g protein.

(Fig. 1a) which, when estimated by electrophoresis on gels using several different polyacrylamide concentrations (Hedrick & Smith, 1968), had a molecular weight of 565000. (The corresponding value for the *Nostoc* sp. enzyme was 575000.) The approximate molecular weights of the other three bands (Fig. 5a) estimated by this method were, in order of increasing electrophoretic mobility, 510000, 310000 and 130000. The latter two species might represent a half molecule, and a dimer or trimer, respectively. The 510000 molecular weight species might represent a conformer of the native enzyme or, less likely, a molecule which has lost one or two sub-units.

Alternatively, when the fully active enzyme was dialysed for 6 to 8 h against buffer A and biosynthetic activity and the electrophoretic pattern of the enzyme on gels were then determined, enzyme activity was decreased by 79% (Table 3) and this was again accompanied by the appearance on gels of the three bands noted above. Such deactivated preparations could be reactivated to a maximum of 59% of the activity of the fully active enzyme by adding both 2-mercaptoethanol and substrates of the biosynthetic assay. Neither 2-mercaptoethanol nor substrates alone caused reactivation. Indeed, the addition of 2-mercaptoethanol alone caused a further loss of activity and a loss of the capacity for

Fig. 6. Effect of various concentrations of 2-mercaptoethanol on the biosynthetic activity of purified glutamine synthetase from *A. cylindrica*. The enzyme was purified using buffer A. (a) Sodium glutamate, to start the reaction, and 2-mercaptoethanol (2-ME) were added at the times arrowed. The final concentrations of 2-mercaptoethanol were 80 mM (-----), 40 mM (—) and 20 mM (-----); no 2-mercaptoethanol (—). (b) Effect of period of incubation with substrates and 2-mercaptoethanol (20 mM, ○; 40 mM, ●; 80 mM, □) on the biosynthetic activity of the *A. cylindrica* enzyme. The control (■) did not contain 2-mercaptoethanol.

Table 3. *Effects of 2-mercaptoethanol and EDTA on the biosynthetic activity of purified glutamine synthetase from A. cylindrica*

The purified glutamine synthetase was untreated (1) or dialysed (2 to 6) against 50 mM-Tris/HCl, pH 7.5, for 12 h, and the biosynthetic activity was then determined under the conditions indicated. The reactions were initiated by adding glutamate. Preincubations (before addition of glutamate) were carried out at 30 °C for 10 min. 2-Mercaptoethanol was added to a concentration of 40 mM, EDTA to 8 mM and substrates were added at normal assay concentrations. Activities are expressed as $\mu\text{mol product formed min}^{-1} (\text{ml enzyme})^{-1}$.

Treatment	Activity	Percentage of control activity
1. Undialysed enzyme (no 2-mercaptoethanol)	7.05	100
2. Dialysed enzyme (no 2-mercaptoethanol)	1.45	21
3. 2-Mercaptoethanol added after substrates and glutamate	4.05	58
4. Incubated with substrates and 2-mercaptoethanol before adding glutamate	2.42	34
5. Incubated with 2-mercaptoethanol before adding substrates and glutamate	0.97	14
6. Incubated with 2-mercaptoethanol and EDTA before adding substrates and glutamate	4.11	59

reactivation on the addition of substrates, even although it resulted in an electrophoretic pattern which showed only the band characteristic of the native enzyme. Thus, 2-mercaptoethanol could bring about reassociation to form the dodecamer, but alone it did not reactivate the enzyme. Addition of EDTA together with 2-mercaptoethanol (Table 3) prevented the further loss of activity which occurred in the absence of substrates and, in this case, reactivation could be brought about by the subsequent addition of substrates. Addition of EDTA alone did not bring about reactivation of the enzyme or alter the electrophoretic pattern. Also, the addition of substrates alone did not alter the electrophoretic pattern. Prolonged dialysis (more than 24 h) of the enzyme against buffer A caused irreversible inactivation of the enzyme and the appearance of at least six bands on gel electrophoresis.

DISCUSSION

The success of the procedure detailed here for purifying glutamine synthetase to homogeneity depended on Mg^{2+} , glutamate, EDTA and 2-mercaptoethanol being present throughout the purification. The biosynthetic activities of the purified enzymes were slightly higher than that of the enzyme from *Anabaena* CA (Stacey *et al.*, 1977) but were much lower than those of the enzymes purified from heterotrophic prokaryotes (Woolfolk *et al.*, 1966; Deuel *et al.*, 1970; Wedler & Hoffmann, 1974). The transferase activities of the purified enzymes were lower than that of the *Anabaena* CA enzyme, though this might be due to different assay conditions.

The molecular weight of glutamine synthetase from *A. cylindrica* was estimated to be 591000 by sedimentation analysis, the most satisfactory method of assay, and, like the *Nostoc* sp. enzyme, it was composed of 12 identical sub-units of 49000 to 50000 molecular weight, arranged in two superimposed hexagonal rings. The two cyanobacterial enzymes thus resembled the enzyme from other prokaryotes, e.g. *Escherichia coli* (Valentine *et al.*, 1968; Ginsburg & Stadtman, 1973), *Bacillus subtilis* (Deuel *et al.*, 1970), *Bacillus stearothermophilus* (Wedler & Hoffmann, 1974) and *Anabaena* CA, all of which have molecular weights of approximately 600000 and are composed of 12 identical sub-units, each of about 50000 molecular weight. They differed from the enzymes from various eukaryotes, e.g. *Pisum sativum* (O'Neal & Joy, 1973), *Candida utilis* (Sims *et al.*, 1974a), the cytosol of soya-bean root nodules (McParland *et al.*, 1976) and of *Lemna minor* (Rhodes *et al.*, 1978), which have molecular weights of 330000 to 390000 and eight sub-units each of about 45000 molecular weight arranged in two sets of planar tetramers (McParland *et al.*, 1976).

Glutamine synthetase may be regulated by adenylation-deadenylation as in Gram-negative bacteria (Tronick *et al.*, 1973), by feedback inhibition as in both prokaryotes and eukaryotes (see Prusiner & Stadtman, 1973), by end-product inhibition by glutamine as in *Bacillus subtilis* (Deuel & Stadtman, 1970), by a ligand-induced conformational change followed by dissociation as in *Candida utilis* (Sims *et al.*, 1974a, b) and by a ligand-induced conformational change resulting in reversible enzyme deactivation as in *Lemna minor* (Rhodes *et al.*, 1978). In agreement with our previous findings (Dharmawardene *et al.*, 1973; Stewart *et al.*, 1975; Rowell *et al.*, 1977) we have been unable to demonstrate adenylation-deadenylation (Ginsburg & Stadtman, 1973) of either cyanobacterial enzyme, even after adding cetyltrimethylammonium bromide to the cells, a technique which stabilizes the adenylation state of the *Rhodospseudomonas capsulata* enzyme (Johansson & Gest, 1977). However, we have observed a reversible deactivation of *A. cylindrica* glutamine synthetase which resembles in some respects what has been found with the enzymes of the eukaryotes *Candida utilis* (Sims *et al.*, 1974a, b) and *Lemna minor* (Stewart & Rhodes, 1977; Rhodes *et al.*, 1978). For example, the requirements of the *A. cylindrica* enzyme for stabilization (Mg^{2+} , glutamate, EDTA and 2-mercaptoethanol) and for reactivation (2-mercaptoethanol and substrates of the biosynthetic reaction) were similar to those reported by Rhodes *et al.* (1978) for *in vitro* deactivated *Lemna minor* glutamine synthetase (Mg^{2+} , glutamate, ATP and a thiol reagent). Furthermore, the deactivation of purified *A. cylindrica* enzyme and the accompanying appearance of three bands on gels, one of which was possibly a half molecule and another a dimer or trimer (see Fig. 5a) suggests that dissociation proceeded via a rigid pattern, as with the *Candida utilis* enzyme (Sims *et al.*, 1974a, b). Such a mechanism has not hitherto been reported for a N_2 -fixing organism.

The reasons why the maximum reactivation of the enzyme observed was only 59% of that prior to deactivation are uncertain but the conditions which we used to reactivate the enzyme *in vitro* might have been sub-optimal. It is not possible, at present, to say whether the recovery in activity noted was due to reassociation of the dissociated forms of the enzyme, or whether it was simply dependent upon conformational changes in the dodecameric form of the enzyme (see Fig. 5).

The reversible deactivation of glutamine synthetase from *A. cylindrica* might represent an example of a hysteretic enzyme (Frieden, 1970) which shows a slow response (relative to the catalytic reaction) to rapid changes in ligand concentration. Although caution must be exercised in extrapolating *in vitro* data to the situation *in vivo*, we have evidence, which will be presented elsewhere, that the *A. cylindrica* enzyme can be deactivated and reactivated *in vivo* and that the enzyme deactivated *in vivo* can be reactivated *in vitro* as described here. We have been unable to obtain evidence for a reversible deactivation of the purified enzyme from *Nostoc* sp.: this suggests that there may be some diversity in the mechanisms of regulation of glutamine synthetase in different cyanobacteria.

This work was supported by the Science Research Council and the Brazilian Agricultural Research Corporation. We thank Dr P. Cohen, Department of Biochemistry, University of Dundee, for carrying out the ultracentrifugation and for supplying fatty acid synthetase and phosphorylase kinase. We also thank Gail Alexander and Margaret Laing for technical assistance.

REFERENCES

- ALLEN, M. B. & ARNON, D. I. (1955). Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiology* **30**, 366–372.
- BROWN, C. M. & HERBERT, R. A. (1977*a*). Ammonia assimilation in purple and green sulphur bacteria. *FEMS Microbiology Letters* **1**, 39–42.
- BROWN, C. M. & HERBERT, R. A. (1977*b*). Ammonia assimilation in members of the Rhodospirillaceae. *FEMS Microbiology Letters* **1**, 43–46.
- BROWN, C. M., MACDONALD-BROWN, D. S. & MEERS, J. L. (1974). Physiological aspects of microbial inorganic nitrogen metabolism. *Advances in Microbial Physiology* **11**, 1–52.
- DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, 404–427.
- DEUEL, T. F. & STADTMAN, E. R. (1970). Some kinetic properties of *Bacillus subtilis* glutamine synthetase. *Journal of Biological Chemistry* **245**, 5206–5213.
- DEUEL, T. F., GINSBURG, A., YEH, J., SHELTON, E. & STADTMAN, E. R. (1970). *Bacillus subtilis* glutamine synthetase. Purification and physical characterisation. *Journal of Biological Chemistry* **245**, 5195–5205.
- DHARMAWARDENE, M. W. N., HAYSTEAD, A. & STEWART, W. D. P. (1973). Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica*. *Archiv für Mikrobiologie* **90**, 281–295.
- FRIEDEN, C. (1970). Kinetic aspects of regulation of metabolic processes: the hysteretic enzyme concept. *Journal of Biological Chemistry* **245**, 5788–5799.
- GINSBURG, A. & STADTMAN, E. R. (1973). Regulation of glutamine synthetase in *Escherichia coli*. In *The Enzymes of Glutamine Metabolism*, pp. 9–43. Edited by S. Prusiner & E. R. Stadtmann. New York and London: Academic Press.
- HEDRICK, J. L. & SMITH, A. J. (1968). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Archives of Biochemistry and Biophysics* **126**, 155–164.
- JOHANSSON, B. C. & GEST, H. (1977). Adenylation/deadenylation control of the glutamine synthetase of *Rhodospseudomonas capsulata*. *European Journal of Biochemistry* **81**, 365–371.
- KINGDON, H. S., HUBBARD, J. & STADTMAN, E. R. (1968). Regulation of glutamine synthetase. XI. The nature and implications of a lag phase in the *E. coli* glutamine synthetase reaction. *Biochemistry* **7**, 2136–2142.
- LADHA, J. K., ROWELL, P. & STEWART, W. D. P. (1978). Effects of 5'-hydroxylysine on acetylene reduction and NH_4^+ assimilation in the cyanobacterium *Anabaena cylindrica*. *Biochemical and Biophysical Research Communications* **83**, 688–696.
- LAYNE, E. (1957). Spectrophotometric and turbidometric methods for measuring proteins. *Methods in Enzymology* **3**, 447–454.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MCPARLAND, R. H., GUEVARA, J. G., BECKER, R. R. & EVANS, H. J. (1976). The purification and properties of the glutamine synthetase from the cytosol of soya-bean root nodules. *Biochemical Journal* **153**, 597–606.
- MEEKS, J. C., WOLK, C. P., THOMAS, J., LOCKAU, W., SHAFFER, P. W., AUSTIN, S. M., CHIEN, W.-S. & GALONSKY, A. (1977). The pathways of assimilation of $^{15}\text{NH}_4^+$ by the cyanobacterium *Anabaena cylindrica*. *Journal of Biological Chemistry* **252**, 7894–7900.
- MOORE, S. (1963). On the determination of cystine as cysteic acid. *Journal of Biological Chemistry* **238**, 235–237.
- NAGATANI, H., SHIMIZU, M. & VALENTINE, R. C. (1971). The mechanism of ammonia assimilation in nitrogen-fixing bacteria. *Archiv für Mikrobiologie* **79**, 164–175.
- O'NEAL, D. & JOY, K. W. (1973). Glutamine synthetase of pea leaves. I. Purification, stabilisation and pH optima. *Archives of Biochemistry and Biophysics* **159**, 113–122.

- OWNBY, J. D. (1977). Effects of amino acids on methionine-sulphoximine-induced heterocyst formation in *Anabaena*. *Planta* **136**, 277–279.
- PAMILJANS, V., KRISHNASWAMY, P. R., DUMVILLE, G. & MEISTER, A. (1962). Studies on the mechanism of glutamine synthetase: isolation and properties of the enzyme from sheep brain. *Biochemistry* **1**, 153–158.
- PRUSINER, S. & STADTMAN, E. R., (editors) (1973). *The Enzymes of Glutamine Metabolism*. New York and London: Academic Press.
- RAO, V. R., DARROW, R. A. & KEISTER, D. L. (1978). Effect of oxygen tension on nitrogenase and on glutamine synthetases I and II in *Rhizobium japonicum* 61A76. *Biochemical and Biophysical Research Communications* **81**, 224–231.
- RHODES, D., SIMS, A. P. & STEWART, G. R. (1978). Glutamine synthetase and the control of nitrogen assimilation in *Lemna minor* L. In *Nitrogen Assimilation of Plants* (in the Press). Edited by E. J. Hewitt & C. V. Cutting. New York and London: Academic Press.
- ROWELL, P., EN TICOTT, S. & STEWART, W. D. P. (1977). Glutamine synthetase and nitrogenase activity in the blue-green alga *Anabaena cylindrica*. *New Phytologist* **79**, 41–54.
- SAWHNEY, S. K. & NICHOLAS, D. J. D. (1978). Some properties of glutamine synthetase from *Anabaena cylindrica*. *Planta* **139**, 289–299.
- SCHACHMAN, H. K. (1957). Ultracentrifugation, diffusion and viscometry. *Methods in Enzymology* **4**, 32–103.
- SIMS, A. P., TOONE, J. & BOX, V. (1974a). The regulation of glutamine synthesis in the food yeast *Candida utilis*: the purification and subunit structure of glutamine synthetase and aspects of enzyme deactivation. *Journal of General Microbiology* **80**, 485–489.
- SIMS, A. P., TOONE, J. & BOX, V. (1974b). The regulation of glutamine metabolism in *Candida utilis*: mechanisms of control of glutamine synthetase. *Journal of General Microbiology* **84**, 149–162.
- SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Analytical Chemistry* **30**, 1190–1206.
- SPIES, J. R. & CHAMBERS, D. C. (1949). Chemical determination of tryptophan in proteins. *Analytical Chemistry* **21**, 1249–1266.
- STACEY, G., TABITA, F. R. & VAN BAALEN, C. (1977). Nitrogen and ammonia assimilation in the cyanobacteria: purification of glutamine synthetase from *Anabaena* sp. strain CA. *Journal of Bacteriology* **132**, 596–603.
- STANIER, R. Y., KUNISAWA, R., MANDEL, M. & COHEN-BAZIRE, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews* **35**, 171–205.
- STEWART, G. R. & RHODES, D. (1977). Control of enzyme levels in the regulation of nitrogen assimilation. In *Regulation of Enzyme Synthesis and Activity*, pp. 1–22. Edited by H. Smith. London: Academic Press.
- STEWART, W. D. P. & ROWELL, P. (1975). Effects of L-methionine-DL-sulphoximine on the assimilation of newly fixed NH_3 , acetylene reduction and heterocyst production in *Anabaena cylindrica*. *Biochemical and Biophysical Research Communications* **65**, 846–856.
- STEWART, W. D. P., HAYSTEAD, A. & DHARMAWARDENE, M. W. N. (1975). Nitrogen assimilation and metabolism in blue-green algae. In *Nitrogen Fixation by Free-Living Micro-organisms*, I.B.P. vol. 6, pp. 129–158. Edited by W. D. P. Stewart. Cambridge: Cambridge University Press.
- THOMAS, J., MEEKS, J. C., WOLK, C. P., SHAFFER, P. W., AUSTIN, S. M. & CHIEN, W.-S. (1977). Formation of glutamine from ^{15}N ammonia, ^{15}N dinitrogen, and ^{14}C glutamate by heterocysts isolated from *Anabaena cylindrica*. *Journal of Bacteriology* **129**, 1545–1555.
- TRONICK, S. R., CIARDI, J. E. & STADTMAN, E. R. (1973). Comparative biochemical and immunological studies of bacterial glutamine synthetases. *Journal of Bacteriology* **115**, 858–868.
- VALENTINE, R. C., SHAPIRO, B. M. & STADTMAN, E. R. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry* **7**, 2143–2152.
- WEBER, K. & OSBORN, M. (1969). The reliability of molecular weight determinations by dodecyl sulphate–polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **244**, 4406–4412.
- WEDLER, F. C. & HOFFMANN, F. M. (1974). Glutamine synthetase of *Bacillus stearothermophilus*. I. Purification and basic properties. *Biochemistry* **13**, 3207–3214.
- WOLK, C. P., THOMAS, J., SHAFFER, P. W., AUSTIN, S. M. & GALONSKY, A. (1976). Pathway of nitrogen metabolism after fixation of ^{15}N -labelled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*. *Journal of Biological Chemistry* **251**, 5027–5034.
- WOOLFOLK, C. A., SHAPIRO, B. & STADTMAN, E. R. (1966). Regulation of glutamine synthetase. I. Purification and properties of glutamine synthetase from *Escherichia coli*. *Archives of Biochemistry and Biophysics* **116**, 177–192.
- YPHANTIS, D. A. (1964). Equilibrium ultracentrifugation of dilute solution. *Biochemistry* **3**, 297–317.