The Effects of Exogenous Amino Acids on Growth and Nitrogenase Activity in the Cyanobacterium *Anabaena cylindrica* PCC 7122

By DAVID M. RAWSON

*Applied Microbiology Group, Department of Science, Luton College of Higher Education,
Park Square, Luton LU1 3JU, Bedfordshire, UK*

(Received 14 March 1985; revised 3 June 1985)

Growth and nitrogenase activity were followed in batch cultures of *Anabaena cylindrica* PCC 7122 supplemented with a range of protein amino acids and urea at concentrations of 1 and 10 mM. Alanine, arginine, asparagine, aspartate, glutamate, glutamine and serine caused reductions in nitrogenase activity and showed evidence of acting as sources of nitrogen. Suppression of nitrogenase by other amino acids was accompanied by reduced growth. A number of amino acids appeared to be toxic at high concentrations. Cysteine and histidine were toxic at low concentrations.

INTRODUCTION

Cyanobacterial nitrogen metabolism has been widely investigated with regard to nitrogen fixation (Allen & Arnon, 1955; Gallon, 1980) and the assimilation of inorganic nitrogen sources (Haystead *et al.*, 1973; Batt & Brown, 1974; Rowell *et al.*, 1977). Amino acid uptake has been noted in several studies (Lee-Kaden & Simonis, 1982; Thomas *et al.*, 1982) and feed-back inhibition by amino acids of enzymes involved in NH_4^+ assimilation has been reported (Rowell *et al.*, 1977; Stacey *et al.*, 1979).

Studies of the ability of cyanobacteria to utilize amino acids as sole nitrogen sources (Neilson & Larsson, 1980; Vashampayan, 1982) show highly variable results from different species, and an apparent poor ability amongst photoautotrophs (Neilson & Doudoroff, 1973). Previous workers have used low amino acid concentrations, usually below 1 mM, and only occasionally have reported on the effect of amino acids on nitrogenase activity.

Possibly the identification of an interrupted tricarboxylic acid cycle in cyanobacteria (Pearce *et al.*, 1969) and the predominance of photoautotrophy have dissuaded surveys of amino acid utilization of the type commonly made of other bacteria and eukaryotic algae. The need for a systematic study of single amino acid utilization and investigation of reported toxic effects was recognized by Stanier & Cohen-Bazire (1977).

This paper reports the results of a survey of the effects of protein amino acids and urea at two concentrations on the growth and nitrogenase activity of the obligate photoautotroph *Anabaena cylindrica* PCC 7122 grown in batch culture under continuous illumination.

METHODS

Organism and culture conditions. *Anabaena cylindrica* (PCC 7122), obtained from the Pasteur Culture Collection, Paris, France (Rippka *et al.*, 1979), was maintained on agar plates made from BG-11₀ medium (nitrate-free BG-11 medium after Rippka *et al.*, 1979) supplemented with 1.5% (w/v) bacteriological agar. Liquid starter cultures were prepared by transfer of inoculum from plate cultures to 50 or 100 ml sterile BG-11₀ medium in conical flasks. Such cultures were grown to late exponential phase (OD_{663} 1.5–1.8), when a single flask culture was used to inoculate all flasks in an experimental run. Investigations of amino acid treated and control cultures were made from cultures grown in 50 ml BG-11₀ medium in 250 ml conical flasks under axenic conditions. All amino acid, urea, NH_4Cl and buffer additions were filter-sterilized. All liquid cultures were maintained at 25 °C, with 5.0 W m⁻² illumination

from warm white fluorescent tubes, at 100 oscillations min^{-1} in an orbital shaker. The effect of amino acids was assayed under both buffered and unbuffered conditions; buffered cultures were kept at pH 7.3 with 5 mM-HEPES, a concentration which kept the pH constant and which has been shown to have little effect on cell structure and nitrogenase activity (Smith *et al.*, 1983).

Controls, not supplemented with amino acids or urea, but under otherwise identical conditions, were run with each experimental batch and all results are compared with the appropriate control cultures.

Checks for culture purity. The axenic nature of all cultures was checked by plating onto tryptone soya sugar agar, brain heart infusion agar, and BG-11₀ agar, and incubating for 5 d at 25 °C. Visual checks by phase contrast microscopy were also made.

Measurement of growth. This was followed turbidimetrically at 663 nm using a 1 cm path length on 1 ml samples following nitrogenase assay.

Nitrogenase assay. The acetylene reduction method of Stewart *et al.* (1967) was used.

Chemicals. The purest grade of commercially available chemicals was used throughout. Amino acids were from Sigma, all other compounds were from BDH.

RESULTS

In *Anabaena cylindrica* glutamine synthetase has been shown to be the main route of NH_4^+ assimilation (Rowell *et al.*, 1977). When nitrogen-fixing cultures were supplemented with NH_4^+ there was a rapid decline in nitrogenase activity accompanied by an increase in the growth rate (Fig. 1a). This rapid inhibition of nitrogenase is still not fully understood but NH_4^+ is not the sole inhibitor and Stewart (1980) suggests that glutamine synthetase and/or its product is involved.

Urea elicited the same response as NH_4^+ (Figs 1b and 1c), with an extended period of nitrogenase suppression, confirming the opinion of Neilson & Larsson (1980) that both atoms of N are utilized. Whilst statistical analysis showed the growth rate of NH_4Cl -supplemented cultures to be significantly ($P < 0.001$) higher than in the control over the 8 d period, urea only caused such an effect over the first 5 d of culture growth. Only minor variations were seen in the length of the lag period.

Unsupplemented BG-11₀-grown cultures had a peak of nitrogenase activity between 45 and 60 h, and reached an OD_{663} of approximately 2.0 within 192 h. Unbuffered control cultures increased in pH, reaching 9.0 ± 0.2 by 192 h, and had a slightly higher growth rate than cultures buffered at pH 7.3. Additions of amino acids to unbuffered cultures resulted in a range of pH values as measured at day 8 of the experimental run; the majority of amino acids had a buffering effect. Highest pH values were found in cultures supplemented with 10 mM concentrations of arginine (9.2–9.4), glutamine (8.3–8.7), proline (8.2–8.3) and alanine (7.8–8.0). All other amino acid supplements gave pH values between 7.0 and 7.5, with the exception of cysteine (6.5–6.6). This variation in pH between unbuffered control and amino acid supplemented cultures makes comparison difficult, and unless otherwise stated the results described here refer to buffered conditions.

The patterns of nitrogenase activity and growth in buffered and unbuffered cultures with and without supplements of urea and NH_4^+ were used as points of reference when considering the effect of exogenous protein amino acids on cultures of *A. cylindrica*.

A reduction in nitrogenase activity accompanied by growth equivalent to or above control growth, as seen in cultures supplemented with NH_4^+ and urea, was taken as evidence that the amino acids were acting as a nitrogen source.

Table 1 summarizes the results obtained with 20 amino acids and urea on growth and nitrogenase activity in *A. cylindrica*.

No amino acids reduced nitrogenase activity whilst maintaining growth at or above control levels to the extent shown by NH_4^+ and urea. However, cultures supplemented with arginine, aspartate, glutamine, glutamate (Fig. 2) and serine all had lower nitrogenase activities whilst culture growth was comparable to that of the control. This indicates that these amino acids were able to satisfy in part the nitrogen requirements of *A. cylindrica*. In the case of arginine this effect was most marked at 1 mM, the reduction in nitrogenase activity being less marked at 10 mM, when the growth rate fell below that of the control over the second half of the experimental period.

Fig. 1

Fig. 1. Nitrogenase activity (■, ▲, ●) and growth (□, △, ○) of *Anabaena cylindrica* in control cultures (●, ○) and in cultures supplemented with (a) 1 mM- NH_4Cl (■, □), (b) 1 mM-urea (▲, △) and (c) 10 mM-urea (▲, △). All cultures were buffered at pH 7.3 with HEPES.

Fig. 2

Fig. 2. Nitrogenase activity (▲, ●) and growth (△, ○) of *Anabaena cylindrica* in control cultures (●, ○) and in cultures supplemented with (a) 1 mM-glutamate (▲, △) and (b) 10 mM-glutamate (▲, △). All cultures were buffered at pH 7.3 with HEPES.

Asparagine, glutamine, glutamate and serine caused more pronounced effects at 10 mM. However, whilst there was little reduction in nitrogenase activity in cultures supplemented with 1 mM-glutamate, there was a statistically significant increase in growth rate above that of the control. Similar effects were obtained with both buffered and unbuffered cultures. Little effect was noted with alanine at 1 mM, whilst at 10 mM buffered cultures had a lower nitrogenase activity and growth rate than the control; unbuffered cultures had a growth rate similar to that of the control but a lower nitrogenase activity.

Asparagine and glutamine are amides which show considerable dissociation into aspartate/glutamate and NH_4^+ . In these cases it was not possible to distinguish between the suppression of nitrogenase caused by the amide or the NH_4^+ dissociation product. The results obtained with glutamate are interesting when compared with those noted by Chapman & Meeks (1983) with *Anabaena variabilis* where an exogenous level of 0.5 mM-glutamate inhibited growth.

A number of amino acids caused a reduction of nitrogenase activity accompanied by reduced growth. Cultures supplemented with isoleucine, phenylalanine and valine at 1 mM were not significantly affected but had a pronounced depression of nitrogenase activity and growth at amino acid concentrations of 10 mM.

Leucine, glycine and lysine at 1 mM and 10 mM caused a reduction of nitrogenase activity, as did tyrosine at 1 mM and 2 mM, but this was only accompanied by a major depression in growth at the higher concentrations (Fig. 3), suggesting possible utilization as nitrogen sources at the lower concentrations.

Proline-supplemented cultures showed little sign of perturbation during the first 100 h of culture, but by 190 h nitrogenase activity fell to zero at a proline concentration of 10 mM, and this was accompanied by changes in filament morphology as the cells became spherical and filament length was reduced to short 5–20 cell chains.

Threonine, methionine and tryptophan suppressed nitrogenase and prevented growth at a concentration of 10 mM, whilst with methionine and tryptophan at 1 mM a reduced nitrogenase

Table 1. *Effect of amino acids and urea on growth and nitrogenase activity of Anabaena cylindrica*

Results are from 50 ml batch cultures with 5 mM-HEPES buffer at pH 7.3 and the compounds indicated. The percentages are the means of at least three observations.

Addition	Concn (mM)	Day...	OD ₆₆₃ (percentage of control)				Nitrogenase activity (percentage of control)			
			2	4	6	8	2	4	6	8
L-Alanine	1		103	100	100	86	79	81	115	77
	10		85	70	69	77	78	85	86	90
L-Arginine	1		92	123	101	104	102	80	56	0
	10		121	101	77	74	128	93	84	69
L-Asparagine	1		103	95	102	96	91	85	98	85
	10		88	94	94	96	58	80	40	42
L-Aspartate	1		88	96	103	103	78	87	70	70
	10		108	84	81	86	108	62	69	69
L-Cysteine	1		40	15	19	20	0	0	1	17
	10		50	13	5	2	0	0	0	0
Glycine	1		96	94	88	82	90	86	84	68
	10		53	38	26	34	45	34	35	35
L-Glutamate	1		104	106	115	106	81	84	89	80
	10		108	102	98	108	95	55	52	63
L-Glutamine	1		100	99	91	93	98	100	97	74
	10		118	97	80	79	93	41	14	0
L-Histidine	1		62	38	12	10	10	0	0	0
	10		46	5	3	1	10	0	0	0
L-Isoleucine	1		100	95	104	82	99	103	108	86
	10		76	78	81	78	39	98	66	61
L-Leucine	1		104	115	76	82	47	21	74	104
	10		80	45	31	45	16	27	63	84
L-Lysine	1		92	90	94	83	108	100	86	22
	10		92	71	62	60	90	90	63	0
L-Methionine	1		86	88	87	73	42	81	110	86
	10		63	20	19	20	3	3	10	15
L-Proline	1		107	112	104	83	105	95	68	60
	10		87	101	94	89	105	108	47	0
L-Phenylalanine	1		114	94	91	96	80	98	117	91
	10		123	88	77	88	48	65	83	97
L-Serine	1		93	92	91	90	102	95	112	90
	10		92	103	100	99	82	92	43	42
L-Threonine	1		92	97	100	100	91	92	35	88
	10		58	16	13	15	9	0	5	8
L-Tryptophan	1		79	68	64	60	43	50	51	45
	10		61	19	13	4	0	0	0	0
L-Tyrosine	1		76	53	66	73	2	28	91	112
	2		64	25	19	24	13	9	15	0
L-Valine	1		107	94	91	84	90	98	95	54
	10		66	59	74	78	18	104	111	67
Urea	1		112	138	109	108	14	0	21	116
	10		147	126	122	113	3	1	0	0

activity was accompanied by only a slight reduction in growth, suggesting their ability to act as nitrogen sources at lower concentrations. Threonine did not show such a clear utilization by *A. cylindrica* at 1 mM during the first 100 h of culture but there was evidence of its utilization as a nitrogen source between 100 and 200 h of culture.

Only two amino acids, cysteine (Fig. 4) and histidine, appeared to be toxic at both 1 and 10 mM, suppressing growth and nitrogenase activity under buffered and unbuffered conditions.

Fig. 3

Fig. 4

Fig. 3. Nitrogenase activity (▲, ●) and growth (△, ○) of *Anabaena cylindrica* in control cultures (●, ○) and in cultures supplemented with (a) 1 mM-leucine (▲, △) and (b) 10 mM-leucine (▲, △). All cultures were buffered at pH 7.3 with HEPES.

Fig. 4. Nitrogenase activity (▲, ●) and growth (△, ○) of *Anabaena cylindrica* in control cultures (●, ○) and in cultures supplemented with (a) 1 mM-cysteine (▲, △) and (b) 10 mM-cysteine (▲, △). All cultures were buffered at pH 7.3 with HEPES.

DISCUSSION

Whilst exogenous glutamine has been shown previously to cause inhibition of nitrogenase in *A. cylindrica* (Rowell *et al.*, 1977), it was thought that other major pool amino acids did not (Stewart, 1980). It is clear from the results described here that several amino acids have an effect on nitrogenase, with some being capable of acting as a source of nitrogen. The response of *A. cylindrica* to individual exogenous amino acids was influenced by concentration, as seen in the variable effects at 1 and 10 mM in buffered cultures. Amino acid uptake is probably affected by pH, and amino acid supplements to unbuffered cultures were shown to affect pH. This makes comparison with control cultures difficult under unbuffered conditions, although with the majority of amino acids investigated similar patterns of response were seen in buffered and unbuffered conditions.

The results obtained with arginine, glutamate, methionine, phenylalanine and proline are very close to those obtained by Thomas *et al.* (1982) with *Gloeocapsa* sp., suggesting that nitrogenase inhibition by amino acids in different cyanobacteria may be less variable than was previously thought.

Amino acid toxicity in bacterial autotrophs has generally been attributed to imbalance of amino acid metabolism (Eccleston & Kelly, 1972). It is particularly interesting to note that Kelly (1969) explained phenylalanine toxicity in *Thiobacillus neapolitanus* as due to phenylalanine acting as an end-point inhibitor of the first enzyme of the branched pathway for aromatic amino acid biosynthesis. Such an explanation may be applicable to cyanobacteria. Hall *et al.* (1982) showed that 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase, the first step unique to the aromatic amino acid pathway, was inhibited by 55% in the presence of 0.5 mM-phenylalanine in *Anabaena* 7122. Inhibition by tyrosine was much less at 9%, with no inhibition being caused by tryptophan. Also a 58% inhibition of shikimate/NADP dehydrogenase was caused by a mixture of phenylalanine, tyrosine and tryptophan, each at 0.5 mM.

As reductions in DAHP synthase and shikimate dehydrogenase activities will result in reduced aromatic amino acid production, end-point inhibition may help explain the results reported here with tyrosine, tryptophan and phenylalanine. In all three cases inhibitory effects were seen only at high concentrations.

I thank N. Hudson for his valuable help with computer software, and A. J. W. Love and R. Driver for helpful discussions.

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.
- BATT, T. & BROWN, D. H. (1974). The influence of inorganic nitrogen supply on amination and related reactions in the blue-green alga, *Anabaena cylindrica* Lemm. *Planta* **116**, 27–37.
- CHAPMAN, J. S. & MEEKS, J. C. (1983). Glutamine and glutamate transport by *Anabaena variabilis*. *Journal of Bacteriology* **156**, 122–129.
- ECCELESTON, M. & KELLY, D. P. (1972). Assimilation and toxicity of exogenous amino acids in the methane-oxidizing bacterium *Methylococcus capsulatus*. *Journal of General Microbiology* **71**, 541–554.
- GALLON, J. R. (1980). Nitrogen fixation by photoautotrophs. In *Nitrogen Fixation*, pp. 197–238. Edited by W. D. P. Stewart & J. R. Gallon. London: Academic Press.
- HALL, G. C., FLICK, M. B., GHERNA, R. L. & JENSEN, R. A. (1982). Biochemical diversity for biosynthesis of aromatic amino acids among cyanobacteria. *Journal of Bacteriology* **149**, 65–78.
- HAYSTEAD, A., DHARMAWARDENE, M. W. N. & STEWART, W. D. P. (1973). Ammonia assimilation in a nitrogen fixing blue-green alga. *Plant Science Letters* **1**, 439–445.
- KELLY, D. P. (1969). Regulation of chemoautotrophic metabolism. III. DAHP synthase in *Thiobacillus neapolitanus*. *Archiv für Mikrobiologie* **69**, 360–369.
- LEE-KADEN, J. & SIMONIS, W. (1982). Amino acid uptake and energy coupling dependent on photosynthesis in *Anacystis nidulans*. *Journal of Bacteriology* **151**, 229–236.
- NEILSON, A. H. & DOUDOROFF, M. (1973). Ammonia assimilation in blue-green algae. *Archiv für Mikrobiologie* **89**, 15–22.
- NEILSON, A. H. & LARSSON, T. (1980). The utilization of organic nitrogen for growth of algae: physiological aspects. *Physiologia plantarum* **48**, 542–553.
- PEARCE, J., LEACH, C. K. & CARR, N. G. (1969). The incomplete tricarboxylic acid cycle in the blue-green alga *Anabaena variabilis*. *Journal of General Microbiology* **55**, 371–378.
- RIPPKA, R., DERUELLES, J., WATERBURY, J. B., HERDMAN, M. & STANIER, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* **111**, 1–61.
- ROWELL, P., ENTICOTT, S. & STEWART, W. D. P. (1977). Glutamine synthetase and nitrogenase activity in the blue-green alga *Anabaena cylindrica*. *New Phytologist* **79**, 41–54.
- SMITH, G. D., MACKEY, E. J. & DADAY, A. (1983). Buffer and salt damage to the filamentous cyanobacterium *Anabaena cylindrica*. *Journal of General Microbiology* **129**, 3099–3102.
- STACEY, G., VAN BAALEN, C. & TABITA, F. R. (1979). Nitrogen and ammonia assimilation in the cyanobacteria: regulation of glutamine synthetase. *Archives of Biochemistry and Biophysics* **194**, 457–467.
- STANIER, R. Y. & COHEN-BAZIRE, G. (1977). Phototrophic prokaryotes: the cyanobacteria. *Annual Review of Microbiology* **31**, 225–274.
- STEWART, W. D. P. (1980). Some aspects of structure and function in N_2 -fixing cyanobacteria. *Annual Review of Microbiology* **34**, 497–536.
- STEWART, W. D. P., FITZGERALD, G. P. & BURRIS, R. H. (1967). In situ studies on N_2 fixation using the acetylene reduction technique. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 2071–2078.
- THOMAS, J. H., MULLINEAUX, P. M., CRONSHAW, A. D., CHAPLIN, A. E. & GALLON, J. R. (1982). The effect of structural analogues of amino acids on ammonia assimilation and acetylene reduction (nitrogen fixation) in *Gloeocapsa* (*Gloeotheca*) sp. CCAP 1430/3. *Journal of General Microbiology* **128**, 885–893.
- VASHAMPAYAN, A. (1982). Amino acid nutrition in the blue-green alga *Nostoc muscorum*. *New Phytologist* **90**, 545–549.