Synechococcus mutants resistant to an enamine mechanism inhibitor of glutamate-1-semialdehyde aminotransferase

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Abstract An enamine mechanism-based inactivator of mammalian δ-aminobutyric acid aminotransferase, 4-amino 5-fluoropentanoic acid is a potent inhibitor of cell growth and pigment formation in the cyanobacterium *Synechococcus* PCC 6301. It was demonstrated that 4-amino 5-fluoropentanoic acid inhibits the aminolaevulinate synthesis at glutamate 1-semialdehyde aminotransferase and that in the mutant obtained by exposing cells to 40 μM 4-amino 5-fluoropentanoic acid, this enzyme was insensitive to the inhibitor. The specific activity of glutamate 1-semialdehyde aminotransferase in cell extracts was lower in the mutant, although the cell growth rate was unaffected. The decrease in sensitivity to 4-amino 5-fluoropentanoic acid in the mutant is due to a structural gene mutation, a single base change in the *hem*L gene resulting in a S162T substitution in the gene product.

Key words: 4-Amino 5-fluoropentanoic acid; Cyanobacterium; Glutamate 1-semialdehyde aminotransferase; Tetrapyrroles; *Synechococcus* 6301

1. Introduction

Tetrapyrroles are synthesised from δ-aminolaevulinic acid (ALA), itself derived in oxygenic photosynthetic organisms and some others from glutamate via the C_5-pathway [1]. The final step is catalysed by glutamate 1-semialdehyde aminotransferase (GSA-AT) (EC 5.4.3.8), which interconverts the amino and oxo functions of glutamate 1-semialdehyde ($OCH.CH(NH_2).CH_2CH_2COOH$) to yield ALA ($NH_2.CH_2.CO.C.CH_2.COOH$). Several compounds characterised initially as mechanism-based inactivators of γ-aminobutyric acid (GABA) transaminase (EC 2.6.1.19) have also proved to be effective inhibitors of tetrapyrrole biosynthesis in higher plants. These have included 3-amino 2,3 dihydrobenzoic acid (gabaculine) [2], 4-aminohex-5-ynoate (acetylenic GABA) [3] and 4-amino-5-fluoropentanoic acid (AFPA) [4]. Of these, gabaculine has been the most widely used and apart from higher plants, its inhibition of tetrapyrrole biosynthesis in cyanobacteria [5] has also been well-characterised. Gabaculine inhibits the final step in ALA formation, catalysed by GSA-AT [6]. Unusually, the catalytically active form of this aminotransferase has pyridoxamine phosphate as a cofactor [7]. The pyridoxaldimine phosphate form of the enzyme which gabaculine actually reacts with, yielding a tightly associated *m*-carboxyphenylpyridoxamine phosphate adduct, has been implicated in suggested catalytic mechanisms [8,9]. While acetylenic GABA probably reacts similarly by an isomerisation-Michael addition mechanism [10], the mode of inactivation of GABA-AT by AFPA is through Schiff base formation with enzyme bound pyridoxal phosphate, halide elimination and enamine formation [11].

Given this quite different mechanism of action of AFPA on GABA-AT compared to gabaculine, it was of interest to see whether AFPA would similarly inhibit the tetrapyrrole biosynthesis in a cyanobacterium and by inhibiting GSA-AT. Previously, we have isolated a gabaculine-resistant mutant of *Synechococcus* 6301 [12] and characterised the structural changes in GSA-AT from this compared to the enzyme from wild-type cells [13]. Because of the insights into structure-function relationships that such studies can potentially yield, we further wished to complement these other investigations by obtaining and characterising AFPA-resistant mutants of *Synechococcus* 6301.

2. Materials and methods

2.1. Cell culture

Synechococcus PCC 6301 was grown photoautotrophically [14] either as a batch culture or as a continuous culture. For the latter, in 800 ml culture volume maintained at a low dilution rate (0.015 h^{-1}), the cell density attained was about 200 Klett units (K) at wavelengths greater than 750 nm after several days.

2.2. Preparation of protein extracts

Cells were recovered from cultures by centrifugation at 15 000 × *g* for 15 min at 4°C, washed twice with 0.1 M Tricine-HCl buffer, pH 7.9, containing 0.3 M glycerol and 1 mM DTT and resuspended at 1 g wet weight of cell material/2.0 ml buffer. The cells were broken by three passages through a chilled French press with additions to buffers immediately before the initial passage and after each cycle of cell breakage of PMSF and 1 *o*-phenanthroline, both at a final concentration of 1 mM. The supernatant after centrifugation at 35 000 × *g* for 20 min and then 180 000 × *g* for 60 min each at 4°C was passed in 1.0 ml fractions through NAP-10 Sephadex columns to obtain the protein fraction used subsequently in enzyme assays.

2.3. Assays

The assay of the GSA-AT was as in [15] except that the initial incubations were in Bistris buffer, pH 6.9. Chlorophyll *a* and protein in cells and extracts were estimated as in [5].

2.4. Gene sequencing

The *hemL* gene for *Synechococcus* 6301 K40 was sequenced by dye termination with Amplitaq DNA polymerase F3 on a 377 ABI automated DNA sequencer using the primers:

M13 forward (−40)	(5′-GTTTTCCCAGTCACGAC-3′)
M13 reverse	(5′-CAGGAAACAGCTATGAC-3′)

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Abbreviations: ALA, δ-aminolaevulinic acid; AFPA, 4-amino 5-fluoropentanoic acid; GSA-AT, glutamate 1-semialdehyde aminotransferase (glutamate 1-semialdehyde 2,1-aminomutase); K5 etc., mutant obtained through acclimatisation to 5 μM AFPA etc.

PII: S0014-5793(99)00465-2

Internal *hem*L primers for the sense (S) and antisense (X) strands in the same orientation (5′-3′, start nucleotide numbers in parentheses) were:

S2	(180)	GACTACGTCGGCACCTGG
S3	(435)	CACGCTGACATGTTCTTG
S4	(693)	GAAATCACGCTGGAGCAT
S5	(1008)	CGCCTGAGTGATGGTTTG
X2	(1038)	CGCATGACCGGTTTCTTG
X3	(767)	GCCAAACTTTTCTTGCAC
X4	(480)	GGTCGCCACCCCAGACCC
X5	(156)	GCGATTGCCGTCCACATC

All bases were sequenced in at least two reactions on both strands with different primers.

3. Results and discussion

When the continuous culture was at a steady state of cell density, 5 μM AFPA was added both to the culture and to the medium reservoir. This addition resulted in a decrease in the culture density over 10 days with a concomitant change in the chlorophyll content which decreased from 14 to 1 μg/ml (Fig. 1). Thereafter, the cell density and pigment content increased steadily over the succeeding 10 days until a steady culture density close to the original value was reached, although the chlorophyll content was not fully restored. This recovery indicated the selection of an AFPA tolerant spontaneous mutant(s) and cells sampled at this point gave the strain designated as *Synechococcus* 6301-K5. This culture was pure by standard microbiological tests.

The continuous culture was then adjusted to 10 μM AFPA and the selection process re-iterated to give strain K10. Exposures in turn to 20 μM and 40 μM AFPA gave strains K20 and K40. In all, the continuous culture was maintained for 3 months before being discontinued after the selection of strain K40. After each of these successive additions of AFPA, the fall in cell density and in the chlorophyll content was not as severe as seen initially on adjustment to 5 μM

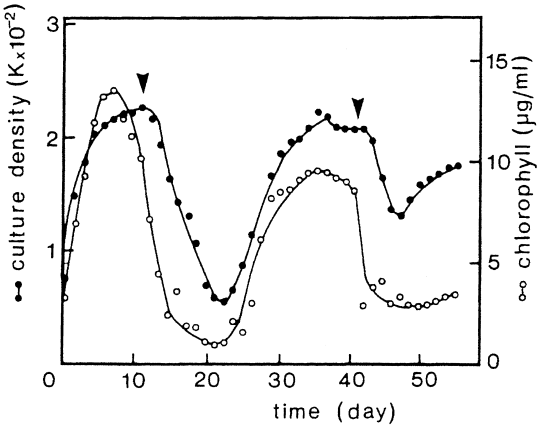

Fig. 1. The effect of addition of AFPA to a continuous culture of *Synechococcus* 6301. At the steady state of culture density, AFPA was added to a final concentration of 5 μM and the culture density (●) and chlorophyll content (○) were monitored over the period of decline and recovery. Cells at 40 days following recovery represented the AFPA tolerant strain K5. The AFPA concentration was adjusted to 10 μM and the culture density and pigment concentration similarly monitored, with further adjustments (not shown) to 20 μM and finally 40 μM AFPA. Cells following recovery from the last addition gave strain K40.

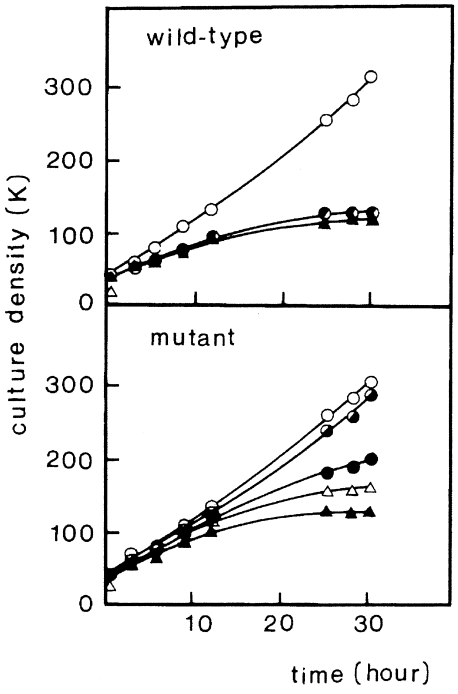

Fig. 2. The effect of AFPA on the growth of *Synechococcus* 6301-K5. The data are for batch cultures of wild-type cells and mutant K5, respectively, in the absence of AFPA (○) and after additions of 5 (◑), 10 (●), 25 (△) and 50 (▲) μM AFPA.

AFPA and the chlorophyll content remained somewhat depressed on recovery compared to the cell density. As a consequence, in the final culture, after recovery from an adjustment to 40 μM AFPA, the chlorophyll content was only 50% of that in the initial steady state.

The degree of AFPA tolerance possessed by the selected strains was assessed in terms of growth in a series of batch cultures. The data for K5 and K40 are presented (Fig. 2 and 3). For the wild-type strain, growth in the absence of AFPA was sustained at a high rate over 30 h whereas supplementation with 5 μM, 10 μM (not shown) or 20 μM AFPA caused a progressive decrease in the cell growth that was essentially complete after 20 h. In contrast, the mutants showed a degree of tolerance that accorded with their selection pressure. Thus K5 showed only a small decline in growth with 5 μM AFPA (Fig. 2), a 50% decrease with 10 μM AFPA, while higher concentrations caused a severely decreased growth rate leading ultimately to a cessation of growth. With K40, the cell growth was unaffected by 40 μM or 80 μM AFPA (Fig. 3). The behaviour of strains K10 and K20 were intermediate and these data are not presented.

The amounts of chlorophyll in batch cultures were also determined and are shown for K5 and K40 relative to the wild-type strain, where 5 μM AFPA was sufficient to severely decrease the chlorophyll content of the culture (Fig. 4). Mutant K5 cells exposed to 5 μM AFPA retained about 75% of the chlorophyll of the untreated culture. At 10 μM, the AFPA chlorophyll content was decreased substantially and to an even greater extent at higher inhibitor concentrations. *Synechococcus* 6301-WT was observed [5] to respond to an enforced decrease in the synthesis of chlorophyll resulting from exposure to gabaculine by a reduction in the growth rate and an increase in cell size and this also is the case

with AFPA. In respect of growth characteristics (Fig. 3) and the chlorophyll content (Fig. 4), batch cultures of *Synechococcus* 6301-K40 behaved identically to the parent strain. However, for mutant K40, there was no decline in chlorophyll at culture concentrations of 10 μM or 20 μM AFPA and only small decreases at 40 μM and 80 μM AFPA.

The biochemical basis of the AFPA tolerance of mutant K40 was investigated by screening cell extracts for sensitivity of the GSA-AT to AFPA (Table 1). This enzyme in the wild-type showed a high sensitivity to AFPA in vitro with 0.5 μM AFPA decreasing the GSA-AT activity by some 20% and 1.0 μM AFPA by 60%. At 5.0 μM and higher concentrations of AFPA, an enzyme activity was barely detectable. In accompanying experiments, extracts of mutant K40 were unaffected by concentrations of AFPA as high as 100 μM and even at 200 μM approximately 50% of the GSA-AT activity remained. These data are informative in a number of respects. Firstly, tolerance towards AFPA is not due to a change in cell permeability to the inhibitor or to induction in vivo of an enzyme degrading AFPA. Secondly, AFPA-resistance has not been achieved through over-expression of the GSA-AT gene, as was the case for *Chlamydomonas* mutants tolerant to gabaculine [16]. Indeed, as judged by these data (Table 1), the K40 mutant possessed significantly less GSA-AT activity than the wild-type. This suggested that the basis of tolerance to AFPA has been the selection of a mutant with an altered GSA-AT, less sensitive to AFPA. This enzyme could have either a lower specific activity than the wild-type enzyme or the gene is 'under-expressed' in the mutant.

Sequencing of the *hem*L gene in *Synechococcus* 6301 K40 identified a single structural gene mutation resulting in a T to A substitution at position 1228 in the wild-type nucleotide sequence. This will result in a change of Ser-162 to Thr in the gene product. This is in a part of the sequence that is conserved in the enzyme from various sources [17]. In addition, Ser-162 is in a region (residues 153–181) where recent crystallographic studies of the GSA-AT α_2-homodimer [18] have shown asymmetry in cofactor binding and in the mobilities of these residues in the two subunits.

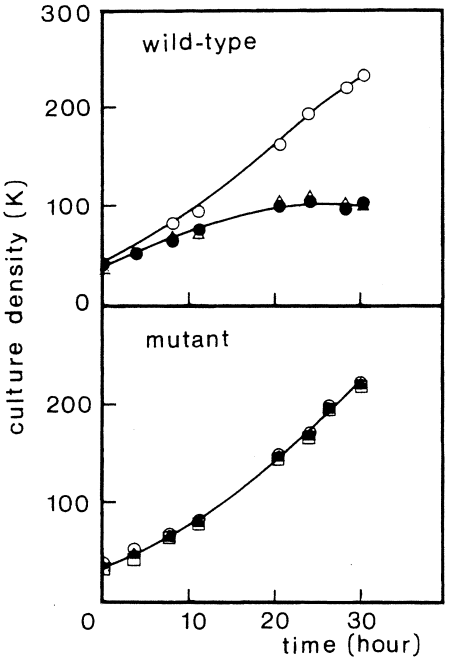

Fig. 3. The effect of AFPA on the growth of *Synechococcus* 6301-K40. The data are for batch cultures of wild-type cells and mutant K40, respectively, in the absence of AFPA (○) and after additions of 10 (●) and 20 (△) μM AFPA for wild-type cultures and of 40 (▲) and 80 (□) μM AFPA for strain K40.

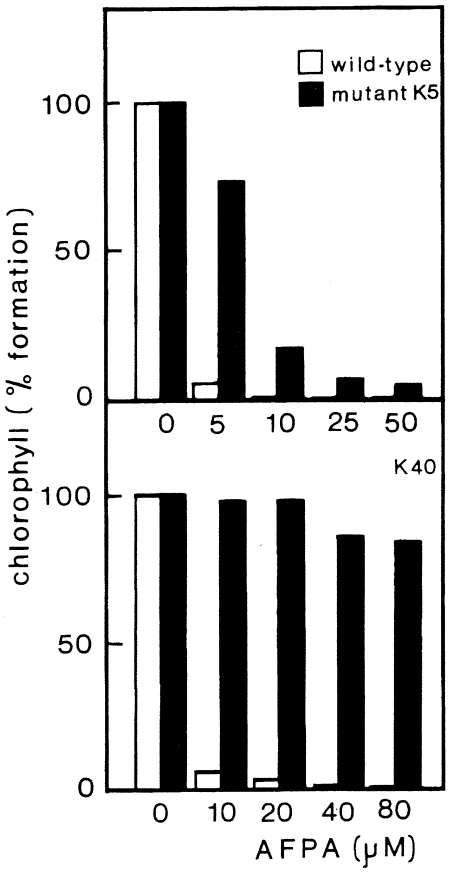

Fig. 4. The effect of AFPA on the chlorophyll content in mutants K5 and K40, respectively, (■) compared to the wild-type strain (□). Chlorophyll synthesis is expressed as a percentage of that in the culture grown for the same time (30 h) in the absence of AFPA.

Mutant GSA-AT obtained by factor Xa cleavage of a malE/*hemL* K40 fusion protein produced by the expression of a pMalc2-*hemL* K40 construct in *Escherichia coli* showed a strong resistance to AFPA ($I_{50} \gg 100$ μM), sensitivity to gabaculine (I_{50} of 6 μM, closely similar to wild-type enzyme) and a catalytic efficiency that was only 6% of that of the wild-type enzyme, derived from a 3-fold increase in K_m and a decrease in V_{max} of 83%.

The change in strain K40 is thus in a different region of GSA-AT than those found with an earlier mutant, *Synechococcus* 6301 GR6, where tolerance to another aminotransferase inhibitor, gabaculine, was due to dual structural gene mutations resulting in a deletion of Ser-7, Pro-8 and Phe-9 near the N-terminus in addition to a M248I substitution. In this mutant, construction of a suicide vector for the *hemL* gene to give replacement by homologous recombination of the wild-type gene in *Synechococcus* PCC7942 demonstrated that resistance to the inhibitor was dependent on both the structural changes [19]. For this gabaculine-resistant mutant, there was also some tolerance to AFPA [12], but for *Synechococcus*

Table 1
Effect of AFPA on the GSA-AT activity in extracts of wild-type *Synechococcus* 6301 and the derived mutant K40

AFPA (μM)		0	0.5	1	5	10	20	100	200
GSA-AT activity	Wild-type	417	325	259	75	58	42	n.d.	n.d.
	Mutant K40	200	–	–	224	250	200	182	100

GSA-AT activity is expressed as pmol ALA produced/min/mg protein. n.d.; not detectable.

6301-K40, neither batch cultures nor GSA-AT in cell extracts show any degree of co-resistance to gabaculine.

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