Inhibition of Bacteriochlorophyll Biosynthesis by Gabaculin (3-Amino, 2,3-dihydrobenzoic Acid) and Presence of an Enzyme of the C$_5$-Pathway of δ-Aminolevulinate Synthesis in *Chloroflexus aurantiacus*

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Biosynthesis of bacteriochlorophyll *c* and *a* in the thermophilic phototrophic prokaryote, *Chloroflexus aurantiacus* Ok-70-fl, was strongly inhibited by the antibiotic gabaculin (3-amino 2,3-dihydrobenzoic acid), an inhibitor of the glutamate-C$_5$-pathway of δ-aminolevulinate (ALA) synthesis. The key enzyme of the Shemin-pathway of ALA formation, ALA synthase (EC 2.3.1.37), was not detected in cell extracts of *Chl. aurantiacus*. However, the extracts catalyzed ALA formation from glutamate 1-semialdehyde, a reaction being highly sensitive to gabaculin.

Introduction

In all organisms studied so far, δ-aminolevulinic acid (ALA) is the precursor of tetrapyrrole compounds (chlorophylls, heme pigments, cobalamins, a.o.). Two different pathways of ALA biosynthesis are known: (a) The "Shemin-pathway", *i.e.* the condensation of succinate and glycine to ALA [1]; and (b) the "C$_5$-pathway" in which glutamate is converted to ALA by the following enzyme-catalyzed steps: (i) activation of glutamate by binding to a specific tRNA; (ii) hydrogenation of the activated glutamate to glutamate 1-semialdehyde; and (iii) transamination of the latter compound to ALA [2, 3]. Research conducted during the last two decades has shown that phototrophic prokaryotes use both pathways. Several species of the phototrophic non-sulfur purple bacteria *(Rhodospirillaceae)* synthesize ALA from succinate and glycine [4−6], whereas cyanobacteria, green sulfur bacteria and purple sulfur bacteria mostly lack ALA synthase (EC 2.3.1.37), the enzyme catalyzing the condensation of succinyl-coenzyme A and glycine to ALA, and synthesize the latter compound *via* the C$_5$-pathway [6−9].

The thermophilic phototrophic prokaryote, *Chloroflexus (Chl). aurantiacus*, has a somewhat ambiguous position with respect to the mechanisms of ALA synthesis. Unlike the cyanobacterium *Anacystis nidulans*, the green sulfur bacterium *Chlorobium limicola* and the non-sulfur purple bacterium *Rhodopseudomonas palustris*, *Chloroflexus aurantiacus* did not incorporate radioactivity into ALA from either [^{14}C]glycine plus succinate or from [^{14}C]glutamate [6]. Therefore, we have reinvestigated the mechanism of ALA synthesis in *Chl. aurantiacus*. It will be shown in this paper that the antibiotic gabaculin (3-amino 2,3-dihydrobenzoic acid), a potent inhibitor of ALA synthesis in cyanobacteria but not in non-sulfur purple bacteria [10], strongly inhibits bacteriochlorophyll synthesis in growing *Chl. aurantiacus* cultures, and that cell extracts of the organism catalyze the conversion of glutamate 1-semialdehyde to ALA.

Materials and Methods

Organisms and culture conditions

Chl. aurantiacus Ok-70-fl was obtained from Dr. Karin Schmidt, Institute for Microbiology, University of Göttingen. The organism was grown photosynthetically (conditions as described in [11]) at 52−55 °C in a yeast extract containing medium (S medium according to [12]).

Rhodobacter (Rhb). sphaeroides (formerly called *Rhodopseudomonas sphaeroides*) DSM 636 was obtained from the German Collection of Microorganisms, Braunschweig. The organism was grown photosynthetically at 30−32 °C in a malate-(NH$_4$)$_2$SO$_4$-medium [13] supplemented with a vitamin mixture [14].

Determination of bacteriochlorophyll and protein content of cells

Bacteriochlorophyll (BChl) *c* and *a* was determined in acetone−methanol (7:2 mixtures) extracts of cells using the specific absorption coefficients $\varepsilon_{666} = 86$ liter $\cdot$ g^{-1} $\cdot$ cm^{-1} (BChl *c*) and $\varepsilon_{769} = 75$ liter $\cdot$ g^{-1} $\cdot$ cm^{-1} (BChl *a*) [15, 16]. The protein content of cells was assayed by a biuret method [17].

Preparation of cell extracts

Bacterial cells harvested at the end of the logarithmic growth phase were washed once in 50 mM Tris-HCl, pH 8.2 *(Chl. aurantiacus)* or pH 7.2 *(Rhb. sphaeroides)* and resuspended (either immediately or after storage at −18 °C) in the same buffer supplemented with 10 mM MgCl$_2$ and 2 mM mercapto-ethanol (for ALA synthase assay). Cell homogenates were prepared by passing the suspensions twice through a French pressure cell (20,000 psi) and were freed of intact cells and cell debris by centrifugation at $15,000 \times g$ (4 °C) for 40 min. Protein concentrations of cell extracts were determined according to [18].

Determination of δ-aminolevulinic acid (ALA)

For separation of ALA from substances interfering with its colorimetric assay, test mixtures were treated with a cation exchanger (Dowex 50W−X8, Na$^+$-form) contained in small polypropylene columns ("Econo" columns, Biorad, München) [19].

ALA eluted from the columns was converted with ethylacetoacetate to ALA-pyrrole which was assayed colorimetrically with Ehrlich's reagent (*p*-dimethyl-aminobenzaldehyde) [20].

Enzyme assays

ALA synthase (EC 2.3.1.37) activity of *Rhb. sphaeroides* extracts was determined at 37 °C in a reaction mixture (1 ml) described in [21]. *Chl. aurantiacus* extracts were tested at 52 °C under almost identically conditions (Tris-HCl buffer of pH 8 instead of pH 7.2). The enzyme reaction was stopped after maximally 90 min by adding 0.1 ml of 1 M citric acid. Precipitated protein was removed by centrifugation and ALA in the supernatant assayed as described above.

Glutamate 1-semialdehyde aminotransferase (EC 5.4.3.8) activity of *Chl. aurantiacus* extracts was assayed at 52 °C in the following reaction mixture (1.1 ml): 100 mM imidazole-HCl, pH 8; 2.5 mM dithioerythritol; 5 mM MgCl$_2$; 10 mM Na-levulinate; 0.7−1.4 mg of a glutamate 1-semialdehyde preparation; 10 vol.% glycerol; 4−10 mg *Chl. aurantiacus* protein; and 10 μM gabaculin where indicated. The enzyme reaction was stopped after 10 min and ALA was determined as described above.

ALA dehydratase (EC 4.2.1.24) activity was determined in 3 ml reaction mixtures [22] at 37 °C *(Rhb. sphaeroides)* or 52 °C *(Chl. aurantiacus)*. The reaction product, porphobilinogen (PBG), was assayed colorimetrically as PBG-pyrrole with Ehrlich's reagent [20].

Enzyme activities are expressed in terms of units (U). 1 unit (1 μmol/min) is equivalent to 16 nkat.

Chemicals

δ-Aminolevulinic acid, coenzyme A (CoA), coomassie brillant blue, dithioerythritol, Dowex 50W−X8, Ehrlich's reagent, and gabaculin were obtained from Serva, Heidelberg; ethylacetoacetate and levulinic acid from Sigma, München; ATP, pyridoxal-5'-phosphate and succinyl-CoA-synthetase from Boehringer, Mannheim. A preparation of glutamate 1-semialdehyde-hydrochloride was kindly provided by Dr. C. G. Kannangara, Carlsberg Laboratory, Copenhagen. All other chemicals were obtained from Merck, Darmstadt.

Results and Discussion

Gabaculin (3-amino 2,3-dihydrobenzoic acid), an "antibiotic" produced by *Streptomyces toyocaensis*, was originally introduced as a neurotropic inhibitor of brain γ-aminobutyrate (GABA) transaminase [23]. The diagnostic value of gabaculin in studies of the enzymatic mechanism of ALA biosynthesis rests with the fact that also the C$_5$-pathway enzyme, glutamate-1-semialdehyde transaminase, is strongly and specifically inhibited [10, 24]. As shown in Table I, gabaculin (at a concentration of 2 μM) almost completely arrested the synthesis of bacteriochlorophyll (BChl) in a photosynthetically growing culture of *Chl. aurantiacus*.

Since cellular growth was not completely blocked in the presence of the inhibitor (due to the fact that saturating light intensity was used to illuminate the culture) the specific BChl content of the inhibited

Table I. Effect of gabaculin (3-amino 2,3-dihydrobenzoic acid) on bacterio-
chlorophyll synthesis and photosynthetic growth of *Chloroflexus aurantiacus* Ok-70-
fl. Cultures were grown photosynthetically using 1 1-screw cap bottles filled to 4/5 of
the total volume with S-medium [12] and flushed with sterile N_2 *via* cotton-stop-
pered gas in-and outlets. Samples were withdrawn *via* a glass-tube reaching to the
bottom of the flask.

	Growth stage	Protein content [mg/ml]	Total BChl content [μg/ml]	Spec. BChl content [μg/mg protein]
Control culture without gabaculin	initial	0.035	0.45	12.8
	after 112 h	0.38	18	47.4
Culture with 2 μM gabaculin	initial	0.053	0.50	9.5
	after 112 h	0.20	0.54	2.7

culture decreased from initially 9.5 to 2.7 μg/mg pro-
tein (compared to an increase from 12.8 to 47.4 μg/
mg protein in a non-inhibited culture). Note, that the
ratio of BChl *c*/BChl *a* (values of 8:1 recorded in non-
inhibited cultures) was only slightly changed in a
gabaculin-treated culture. Thus, synthesis of both
BChl types was affected by the inhibitor.

As expected, control experiments with photo-
synthetically growing cultures of *Rhb. sphaeroides*
(which synthesizes ALA *via* the Shemin-pathway)
showed that gabaculin (up to 10 μM) had no measur-
able effect on the specific BChl-content of the cells
(26 and 25.6 μg BChl *a*/mg protein in control and
inhibitor-containing cultures, respectively) or on the
growth rate. Contrary to results with *Rhb.
sphaeroides* in which levulinic acid (added at a con-
centration of 50 mM) caused a 60% decrease of the
specific BChl-content accompanied by an accumula-
tion of ALA in the culture medium (up to 5 nmol
ALA/ml during the first 20 h of growth), levulinate
(50 mM) caused no ALA excretion in *Chl. auran-
tiacus* cultures.

Under our experimental conditions (see section
Materials and Methods) not even traces of ALA
synthase activity were detected in cell extracts of
Chl. aurantiacus. In control experiments with cell ex-
tracts of photosynthetically grown *Rhb. sphaeroides*,
ALA synthase activities in the range of 1 to 2.5 U/g
protein (= 16 to 40 nkat/g protein) were readily
measured.

A possible cause of the failure to detect ALA
synthase activities in *Chl. aurantiacus* extracts could

have been a very unfavourable activity ratio ALA
synthase/ALA dehydratase. The latter enzyme was
detected at activity levels of 1 to 2 U/g protein in
extracts of *Chl. aurantiacus* (for comparison: ALA
dehydratase activities in cell extracts of *Rhb.
sphaeroides* were in the range of 20 to 25 U/g pro-
tein). Like other ALA dehydratases from photo-
trophic microorganisms [10], the *Chl. aurantiacus*
enzyme was inhibited by levulinic acid. Analysis of
reaction kinetics at various ALA and inhibitor con-
centrations showed that levulinate inhibited the
enzyme competitively with respect to ALA (K_i =
4 mM). However, even when the inhibitor was added
at concentrations of 100 to 200 mM, no ALA forma-
tion was recorded with *Chl. aurantiacus* extracts in
ALA synthase assay mixtures.

Importantly, cell extracts of *Chl. aurantiacus*
catalyzed the conversion of glutamate 1-semialde-
hyde to ALA (Table II), a reaction being highly sensi-
tive to gabaculin. Thus, *Chl. aurantiacus* contains an
aminotransferase (system) similar or identical to the
glutamate 1-semialdehyde aminotransferase of the
C_5-pathway [24].

Our experimental results have made it very likely
that in the thermophilic phototrophic prokaryote,
Chloroflexus aurantiacus, δ-aminolevulinic acid
(ALA) is synthesized *via* the glutamate-C_5-pathway.
Together with previous investigations of other
laboratories [6–9] this finding shows that – with the
exception of some genera of the non-sulfur purple
bacteria [4–6] – the majority of phototrophic pro-
karyotes does not use the Shemin-pathway of ALA

Table II. Conversion of glutamate-1-semialdehyde (GSA) to δ-aminolevulinic acid catalyzed by unfractionated cell extracts of *Chloroflexus aurantiacus* Ok-70-fl. Reaction mixtures (1.1 ml) with 100 mM imidazol-HCl, pH 8; 2.3 mM dithioerythritol; 4.5 mM MgCl$_2$; 10 mM levulinic acid (Na$^+$-salt); 9 vol.% glycerol and 4−10 mg extract protein were incubated for 10 min at 52 °C. ALA in the reaction mixtures was analyzed as described in the Materials and Methods section.

Reaction mixture	nmol ALA formed
500 µl extract + 700 µg GSA	20.7
500 µl extract + 1400 µg GSA	36.3
200 µl extract + 700 µl GSA	17.0
500 µl extract + 700 µg GSA + 10 µM gabaculin	3.2
700 µg GSA	2.5

synthesis. The recent demonstrations of C$_5$-pathway activities in the archaebacterium *Methanobacterium thermoautotrophicum* [25] and the thermophilic *Clostridium thermoaceticum* [26] document that the C$_5$-pathway is much more widespread among organisms than had been expected when this reaction sequence was first described by Beale [2].

Acknowledgements

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