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Effect of Gabaculine on the Synthesis of Heme and Cytochrome *f* in Etiolated Wheat Seedlings[1]

Clare M. Anderson and John C. Gray*

Botany School, University of Cambridge, Cambridge, CB2 3EA, United Kingdom

ABSTRACT

The effect of gabaculine (3-amino 2,3-dihydrobenzoic acid), an inhibitor of tetrapyrrole synthesis, on the accumulation of heme and cytochrome *f* in etiolated wheat (*Triticum aestivum* var Mardler) seedlings has been examined. Gabaculine treatment resulted in decreased amounts of heme and of holocytochrome *f* detected spectroscopically and by peroxidase activity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amount of the cytochrome *f* polypeptide detected immunochemically on Western blots was much less affected by gabaculine treatment, indicating that apocytochrome *f* synthesis was not tightly coupled to heme availability. Gabaculine treatment did not affect the size of the cytochrome *f* polypeptide, indicating that heme addition is not required for proteolytic removal of the presequence.

Cyt *f*, a component of the Cyt *bf* complex, is one of the best characterized intrinsic membrane proteins of the chloroplast thylakoid membrane system. The polypeptide is encoded by the chloroplast genome (1, 30) and has been shown to be synthesized on thylakoid-bound ribosomes (12). The *petA* gene encodes a precursor of Cyt *f* with an *N*-terminal presequence which is believed to direct the protein to the thylakoid membrane for insertion (24, 29). Cyt *f* is a *c*-type Cyt with a heme prosthetic group attached covalently to two cysteine residues by thioether bonds.

Cyt c_1 is the mitochondrial analog of Cyt *f* and it displays a similar structure and function in the Cyt bc_1 complex (13). Cyt c_1 is nuclear-encoded and is synthesized with a long presequence which is removed in two proteolytic processing steps (11, 21, 22). The *N*-terminal region of the presequence targets the protein to the mitochondrial matrix, where it is removed, leaving an intermediate form of the precursor protein, which contains a hydrophobic presequence for targeting to the inner mitochondrial membrane (10, 21, 22). This intermediate form is analogous to the precursor form of apocytochrome *f* in chloroplasts. In yeast and *Neurospora*, heme addition to apocytochrome c_1 is tightly coupled to the second processing event, which generates the mature-sized protein. The intermediate form of Cyt c_1 accumulates in heme-deficient mutants or in wild-type organisms when heme synthesis or attachment is inhibited (10, 21, 22).

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The aim of the present study was to investigate the role of heme in the synthesis of Cyt *f*. The consequences of inhibiting heme synthesis with gabaculine (3-amino 2,3-dihydrobenzoic acid) on the size and the accumulation of the Cyt *f* polypeptide have been examined in wheat (*Triticum aestivum* var Mardler) seedlings. Gabaculine inhibits glutamate semialdehyde aminotransferase, the enzyme catalyzing the synthesis of 5-aminolevulinate (15), the universal precursor of tetrapyrroles in higher plants. Because gabaculine also inhibits Chl synthesis (9, 20), it was decided to use etiolated tissue to ensure that any effect produced by the inhibitor was a result of reduced heme synthesis and not a consequence of the inhibition of Chl synthesis. Wheat was chosen as the plant material because Cyt *f* accumulates in leaves of dark-grown cereal seedlings (23, 25).

MATERIALS AND METHODS

Plant Material

Wheat seeds (*Triticum aestivum* var Mardler) were obtained from the Institute of Plant Science Research, Cambridge Laboratory. Following overnight imbibition in distilled water or the appropriate concentration of gabaculine (Fluka Chemie AG), the seeds were surface-sterilized by rinsing in 70% ethanol and then in a solution of 10% sodium hypochlorite (8% available Cl) for 10 min each. The seeds were then washed several times in distilled water to remove the bleach. Approximately 20 seeds were germinated in 60 mL sterilin vials on 5 mL of 0.8% (w/v) agar containing gabaculine (to pH 7.5 with KOH). Microbial contamination of the seeds was prevented by including an antibiotic antimycotic in the agar at a concentration recommended by the manufacturer (Sigma Chemical Co.). The seeds were germinated in complete darkness for 7 d at 20°C.

Preparation and Analysis of Membrane Proteins

Membranes were isolated by grinding 2 g of shoot tissue from each treatment in a mortar with grinding medium (50 mM Hepes-KOH, pH 8.0, 300 mM sorbitol, 10 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM sodium isoascorbate). The homogenate was filtered through eight layers of muslin and then centrifuged at 10000*g* for 15 min to pellet the membranes. Membrane pellets were solubilized by boiling for 3 min in 500 μL of a buffer containing 80 mM Tris-HCl, pH 6.8, 10% glycerol, 10% SDS, 0.002% bromophenol blue, and 5% (v/v)2-mercaptoethanol (added just before use). Protein samples (50 μL) were separated by electrophoresis in 12.5%

polyacrylamide gels with a 5% stacking gel using the buffer system of Laemmli (19). The Cyt *f* polypeptide was detected by immunodecoration of Western blots. Proteins were transferred onto nitrocellulose membranes as described by Towbin *et al.* (27) and the membrane was probed with monospecific antibodies to charlock Cyt *f* (7, 11) followed by ^{125}I-protein A. Cyt *f* was also visualized by staining gels of total membrane protein for heme-associated peroxidase activity (26). The gel was immersed in a mixture of 230 mL of 250 mM sodium acetate (pH 5) and 150 mg of 3,3,5,5-tetramethylbenzidine in 100 mL of methanol. After 1 h in the dark, 1 mL of a 30% hydrogen peroxide solution was added to visualize the bands. In order to quantify the amount of holocytochrome *f* and apocytochrome *f*, autoradiograms of Western blots and gels stained for heme-associated peroxidase activity were scanned on a Molecular Dynamics 300S scanning densitometer.

Heme Determinations

Heme was extracted from samples (500 mg) of shoot tissue according to Werck-Reichart *et al.* (28). Shoots were ground in a mortar with 80% (v/v) acetone containing 100 μM KOH. The homogenate was centrifuged at 5000*g* for 5 min at 4°C and the pellets were dried under vacuum and dissolved in 2 mL of 0.2 M KOH containing 3% sodium cholate. Pyridine (1 mL) was added and the samples were centrifuged at 5000*g* for 5 min. The dithionite-reduced minus oxidized difference spectrum of the pyridine-hemochrome complex in the supernatant was recorded between 500 and 600 nm. Heme content was calculated from the absorbance maximum at 556 nm using an absorption coefficient of 20.7 mM^{-1} cm^{-1} (5). To measure the amount of heme specifically associated with Cyt *f*, visible difference spectroscopy was undertaken as described by Bendall and Rolfe (3). Membranes were isolated from 4 g of shoot tissue as described above, suspended in 5 mL of 10 mM tricine KOH, pH 8.0, using a hand-held Potter homogenizer, and repelleted by centrifugation at 15000*g* for 30 min at 4°C. The membranes were resuspended in 3 mL of a medium containing 300 mM sorbitol, 50 mM phosphate buffer, pH 6.5, and distributed between two cuvettes. The difference spectrum between 538 and 580 nm was recorded 3 min after the addition of 1 mM hydroquinone to one cuvette and 1 min after the addition of 1 mM $K_3Fe(CN)_6$ to the other. The concentration of Cyt *f* in each sample was determined by measuring the peak height at 554 nm above a baseline drawn through the isosbestic points at 543 and 560 nm.

RESULTS

The most obvious effect of gabaculine on germinating wheat seedlings was an inhibition of shoot growth (Table I). The length and fresh weight of shoots decreased with increasing gabaculine concentration. The lengths and fresh weights of the shoots grown in the presence of 0.5 mM gabaculine were only 14 and 15%, respectively, of those of control shoots grown in the absence of the inhibitor. A marked inhibition of growth by gabaculine has also been reported for oats (8). Table I also shows the effect of gabaculine on the amount of extractable heme in wheat leaves. Gabaculine clearly decreased the amount of heme which can be extracted from the shoots. However, heme synthesis seemed to be less sensitive than growth to inhibition by gabaculine. Shoots grown in the presence of 0.5 mM gabaculine contained 30% of the heme present in control shoots. However, gabaculine appeared to have little, if any, effect on the relative protein content of the shoots as shown by electrophoresis of shoot extracts. Tracks loaded with 50 μL of shoot extract (200 mg tissue) showed similar banding patterns of the same intensity when stained with Coomassie blue over the whole range of gabaculine concentrations examined (data not shown).

The amount of holocytochrome *f* in shoots treated with various concentrations of gabaculine was determined from difference spectra recorded between 538 and 580 nm. The results are presented in Table I. Shoots treated with 0.5 mM gabaculine contained 33% of the holocytochrome *f* present in untreated shoots, indicating that gabaculine inhibition of

Table I. *Effect of Gabaculine on Etiolated Wheat Seedlings*

Wheat seedlings were germinated in complete darkness on agar containing three different concentrations of gabaculine for 7 d. Length and fresh weight measurements were made on shoots after excision at the junction with the seed. Heme content of 500 mg of shoot tissue was determined spectrophotometrically (28). Cyt *f* content of membranes from 3 g tissue was determined spectrophotometrically (3). The total amount of Cyt *f* polypeptide was detected immunochemically on Western blots of polyacrylamide gels loaded with 50 μL protein extracts (equivalent to 200 mg tissue), and the amount of holocytochrome *f* was detected by staining identical gels for heme-associated peroxidase activity (26). For the latter two measurements, results are expressed as percentages of the amounts in control plants. Where appropriate, results are given as mean ± SE with number of determinations in parentheses.

	Gabaculine Concentration, mM			
	0	0.1	0.25	0.5
Shoot length, mm	69 ± 5 (12)	23 ± 3 (34)	12 ± 1 (70)	10 ± 1 (84)
Shoot fresh weight, mg	74 ± 7 (12)	31 ± 4 (34)	13 ± 1 (70)	11 ± 1 (84)
Heme content, nmol/g fresh wt	3.53 ± 0.45 (3)	1.74 ± 0.11 (3)	1.56 ± 0.09 (3)	1.15 ± 0.01 (3)
Cyt *f*, pmol/g fresh wt	45	17	21	15
Cyt *f* polypeptide, %	100	83 ± 12 (4)	75 ± 2 (4)	42 ± 5 (4)
Heme-peroxidase activity, %	100	56 ± 7 (4)	22 ± 3 (4)	11 ± 4 (4)

heme associated with Cyt *f* was similar to that of total extractable heme. A more extensive investigation of the effect of gabaculine on the nature of the Cyt *f* polypeptide was carried out by comparing Western blots probed with antibodies to charlock Cyt *f* with SDS-polyacrylamide gels stained for heme-associated peroxidase activity. The Western blot gives a measure of the total amount of Cyt *f* including both the apo- and the holoproteins, whereas the gel stained for heme-associated peroxidase activity detects only the holoprotein. The results of this study are presented in Figure 1. The antibodies recognized a single polypeptide of 38 kD which corresponded to the size of the mature Cyt *f* polypeptide in pea (7, 29). There was no indication of a slower mobility protein corresponding to the precursor of Cyt *f*. The precursor and mature protein of pea Cyt *f* differ in size by 4 kD and are easily separated by SDS-gel electrophoresis (24, 29). With increasing gabaculine concentration there is a decrease in the total amount of immunochemically detectable Cyt *f* polypeptide, but the effect of gabaculine on the amount of the holocytochrome was much greater. To quantify these results, the autoradiograms of Western blots and the gels stained for heme-associated peroxidase activity were scanned on a densitometer. The results of four independent experiments are given in Table I as a percentage of the control tissue. This shows clearly that gabaculine inhibits the accumulation of holocytochrome *f* to a greater extent than apocytochrome *f* accumulation.

The addition of 5-aminolevulinate to the growth medium was able to reverse the inhibitory effect of gabaculine on the accumulation of holocytochrome *f* detected by its heme-associated peroxidase activity on SDS-PAGE. In an experiment in which 0.25 mM gabaculine decreased heme-peroxidase activity to 27% of that in control tissue, the addition of 5-aminolevulinate at concentrations of 0.1 mM, 0.5 mM, and 1 mM, in the presence of 0.25 mM gabaculine, increased the heme-peroxidase activity to 103, 98, and 98%, respectively, of the activity in control tissue. However, the addition of 5-aminolevulinate was not fully able to reverse the inhibitory effect of gabaculine on shoot growth. In this experiment, 5-aminolevulinate was able to restore shoot weight to only 63% of that of control tissue. The inability of 5-aminolevulinate to reverse fully the gabaculine inhibition of seedling growth, while fully reversing the inhibition of holocytochrome *f* accumulation, may indicate that gabaculine has inhibitory effects in the plant on processes other than tetrapyrrole synthesis.

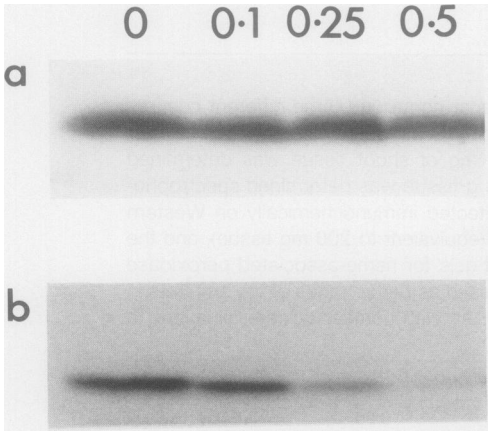

Figure 1. Detection of Cyt *f* polypeptide in membranes of wheat shoots. (a) Total Cyt *f* polypeptide detected on Western blots by incubation with antibodies to charlock Cyt *f* and ^{125}I-protein A. (b) Holocytochrome *f* detected by staining gels for heme-associated peroxidase activity. Gels were loaded with 50 μL of protein extract (equivalent to 200 mg of tissue).

DISCUSSION

The present study has clearly demonstrated that the synthesis and proteolytic processing of Cyt *f* are not tightly coupled to the availability of heme. A recent study of gabaculine effects on the photosynthetic apparatus of *Lolium temulentum* has produced a similar conclusion (6). This is in marked contrast to the synthesis of mitochondrial Cyt c_1, which requires heme addition before the final proteolytic processing step to generate the mature polypeptide in yeast and *Neurospora* (10, 21, 22). In the absence of heme, either in heme-deficient mutants or by the use of levulinic acid as an inhibitor of heme synthesis, yeast accumulates the intermediate precursor form of Cyt c_1 (10, 22). In the present investigation, Cyt *f* from both untreated and gabaculine-treated wheat shoots migrated on SDS-polyacrylamide gels with an apparent mol wt of 38,000, which corresponds to the mol wt of the mature protein (7, 29). No higher mol wt precursor form of Cyt *f* was ever detected on any of the Western blots of protein from gabaculine-treated plants, indicating that Cyt *f* can be processed to the mature size in the absence of heme.

The absence of tight coordination between heme availability and Cyt *f* synthesis is similar to the lack of coordination between phytochrome apoprotein and chromophore synthesis (8, 14, 18). In oats and pea seedlings, gabaculine has been used to inhibit synthesis of the linear tetrapyrrole chromophore (8, 14, 18), but the synthesis of the phytochrome apoprotein is inhibited to a much smaller degree. This lack of coupling between tetrapyrrole and apoprotein synthesis, however, appears not to be a general rule in plants. Werck-Reichart *et al.* (28) have reported a tight coordination between heme and apocytochrome P-450 synthesis in artichoke tubers. Apocytochrome P-450 was not detectable when heme was decreased to 11% of the amount found in control tissue by treatment with 1 mM gabaculine (28). The accumulation of Chl apoproteins in chloroplasts is also dependent on the synthesis of Chl *a*. The synthesis of Chl *a* is required for the accumulation of several chloroplast-encoded Chl *a*-binding proteins in barley chloroplasts (16, 17), and Chl *a* is required to stabilize the apoprotein of the nuclear-encoded light-harvesting Chl *a*/*b*-binding protein in pea and barley (2, 4).

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