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Phytoene Desaturase, the Essential Target for Bleaching Herbicides[1]

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Abstract. **Many bleaching herbicides with different core structures inhibit phytoene desaturase (PD), a membrane-bound enzyme in the carotenogenic pathway catalyzing the hydrogen abstraction step at the first C_{40} precursor of β-carotene. Prospects are good that new PD-active herbicides will be discovered by screening for bleaching activity. Accordingly, interest in PD enzymology and molecular genetics has increased. Although active carotenogenic cell-free systems are available, no isolation of PD has been achieved since the enzyme cannot be detected in its isolated form due to complete loss of activity. A portion of the *Rhodobacter* PD gene was incorporated into an appropriate plasmid which could be expressed in *E. coli*. This system was used to produce an antibody specific against PD from higher plants as well as *Rhodobacter*. All PDs assayed had an apparent molecular weight of 52 to 55 kDa. A *Rhodobacter* gene probe hybridized with a 3.1 kb*Bam*H I fragment from *Aphanocapsa* which allowed us to sequence the PD gene from this cyanobacterium. Its DNA sequence matched with the apparent molecular weight of the PD band in the western blot, and a fusion-gene product was found to be immunoreactive with the *Rhodobacter* PD antibody, *Anacystis* mutants were produced exhibiting cross-resistance against norflurazon and fluorochloridone. Apparently, this resistance is due to an altered PD with concurrent decrease of inhibitor binding affinity. Cloning of the resistant gene into the wild type is in progress.**
Additional index words. **Herbicide resistance, cell-free carotenogenic assays, phytoene desaturase.**

THE CAROTENOGENIC PATHWAY

Chlorophylls and carotenoids are formed by plant-specific pathways, and interruption of biosynthesis by inhibitors causes bleaching of cells and plant foliage (4, 25). Carotenogenesis is affected by many agrochemicals. Carotenoids, namely carotenes and xanthophylls, play an essential role in photosynthesis by protecting chlorophyll against photooxidative destruction by singlet oxygen. This highly reactive oxygen species is formed in excess through excited chlorophyll when the photosynthetic machinery (electron transport and subsequent CO_2 assimilation) operates too slowly to accommodate strong light. Protection against singlet O_2 is achieved when nine or more double bonds are present in the carotenoid molecule (13) as is the case with lycopene, β-carotene, or with the xanthophylls. These molecules physically interact with triplet state chlorophyll and dissipate its energy as heat. If a herbicide prevents carotene and xanthophyll formation, chlorophyll – although formed – will not accumulate either. Consequently leaves emerging after herbicide treatment will be depleted of all colored plastidic pigments.

The mode of action of bleaching herbicides is inhibition of formation of carotenes. Thylakoids having an intact pigment inventory before herbicide treatment are not affected by application of the bleaching herbicide. Typically, bleaching occurs only in newly formed leaves as is well known to herbicide "screeners" in the greenhouse. This phenomenon is in contrast to peroxidative, degradative herbicides like *p*-

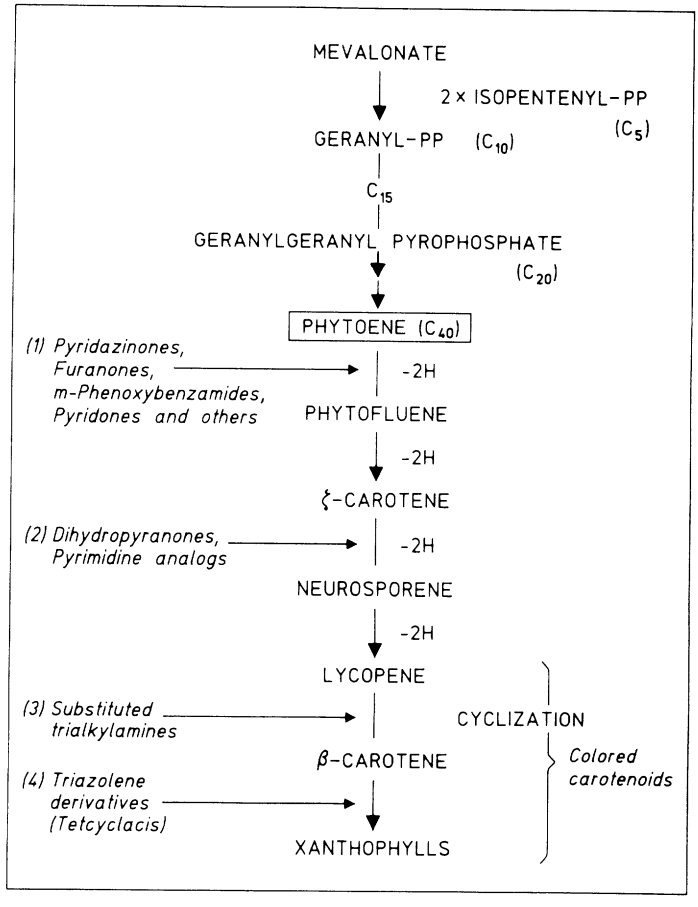

Figure 1. Scheme of the carotenoid biosynthesis pathway and sites of inhibition among the membrane-bound carotenogenic enzymes. The four groups of interfering compounds indicated affect hydrogen abstraction (1, 2), cyclization (3), and xanthophyll formation (4).

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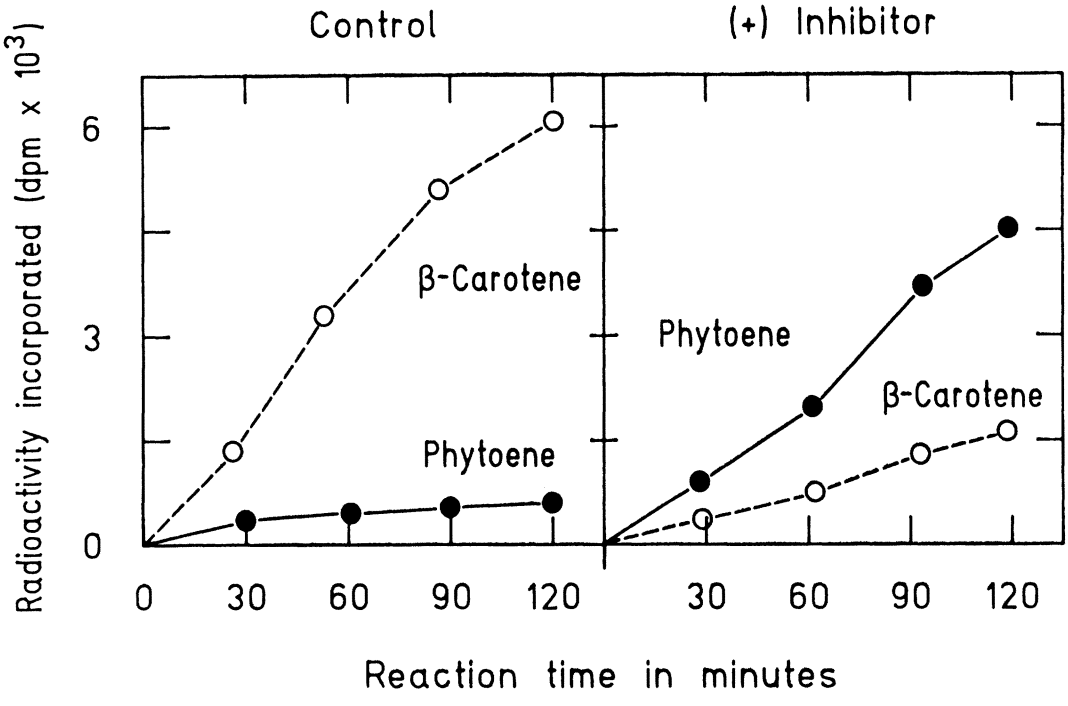

Figure 2. ^{14}C incorporation from mevalonate into phytoene and β-carotene using thylakoids from *Aphanocapsa*. ^{14}C-phytoene was generated with a cell extract of *Phycomyces* C5 car B10(–) present in the same reaction mixture (5, 10). This figure illustrates how a phytoene desaturase inhibitor changes ^{14}C flow. The increase of the dpm ratio of ^{14}C-phytoene to ^{14}C-β-carotene provides a measure of inhibition. (See text for refs.)

nitrodiphenyl ethers (15) or cyclic imides (21). Such compounds interfere with chlorophyll biosynthesis and the accumulating tetrapyrrole precursor(s) leads to rapid radical-induced degradation of membrane constituents including disappearance of plastidic pigments already present in the cell.

The first step in the biosynthesis of carotenoids is the head-to-tail condensation of two molecules of geranylgeranyl pyrophosphate to prephytoene pyrophosphate which is arranged to 15-*cis* phytoene after dephosphorylation (Figure 1). The subsequent pathway to β-carotene involves four desaturation and two cyclization steps. The colored carotenes and xanthophylls are mostly *trans* isomers. Desaturation procedure means a successive formation of conjugated double bonds, alternating from left to right of the (symmetric) center of phytoene (7), and shifting light absorbance from the ultraviolet (phytoene, peak at 286 nm) to the blue (β-carotene, peak at 448 nm; see ref. 24 for spectra of β-carotene precursors). Phytoene has three conjugated double bonds (a "triene") while β-carotene and the xanthophylls have eleven.

Phytoene is dehydrogenated by phytoene desaturase (PD)[3], a membrane-bound enzyme of the thylakoid which can be solubilized by detergents but rapidly loses activity (6). The phytoene desaturase reaction produces ζ-carotene with

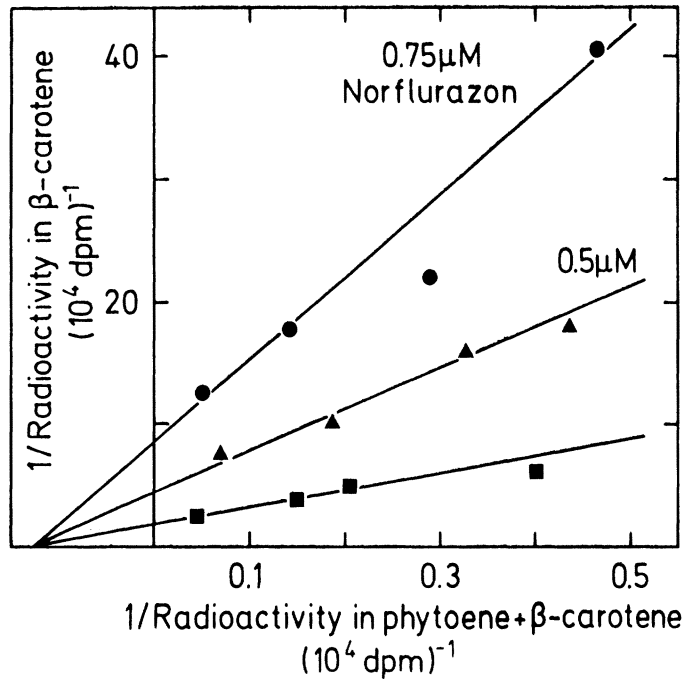

Figure 3. Double-reciprocal plot of the kinetics of ^{14}C incorporation from labeled phytoene into β-carotene using *Anacystis* membranes (28). The radioactivity in both phytoene and β-carotene represents the substrate (phytoene) concentration used for phytoene desaturase. ^{14}C-β-carotene is part of the original phytoene substrate but already converted by the dehydrogenation reaction. The lower line is the control.

[3]Abbreviations: PD, phytoene desaturase; kDa, kilodalton; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

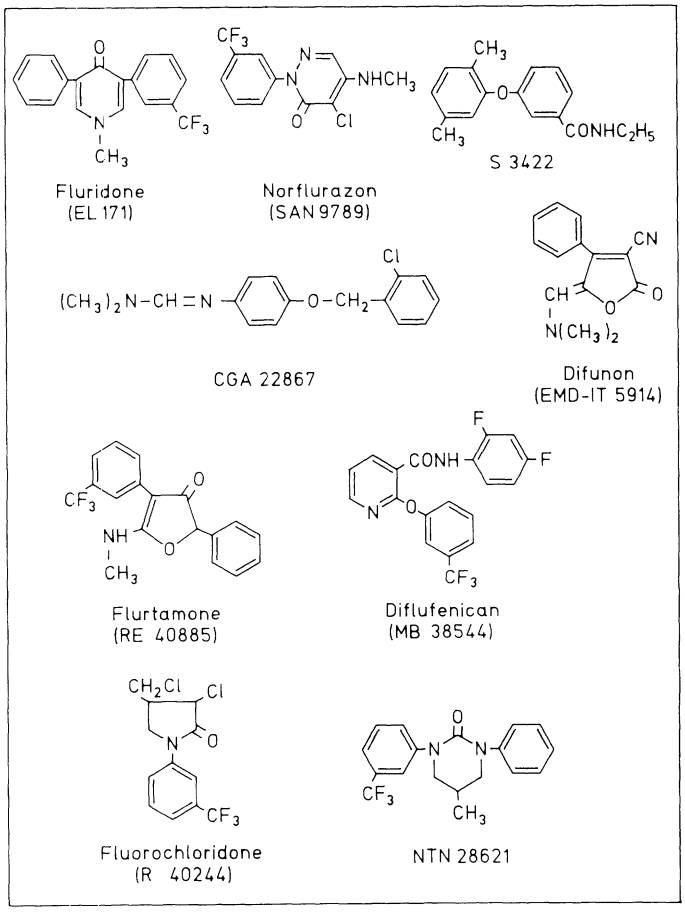

Figure 4. Structures of bleaching compounds that have been shown to inhibit phytoene desaturase (4, 9, 18, 24, 26, 29, and unpublished results). The benzoylcyclohexanediones and the benzoylhydroxypyrazoles are not shown here. Although they induce accumulation of phytoene in treated radish (32) and soybean leaves (19), inhibition of PD has not yet been successfully proven.

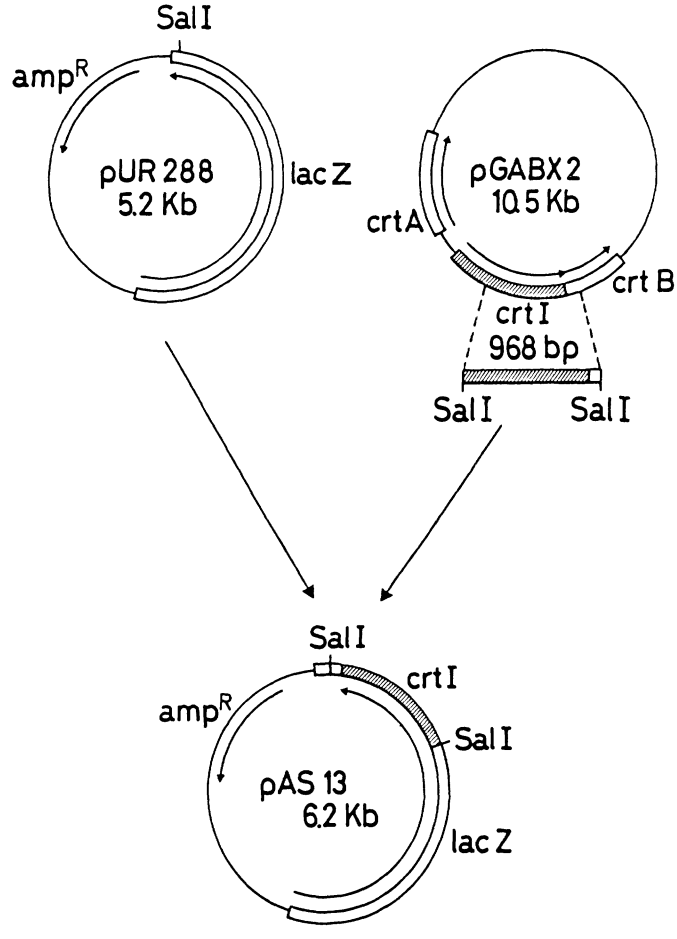

Figure 5. The plasmid pAS13, developed from pGABX2 and pUR288. Part of the crt I gene (which is a part of the phytoene desaturase from *Rhodobacter capsulatus*) was fused N-terminal to the *lacZ* gene of pUR288. This part is equivalent to 921 base pairs (307 amino acids), out of 1575 base pairs of the complete crt I gene. A *Sal* I fragment was used to construct this plasmid. From Schmidt et al. (31).

phytofluene as an intermediate. Inhibitor studies indicate that ζ-carotene desaturation is different from phytoene desaturation (9). Also the next membrane-bound enzymes in the pathway interact with different inhibitors (4, 24).

THE CELL-FREE CAROTENOGENIC SYSTEM

This laboratory was instrumental in developing active carotenogenic cell-free systems demonstrating the site of inhibitors of carotenoid formation along the biosynthetic pathway (9, 26). These systems have used isolated thylakoids from the blue-green algae *Aphanocapsa* (=*Synechocystis*) or *Anacystis* (=*Synechococcus*) that yield β-carotene and even xanthophylls (5, 10, 23, 27). *Chenopodium* chloroplasts were also found active. (See Table 3 of ref. 25.) To assay the membrane-bound enzymatic steps of carotenogenesis ^{14}C-labeled geranylgeranyl pyrophosphate and phytoene are the appropriate starter substrates. These are generated by the carotene-deficient mutant *Fusarium moniliforme SG 4* or *Phycomyces blakesleeanus, C5*, respectively, using ^{14}C-mevalonate. Labeled geranylgeranyl pyrophosphate can also be chemically synthesized in the laboratory with difficulty. A carotenogenic system also has been developed from chromoplasts of daffodil petals, and inhibition of PD by bleaching herbicides demonstrated (18).

As shown in Figure 2, a PD inhibitor can be detected by determining the ^{14}C incorporation into phytoene and β-carotene. The ratio of ^{14}C present in both compounds is taken as a measure of inhibitory activity. Needless to say, precursors and products must be thoroughly purified by thin-layer chromatography (TLC)[3] or high-pressure liquid chromatography (HPLC)[3]. Cell-free studies have shown that inhibitors of phytoene desaturase interfere noncompetitively, although reversibly (28), with PD (Figure 3). As was demonstrated further, the inhibition constant (k_i) in the cell-free system was very similar to the I_{50} value determined with intact cells (17). This indicates that the concentration of a bleaching herbicide applied to an aqueous algae culture is in approximate equilibrium with the herbicide concentration

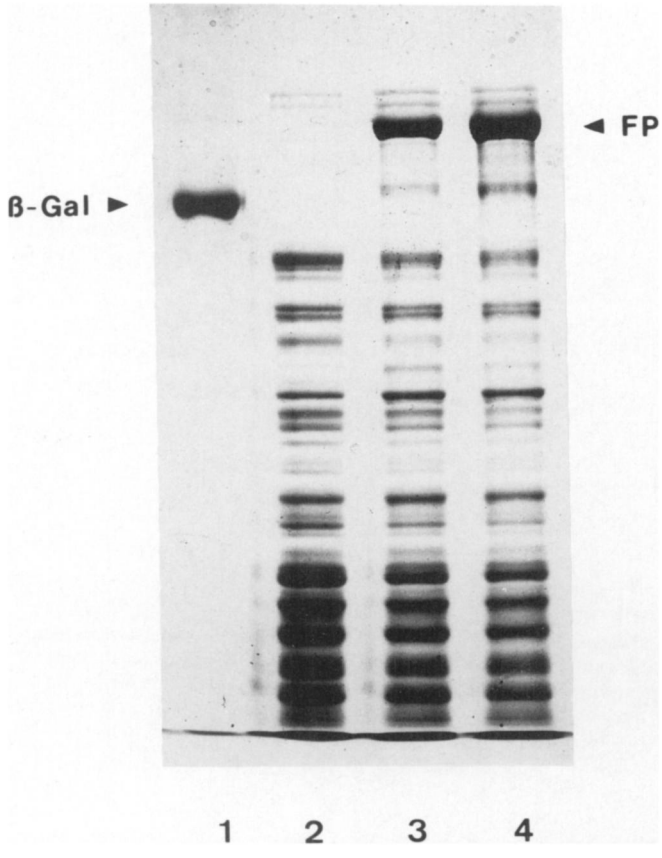

Figure 6. Expression of the fusion protein (FP) of the gene construct of Figure 5. *E. coli* JM 107/pAS13 was incubated with isopropylthiogalactoside (IPTG) to induce formation of the 150 kDa fusion protein after 3 h (lane 3) and 4 h (lane 4); lane 2 no inducer present. From (31). Lane 1, pure β-galactosidase of E. coli.

inside the cell. Thus our cellular algae systems represent reliable assays to determine the inhibitory activity of bleaching herbicides after 1 to 2 days of sterile cultivation (25). Using hexane extracts, the type of β-carotene precursor accumulated (phytoene, ζ-carotene, or lycopene) can be spectroscopically verified (24). Preliminary information on activity and inhibition site in the biosynthetic pathway is most conveniently generated with the intact algal cell. For enzymological studies, the more complicated cell-free carotenogenic system is applied. The cell-free system will also have the capacity to reveal whether a herbicide acts on more than one target in the cell as has been shown for fluometuron or fomesafen (4, 24). It should be emphasized that the carotenogenic pathway from mevalonate to β-carotene was affected identically by various inhibitors for both *Aphanocapsa* thylakoids and higher plant chloroplasts.

PHYTOENE DESATURASE INHIBITORS

Several inhibition sites along the carotenogenic pathway have been identified (indicated by horizontal arrows in Figure 1) including different dehydrogenation steps, the cyclization reaction, and formation of xanthophylls (i.e., introduction of hydroxyl groups into the carotene structure; see refs. 4, 25 for details). Compounds inhibiting steps 1) and 2) of Figure 1 have proven efficacy as herbicides. Undoubtedly, the chemical attack on the first dehydrogenation enzyme has been the most successful as yet for herbicide development. In Figure 4, active "lead" structures of bleaching herbicides from different chemical families are given, all which have been shown to inhibit phytoene desaturase and to cause the accumulation of phytoene in the cell. With such a diversity of inhibitors already known, it is likely that more will be found by random synthesis. Fluorochloridone (16), diflufenican (11, 26), and flurtamone (22, 29) recently have been introduced as new herbicides, and phytoene desaturase inhibition activity of the tetrahydropyrimidinone NTN 28621 has recently been published (3). (For references on the older compounds see ref. 25.)

Table 1. Inhibition of phytoene dehydrogenation in photosynthetic membranes from *Aphanocapsa* (*Synechocystis*) by the antibody against a phytoene desaturase fusion protein (encoded by part of the crt I gene from *Rhodobacter* and the β-galactosidase gene from *E. coli*).

Fractions, (+, −) antibody	Incorporation of ^{14}C			
	Phytoene	ζ–Carotene	Lycopene	β–Carotene
	dpm × 10^2			
Membrane fraction (A):				
(−) antibody	147.9	76.6	Traces	138.8
(+) antibody	149.5	50.1	Traces	145.3
Solubilized fraction (B):				
(−) antibody	5.3	21.5	ND	ND
(+) antibody	36.3	6.9	ND	ND

ND = nondetectable.

After Schmidt et al. (31).

CLONING OF THE RHODOBACTER crt I GENE

More detailed studies on the enzymatic and inhibitor-binding properties of phytoene desaturase are necessary. However, all attempts to isolate the protein from the membrane have failed to produce an active enzyme (6), and the enzyme could not be identified during the preparation steps. Therefore, we used a molecular-cloning approach to get access to the PD gene and its product, the phytoene desaturase. The genes of bacterial carotenoid biosynthesis for the purple bacterium *Rhodobacter capsulatus* are clustered in an 11-kb region (33, 34); the function of several genes has been identified (2, 14) including PD (crt I gene). Other genes encode the prephytoene pyrophosphate synthetase (crt B gene) and the phytoene synthase (crt E gene) (2). Recently, the DNA sequence of the clustered genes has been determined (1). The crt I gene was subcloned into pBR325 resulting in plasmid pGABX2 (1), and a 968 base-pair (bp) fragment (obtained by *Sal* I digestion of that plasmid) was ligated into pUR288 at the 3′-end of its *lac*Z gene (Figure 5;

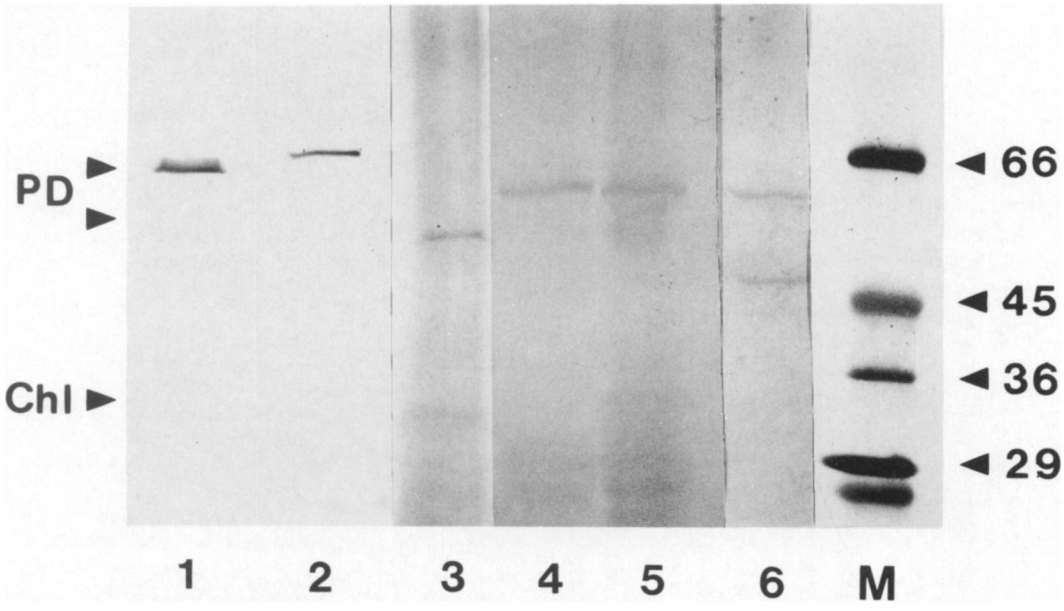

Figure 7. Immunoblots using the antibody against the PD/β-galactosidase fusion protein (Figure 6) with solubilized homogenates from *Rhodobacter capsulatus* (lane 1) *Aphanocapsa* PCC 6714 (lane 2), solubilized thylakoids from *Bumilleriopsis filiformis* (lane 3), *Brassica napus* (rape) (lane 4), and *Spinacia oleracea* (lanes 5, 6); markers (M) are at the right (31). *Bumilleriopsis* yielded a band with an apparent molecular weight of 55 kDa while thylakoids from higher plants, *Aphanocapsa*, and *Rhodobacter* gave 54–65 kDa bands. After freezing and thawing of the samples however, a second band at approximately 52 kDa appeared as shown in lane 6 for thylakoids from spinach. Gel runs are from different experiments.

ref. 31). The fragment in the new vector pAS13 contained 921 bp, i.e. 60% of the PD gene of *Rhodobacter*. This fusion-gene construct could be propagated in an appropriate *E. coli* strain and expressed after induction with isopropylthiogalactoside (IPTG) (Figure 6). The stable 150-kilodalton (kDa)[3] product was electroeluted and a rabbit antibody produced. As shown in Figure 7 the immunodection of PD was positive and monospecific with solubilized protein fractions from *Rhodobacter* and *Aphanocapsa*, and solubilized thylakoids from *Bumilleriopsis, Spinacia*, or *Brassica*, although the immunoreactivity was different. The antibody also was reactive with β-galactosidase. Controls with an antiserum against (bacterial) β-galactosidase were negative in our samples. An apparent molecular weight of 64 kDa was found except for PD from the xanthophycean alga *Bumilleriopsis* which yielded a 55 kDa band. After storing the membranes from other species for some time in the deep freeze (–20 C) with subsequent thawing, two bands were detected, one of them exhibiting an approximate 52 kDa apparent molecular weight (in Figure 7 documented for spinach). It appears that a "linking peptide" of about 10 kDa is tightly associated with the PD and requires a harsher treatment to separate it from the enzyme except in the case of *Bumilleriopsis*.

Table 1 demonstrates interaction of the fusion-gene antibody with the cell-free carotenogenic system. Using the membrane fraction of *Aphanocapsa* (part A of Table 1) formation of phytoene and β-carotene (and ζ-carotene) is not influenced by the antibody. Apparently, it has no access to the reaction site. However, a gentle solubilization with Tween 40 (1%, incubation of membranes for 30 min, 0 C, yielding a supernatant after centrifugation) decreases the control rates and abolishes β-carotene formation, but renders the remaining activity sensitive to the antibody as can be seen by the phytoene accumulation and decreased ^{14}C incorporation into ζ-carotene (part B of Table 1). The good cross-reactivity of our antibody between purple bacterium and spinach points to an enzyme with a conserved structure. Attempts are underway to isolate the enzyme from spinach, and the long-term goal is identification of the higher plant gene. *Bam*H I restriction fragments obtained from chromosomal DNA of *Aphanocapsa* PCC 6714 were hybridized with the *Rhodobacter* PD gene, and a corresponding cyanobacterial gene could be isolated and characterized (30). Aphanocapsa is the photosynthetic cyanobacterium that yielded the first active carotenogenic thylakoid system described above.

MUTANTS RESISTANT TO PHYTOENE DESATURASE INHIBITORS

Mutants were produced from *Anacystis* (*Synechococcus* PCC 7942) and selected for tolerance to various bleaching herbicides including fluridone, difunon, norflurazon, and fluorochloridone. A mutant NFZ 4 exhibits a high degree of resistance to both norflurazon and fluorochloridone but not to fluridone. The resistance factors (=ratio I_{50} value or k_i of the mutant divided by I_{50} or k_i of the wild type) were found as 36 for norflurazon and 32 for fluorochloridone, respectively, and about identical with both intact cells and isolated thylakoids (17). Obviously, the PD reaction itself is resistant to the

bleaching herbicides in those mutants. Another norflurazon-resistant mutant (with a high resistance factor of 76) shows little cross-resistance against fluorochloridone. Characterization of these mutants is underway. The findings on cross-resistance of the PD, although preliminary at the moment, resemble those of the D1 peptide, the membrane-bound herbicide-binding protein of the photosystem-II core complex (20). Apparently, in both cases inhibitors from different chemical families bind at the same region of the polypeptide and interact, at least in part, with the same amino acid residue(s), while other compounds inhibit PD by attaching to other amino acids.

The first cloning of the gene from the NFZ 4 mutant into the wild type by genetic complementation has been accomplished, although the high resistance factor of the mutant has not yet been attained (8).

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