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Xanthophyll Biosynthesis

CLONING, EXPRESSION, FUNCTIONAL RECONSTITUTION, AND REGULATION OF β-CYCLOHEXENYL CAROTENOID EPOXIDASE FROM PEPPER (*CAPSICUM ANNUUM*)*

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Pepper (*Capsicum annuum*) β-cyclohexenyl xanthophyll epoxidase cDNA was cloned and the corresponding enzyme overexpressed and purified from *Escherichia coli*, for investigation of its catalytic activity. The recombinant protein did not directly accept NADPH for epoxidation of cyclohexenyl carotenoids, nor did it operate according to a peroxygenase-based mechanism. Instead, the reducing power of NADPH was transferred to the epoxidase via reduced ferredoxin as shown by reconstitution of epoxidase activity in the presence of NADPH, ferredoxin oxidoreductase, and ferredoxin. Bacterial rubredoxin could be substituted for ferredoxin. The pepper epoxidase acted specifically on the β-ring of xanthophylls such as β-cryptoxanthin, zeaxanthin, and antheraxanthin. The proposed reaction mechanism for epoxidation involves the formation of a transient carbocation. This characteristic allows selective inhibition of the epoxidase activity by different nucleophilic diethylamine derivatives, *p*-dimethylaminobenzenediazonium fluoroborate and *N,N*-dimethyl-2-phenylaziridinium. It was also shown that the epoxidase gene was up-regulated during oxidative stress and when chloroplasts undergo differentiation into chromoplasts in pepper fruit.

Carotenoids serve as accessory pigments in the capture of photon energy (1) and efficiently quench the deleterious effects of triplet chlorophyll and singlet oxygen (2). Carotenoid epoxides, known to occur in plants and alga, display additional roles. First, the cylic deepoxidation of violaxanthin and epoxidation of zeaxanthin represent key mechanisms in the adapting plants and green alga to high light intensity (3, 4). Second, xanthophyll epoxides serve as precursors of the plant hormone abscisic acid (5). Finally when xanthophyll epoxides are converted to the ketoxanthophylls capsanthin and capsorubin, they yield the red color of ripe pepper fruits that tracks the transformation of chloroplasts into chromoplasts (6).

Although molecular oxygen is used for the conversion of carotenes into xanthophylls, the component proteins involved in the formation of 5,6-epoxy carotenoids have not yet been identified (3, 7) as opposed to the deepoxidase, which has been enzymatically characterized (8, 9). A cDNA encoding zeaxanthin epoxidase has been cloned from *Nicotiana plumbaginifolia* using insertional mutagenesis (10). Using this probe, we have cloned and expressed the corresponding carotenoid epoxidase from pepper (*Capsicum annuum*). In this paper, we address the questions concerning the organization of the component proteins responsible for the epoxidation of cyclohexenyl carotenoids, their specificity for different substrates and the effect of different amine derivatives on the potential regulatory control of this enzyme. We also show that the cyclohexenyl epoxidase is subject to developmental and stress regulation at the gene and protein level.

EXPERIMENTAL PROCEDURES

Plant Materials—Pepper plants (*C. annuum* L. cv. Yolo Wonder) were grown under controlled greenhouse conditions until fruits ripened, as characterized by the red color change of the fruits.

Chemical Inhibitor Synthesis and Test—The electrophilic reagents *N,N*-dimethyl-2-phenylaziridinium (DPA)[1] and *p*-dimethylaminobenzene diazonium fluoroborate (DDF) were prepared, respectively, as described previously (11, 12). 2-Diethylaminoethyl-3,4-methylphenylether (MPTA) and 2-(4-chlorophenylthio)triethylamine (CPTA) were a gift from Dr. H. Yokoyama, United States Department of Agriculture, Pasadena, CA. A known concentration of stock solution of CPTA and MPTA was added directly into the reaction medium described below. For affinity inhibition, 25 μg of purified epoxidase in 50 mM potassium phosphate buffer (pH 7.6) were incubated with 25 μM DPA or DDF for the indicated times as specified in the text. In the latter case the mixture was irradiated with 295-nm or 410-nm light provided by a Spectroline lamp for different times as described previously (13). Following DPA and DDF treatment, the mixture was rapidly filtered through an Eppendorf tube containing Sephadex G50 to remove excess reagents. The filtrate was then added to the reaction mixture described below, in order to determine residual epoxidase activity.

Preparation of Plastids—Plastids were isolated as described previously (14), except that in some cases a mixture of protease inhibitors containing bestatin (100 μM), leupeptin (100 μM), aprotinin (1 μM), and phenylmethylsulfonyl fluoride (1 mM) were added to the extraction and purification buffers.

Preparation of Carotenoid Substrates—β-Cryptoxanthin and antheraxanthin were isolated, respectively, from ripe fruits of *Carica papaya* and *Mangifera indica*, while zeaxanthin was isolated from *Escherichia*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X91491.

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[1] The abbreviations used are: DPA, *N,N*-dimethyl-2-phenylaziridinium; CPTA, 2-(4-chlorophenylthio)triethylamine; DDF, *p*-dimethylaminobenzenediazoniumfluoroborate; HPLC, high-performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; MPTA, 2-(4-methylphenoxy)-triethylamine; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; LHCII, light-harvesting pigment-protein complex of photosystem II.

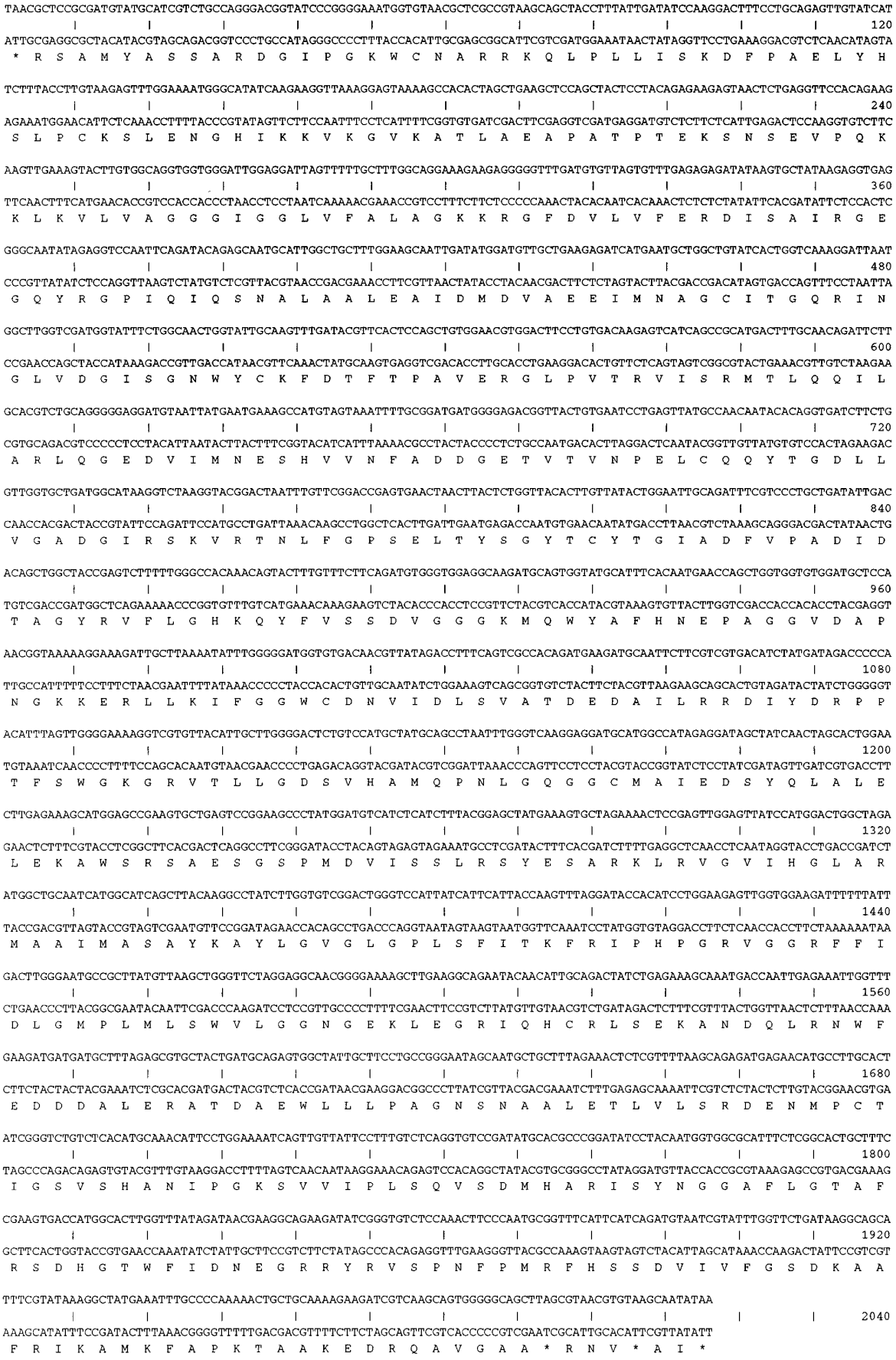

FIG. 1. **Nucleotide and predicted amino acid sequence of *C. annuum* β-cyclohexenyl epoxidase.**

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Cap-CarEpox   K V L V A G G I G G L V F A L A G K K R G F D V L V F E R
Yeast-SqEpox  D A I V I G A G V I G P C V A T G L A R K G K K V L I V E R
Rat-SqEpox    E V I I I G S G V L G S A L A T V L S R D G R T V T V I E R
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FIG. 2. **FAD binding domain of pepper epoxidase.** *A*, comparison of the FAD binding site of *C. annuum* β-cyclohexenyl epoxidase (*Cap-CarEpox*) with that of squalene epoxidase from *S. cerevisiae* (*Yeast-SqEpox*) (29) and *R. norvegicus* (*Rat-SqEpox*) (30). Identical amino acids are *boxed*.

coli expressing *Erwinia herbicola* carotenoid biosynthetic genes (15), and α-carotene was isolated from carrot roots. The normal chloroplast carotenoid β-carotene and lutein were isolated from pepper leaves. In each case the total lipid was extracted with acetone, and saponified according to standard procedures (16). Subsequently, a preliminary fractionation was achieved using a cellulose column (17) that was sequentially eluted with hexane, hexane/acetone (90/10, v/v) to elute, respectively, the carotene fraction and xanthophyll fractions excluding neoxanthin. Further purification of the xanthophyll fractions was carried out by preparative HPLC on a micro-BondaPak C_{18} column using methanol/acetone/water (90:17:3) as the elution solvent (18). The carotene fractions were purified using the same chromatographic adsorbent, except elution was carried out as described previously (19). The identity of the purified carotenoids was determined from published chromatographic and spectral data (16). Following fractionation, samples of each carotenoid solution were evaporated to dryness and stored under argon at −20 °C until used.

In Vitro Epoxidation of Carotenoids—The standard incubation medium contained in a final volume of 500 μl, 50 mM potassium phosphate buffer (pH 7.6), 5 μM FAD, 1 mM NADPH, 25 μM carotenoid substrate, 100 μM mixture of monogalactosyldiglyceride, and digalactosyldiglyceride (1:1) dissolved in 50 μl of acetone/methanol (1:1), 25 μg of spinach ferredoxin or rubredoxin (Sigma), as specified in the text, 25 milliunits of ferredoxin: $NADP^+$ oxidoreductase, 20 units of glucose-6-phosphate deshydrogenase, 2 mM glucose 6-phosphate, 500 μg of bovine serum albumin, and a definite amount enzyme. At the completion the reaction mixture was extracted with an equal volume of chloroform/methanol (2:1) as described previously before analysis by HPLC on a micro-BondaPak C_{18} column or a NovaPak C_{18} column using methanol/acetone/water (90:17:3).

cDNA Library Construction and DNA Techniques—A cDNA library was constructed using a random mixture of mRNAs from light and dark grown pepper seedlings and pepper fruits at green, intermediate, and red stage of ripeness. Total RNA was isolated from the frozen tissues according to a previously described procedure (20). $Poly(A)^+$ RNA was isolated using the PolyATtract® mRNA system (Promega). Five micrograms of $Poly(A)^+$ RNA were used to generate a λgt11 library using the Copy Kit for cDNA synthesis (Invitrogen). The ligated cDNA-vector was packed using the Gigapack Gold packaging extract (Stratagene). Subsequently, the screening procedure was carried out using standard procedures (21) using a radiolabeled *Hin*dIII insert (1360 base pairs) from *N. plumbaginifolia* (10). DNA from positive clones was obtained by PCR amplification using sense and reverse λgt11-specific primers, according to the program: 94 °C (2 min) and 35 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (2 min) followed by 72 °C (5 min). The inserts were isolated by digestion with *Not*I before cloning into pBluescript KS^+. For Northern blot analysis a pepper *Hin*dIII and *Pst*I insert (881 base pairs) was radiolabeled and used as a probe.

Expression of Pepper Epoxidase in E. coli and Purification—The epoxidase devoid of transit peptide sequence was expressed in *E. coli* using QIA*express* pQE vectors (Qiagen). To accomplish this, the sense oligonucleotide CGCGGATCCCGCCACACTAGCTGAAGCTCCAGC and the M13-20 Primer GTAAAACGACGGCCAGT were used to amplify the epoxidase by PCR using the program shown above. Following sequence verification, the PCR product was digested by *Bam*HI and *Sac*I and ligated to the pQE-31 vector. The resulting plasmid was used to transform *E. coli* JM109. Bacterial cells were cultured at 37 °C up to A_{600} of 0.6 before adding 1 mM IPTG. Following 4 h of culture, the cells were harvested and lysed by sonication in 50 mM Tris-HCl buffer (pH 7.6) containing 50 μM FAD. The resulting homogenate was adjusted to 6 M guanidium in the same buffer, before loading onto a metal affinity resin (TALON™, Clontech). The column was washed with the same buffer minus FAD, before eluting the epoxidase with 50 mM Tris-HCl buffer (pH 7.6) containing 50 mM imidazole. The epoxidase containing fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.6) containing 0.25% *n*-octyl-β-D-glucopyranoside and 10% glycerol. Further purification was achieved using a Mono Q column (HR5/20, Pharmacia Biotech Inc.) employing a linear gradient of 0 to 0.3 M NaCl in the same buffer containing 2% glycerol. The fractions containing pure protein were pooled, adjusted to 50% glycerol, and stored at −20 °C.

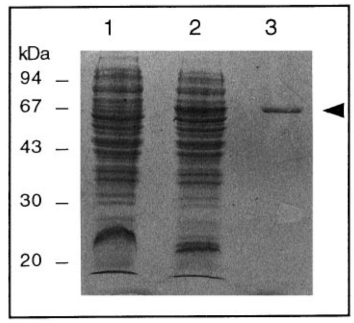


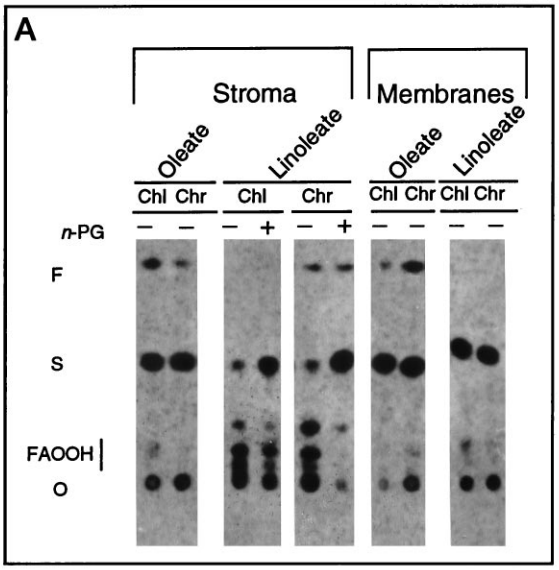
FIG. 3. **SDS-PAGE analysis of *C. annuum* β-cyclohexenyl epoxidase expressed in *E. coli*.** *Lane 1*, uninduced cells; *lane 2*, IPTG induced cells; *lane 3*, purified fractions of the β-cyclohexenyl epoxidase. The molecular mass markers are indicated on the *left*. The position of the β-cyclohexenyl epoxidase is indicated by the *arrow*.

Other Methods—Antibodies were prepared to the epoxidase expressed in *E. coli*, SDS-PAGE, and immunoblotting were carried out as described previously (22, 23). Protein content was determined as described previously (24). Unless otherwise stated the lipoxygenase activity was assessed using [1-^{14}C]oleic acid (56 mCi/mmol) and [1-^{14}C]linoleic acid (55 mCi/mmol) substrates. The 100-μl reaction mixture contained 50 mM Tris-HCl (pH 7.6), 25,000 cpm of oleate or linoleate, and plastid subfractions equivalent to 1 mg of protein. After incubation at 30 °C for 30 min, the reactions were terminated by the addition of 300 μl of acetone, and 100-μl aliquots were applied to silica gel plates developed with hexane:diethyl ether:formic acid (50:50:1). Radioactive spots were detected and quantified using a phosphorimaging system (Fuji). 13-Hydroperoxylinolenic acid was prepared using soybean lipoxygenase and linolenic acid as described previously (25) and quantified from its molar absorption 25,000 liters·mol^{-1} cm^{-1}.

RESULTS AND DISCUSSION

cDNA Cloning of β-Cyclohexenyl Carotenoid Epoxidase and E. coli Expression—The *N. plumbaginifolia* insert isolated by *Hin*dIII insert (1360 base pairs) was radiolabeled and used to screen the pepper cDNA library. Five positive clones were isolated and purified. Their sizes were determined by PCR using the λgt11 primers. The full-length clone (Fig. 1) encodes a protein having an approximate molecular mass of 72 kDa. The amino terminus contains several hydroxylated residues characteristic of other plastid transit peptides (26). The transit peptide could be cleaved in the region VKTLAE to yield the mature epoxidase. The amino terminus contains a consensus FAD binding domain (27, 28) typically found in yeast (29) and rat (30) squalene epoxidases (Fig. 2). The sequence does not contains the typical heme binding motif F*XX*G*XXX*C*X*G (31) or the modified heme domain P*X*V*X*NKQCAG observed in cytosolic (32) and plastidial (33) allene oxide synthases. These data suggest that epoxidation of plant carotenoids does not probably involves P450 cytochrome. Our deduced sequence carries three possible PEST domains (Pro^{253}–Thr^{265}, Ser^{404}–Ser^{416}, Asn^{514}–Glu^{523}) usually observed in rapidly degraded proteins (34). The hydropathy plot (35) of the deduced peptide sequence shows that this epoxidase is moderately hydrophobic. The pepper epoxidase shows 88% identity to the amino acid sequence of *N. plumbaginifolia* (10).

In order to characterize the gene product, the *Bam*HI and*Sac*I product from PCR amplification (see "Experimental Procedures") was cloned into pQE-31 vector, and the resulting



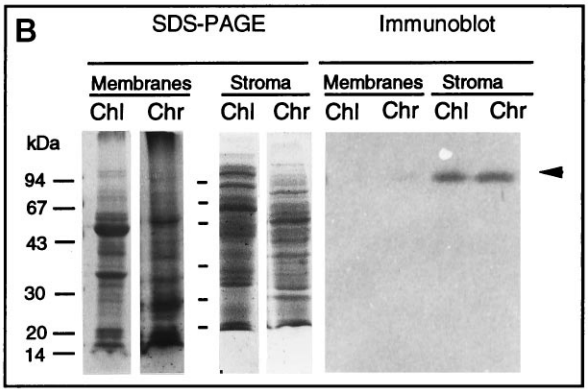


FIG. 4. **Hydroperoxide formation in isolated pepper plastid subfractions.** *A*, thin layer chromatography of the reaction products formed after *in vitro* incubation of the indicated plastid subfractions with [^{14}C]oleate or -linoleate. The abbreviations used for the different subfractions refer to chloroplast (Chl), chromoplast (Chr), *n*-propyl gallate used at 50 μM concentration (*n*-PG), and to the products: untransformed substrate (S), peroxidized fatty acid and related products (FAOOH), origin (O), and the solvent front (*F*). *B*, polyacrylamide gel and immunoblot analysis of lipoxygenase in different pepper plastid subfractions. Proteins were resolved by SDS-PAGE and stained with Coomassie Blue or electrophoretically transferred onto a nitrocellulose sheet before immunoblotting using antibodies raised against recombinant rice lipoxygenase. The *arrow* indicates the position of the plastid lipoxygenase.

plasmid was used to transform *E. coli*. When cell extracts derived from *E. coli* were analyzed by SDS-PAGE, a prominent band corresponding approximately to the molecular mass of the mature epoxidase (65 kDa) was observed following induction with IPTG (Fig. 3). This polypeptide was purified by affinity and Mono Q chromatography with a yield of 40% and was judged to be 95% pure.

Multicomponent Protein Characteristic of Cyclohexenyl Carotenoid Epoxidation—The ability of heterologously expressed and purified epoxidase to catalyze the epoxidation of zeaxanthin was tested in the presence of 1 mM NADPH and molecular oxygen. Under these minimal conditions, no enzymatic formation of epoxy derivatives was observed (results not shown). Two possible reasons for this were then proposed and evaluated. One was that a peroxygenase reaction was required. A second was that an additional electron transport system was needed for the reaction to proceed.

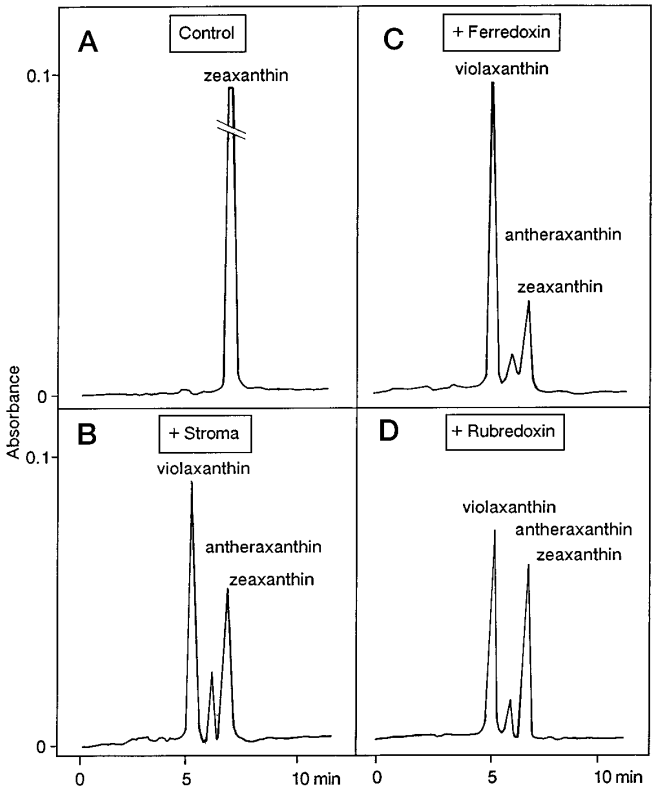


FIG. 5. **HPLC analysis of the reaction products obtained after incubation of *C. annuum* β-cyclohexenyl epoxidase with zeaxanthin.** *A*, reaction products obtained without redox proteins. *B*, reaction products obtained with stroma proteins. *C*, reaction products obtained with ferredoxin. *D*, reaction products obtained with rubredoxin. The incubation and analysis of products were carried out as described under "Experimental Procedures." HPLC detection was by absorption at 440 nm.

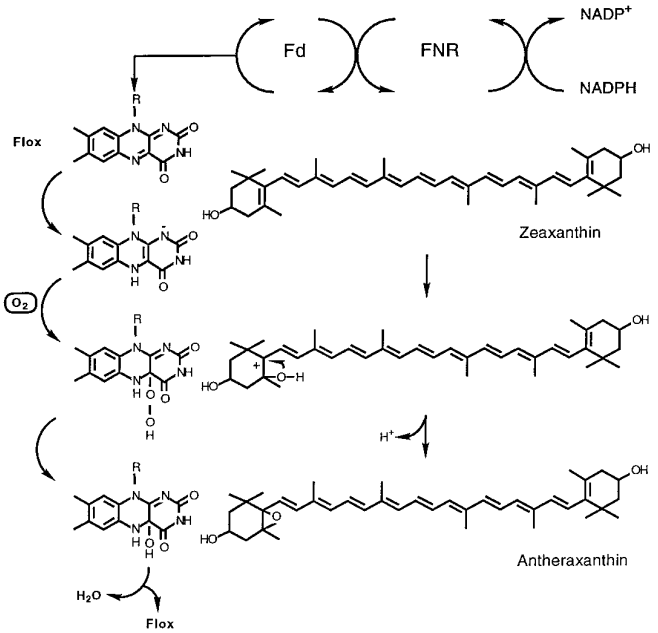


FIG. 6. **Redox cofactors involved in the monooxygenase activity of β-cyclohexenyl carotenoid epoxidase.**

A diagnostic feature of peroxygenase reaction is their need for a hydroperoxide-dependent co-oxygenation (36, 37). The formation of β-carotene 5,6-epoxide may, in fact, rely on such a reaction. Its formation has been observed following chemical

autooxidation by perooxyradicals (38). A similar reaction has been shown during the epoxidation of retinoic acid into 5,6-epoxy retinoic acid by soybean lipoxygenase-2 and -3 (39). In plastids, a prerequisite for this is an endogenous source of hydroperoxide. To evaluate this hypothesis, we examined the capacity of purified pepper plastids to produce hydroperoxylinoleic acid as a co-substrate. Data in Fig. 4*A* show the enzymatic conversion of linoleic acid into peroxylinoleic acid by plastid stroma from purified pepper chloroplast or chromoplasts. Substrate specificity of the reaction suggests that lipoxygenase is involved. The fact that oleic acid is not a substrate argues with other data indicating plant lipoxygenase involvement (40). This is supported by immunological data where antibodies to a putative plastidial rice lipoxygenase (41) positively reacted with the plastidial pepper lipoxygenase (Fig. 4*B*). Based on these evidences, our purified epoxidase was added to a reaction mixture containing exogenous hydroperoxylinoleic (25 μM) as a co-substrate. However, our data indicated that no epoxidation reaction occurred, suggesting that the biosynthesis of xanthophyll epoxides is not a peroxygenase-based mechanism.

Faced with these results we reasoned that zeaxanthin epoxidation could involve several other component proteins. It is noteworthy that squalene epoxidase requires a flavoprotein oxidase and a NADPH-cytochrome P450 reductase (42). We, therefore, attempted to reconstitute the pepper epoxidase by incorporating stromal proteins from pepper chromoplast into the reaction mixture. Under these conditions, a significant epoxidation of zeaxanthin into antheraxanthin and violaxanthin was observed (Fig. 5, *A* and *B*). As the boiled chromoplast stroma failed to sustain the epoxidation reaction, it also demonstrated that stromal protein cofactors were required. To further evaluate this, a reaction mixture was furnished with spinach ferredoxin and ferredoxin oxidoreductase and tested for epoxidase activity. Under these conditions, significant epoxidase activity was observed, as shown by the formation of antheraxanthin and violaxanthin (Fig. 5*C*). We, therefore, concluded that under *in vivo* conditions, the reducing power of NADPH is transferred to zeaxanthin via reduced ferredoxin. This fact is reinforced by the fact that bacterial rubredoxin can substitute for ferredoxin (Fig. 5*D*). The requirement of additional electron transporter is further demonstrated by the fact that *E. coli* synthesizing zeaxanthin could not convert zeaxanthin into antheraxanthin or violaxanthin when transformed with the pepper epoxidase under conditions where both promoters are compatible. In this context, one could note that *E. coli* ferredoxin cannot substitute for spinach ferredoxin (43). Our data suggest that the functioning of the β-cyclohexenyl epoxidase is coupled to the ferredoxin-ferredoxin oxidoreductase and photosystem I in chloroplasts. The same may occur in nongreen plastids, as both ferredoxin and ferredoxin reductase activities have been observed in nonphotosynthetic chromoplasts of red tomato fruits (44). A similar fact can be deduced from nonphotosynthetic bean sprouts (45) and radish roots (46). These data suggest that in nongreen plastids a ferredoxin-ferredoxin oxidoreductase pathway linked to the oxidative pentose phosphate cycle may provide the reducing power. In this context, it is of interest that a nonphotosynthetic ferredoxin gene has recently been characterized from *Citrus* fruit (47). This sequence revealed a typical plastid transit peptide and expression of its gene parallels the synthesis of carotenoid in *Citrus* chromoplasts.

The ability of molecules to be oxidized by hydroperoxide or peracid is a diagnostic feature of the involvement of hydroperoxyflavinilated enzymes in the oxidation of nucleophiles (48). This requirement is fulfilled by the cyclohexenyl ring of carotenoids (38). Therefore, cyclohexenyl carotenoid epoxidase can be classified as a monooxygenase that catalyzes the introduction of molecular oxygen in the presence of NADPH, ferredoxin, and ferredoxin-like reductase. Since the catalytic mechanism of flavoprotein monooxygenases (49) involves the formation of a flavin hydroperoxide enzyme intermediate, the resulting hydroperoxyflavin is thus cleaved to incorporate one oxygen atom into zeaxanthin, while the other is reduced into water (Fig. 6).

Substrate Specificity—The activity of the purified epoxidase was tested against several substrate carotenoids possessing ϵ rings (α-carotene and lutein) and β-rings (β-carotene, β-cryp-

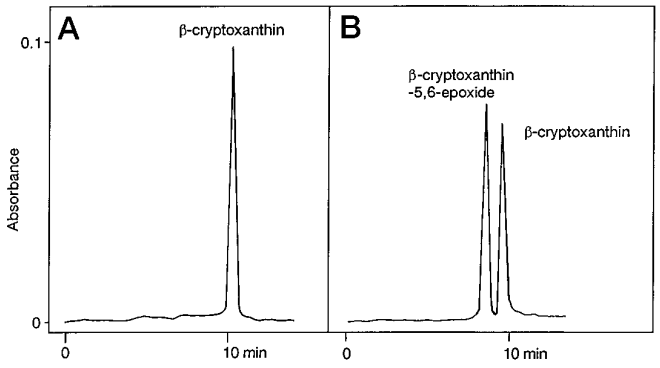


FIG. 7. **HPLC analysis of the reaction products obtained after incubation of *C. annuum* β-cyclohexenyl epoxidase with β-cryptoxanthin.** *A*, heat-denatured β-cyclohexenyl epoxidase. *B*, native β-cyclohexenyl epoxidase. The incubation and analysis of products were carried out as described under "Experimental Procedures." HPLC detection was by absorption at 440 nm.

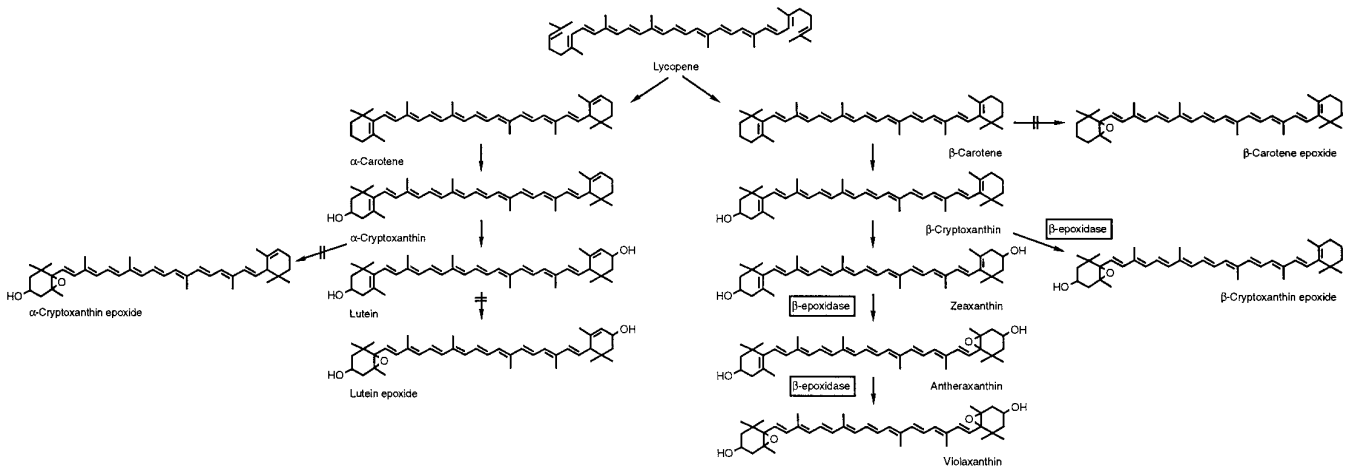


FIG. 8. **Enzymatic steps catalyzed by *C. annuum* β-cyclohexenyl epoxidase.**

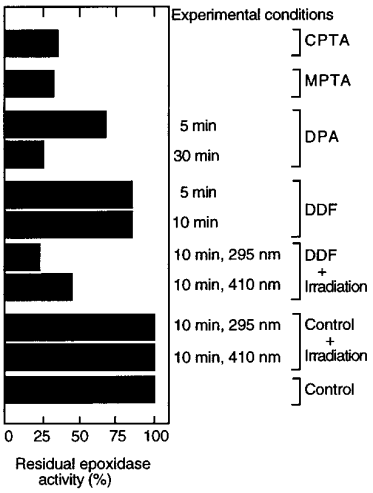


FIG. 9. **Regulation of *C. annuum* β-cyclohexenyl epoxidase by amine derivatives.** The incubations were carried out as described under "Experimental Procedures" using CPTA, MPTA, DPA, and DDF. The preincubation times were as indicated. In the case of DDF, the enzyme was irradiated either at 295 or 410 nm before incubation.

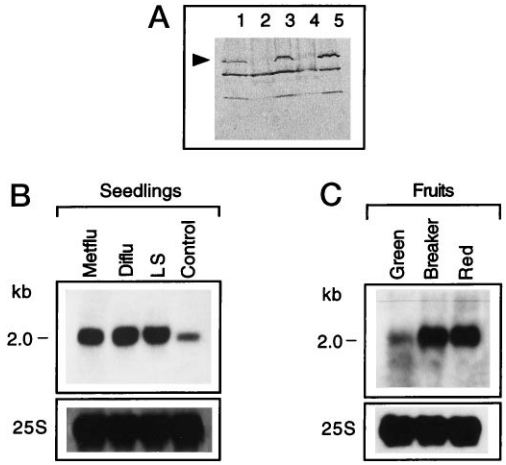


FIG. 10. **Immunoblot and Northern blot analysis of *C. annuum* β-cyclohexenyl epoxidase isolated from fruits and light-grown seedlings.** *A*, immunoblot analysis of β-cyclohexenyl epoxidase from pepper seedlings treated with different herbicides. Western blot was carried out using plastids isolated from control seedlings (*lane 1*) and 20 μM metflurazon (*lane 2*), 5 mM CPTA (*lane 3*), 20 μM LS 80707 (*LS*) (*lane 4*)-treated seedlings and chloroplast membranes from control seedlings (*lane 5*). The position of the mature β-cyclohexenyl epoxidase is indicated by the *arrow*. *B*, RNA gel blot analysis of the expression of the β-cyclohexenyl epoxidase gene from *C. annuum* seedlings. The different lanes refer to *C. annuum* seedlings treated with 20 μM metflurazon (*Metflu*), diflufenican (*Diflu*), LS 80707 (*LS*), and the control (*C*). *C. annuum* 25 S rRNA was used as a control to assess that equal amount of total RNA was blotted. *C*, RNA gel blot analysis of the expression of the β-cyclohexenyl epoxidase gene during the ripening of *C. annuum* fruits taken at green, breaker, and red stages.

toxanthin, and antheraxanthin). The obtained products were subjected to HPLC analysis. The results showed that monohydroxy β-carotene, β-crytoxanthin was epoxidized (Fig. 7, *A* and *B*) as was antheraxanthin while β-carotene was not epoxidized (results not shown). In this context, it is worth noting that β-carotene-5,6-epoxide has been detected in plants under photooxidative conditions. Its formation, however, seems to be nonenzymatic as the optically inactive isomer is detected (50). In examining the specificity of the purified epoxidase, it was observed that α-carotene and lutein, which have β,ϵ-rings, were not epoxidized (results not shown). Thus, the cloned epoxidase appears to be a β-cyclohexenyl epoxidase catalyzing the reactions depicted in Fig. 8.

Regulation of Carotenoid Epoxidase by Amine Derivatives—The epoxidation mechanism displayed in Fig. 6 involves a transient carbocation, which could allow the testing of potential regulators. Secondary and tertiary amine derivatives, which are protonated at physiological pH should compete efficiently with the carbocation generated during catalysis and, thus, inhibit epoxidase activity. These considerations were used in the design of sterol biosynthetic inhibitors (51, 52) and carotenoid cyclase inhibitors (53–55). Based on this, we tested the effect of diethylamine derivatives on epoxidase activity.

When 50 μM CPTA or MPTA were added into the incubation medium, we noted a 65–70% inhibition of epoxidase activity compared with the control (Fig. 9). This suggests that diethylamine derivatives display a novel effect on carotenoid biosynthesis that is reminiscent of nicotine inhibition. Previous data on photosynthetic bacteria show that the introduction of a hydroxyl group at the C1 of lycopene, which yields rhodopin (56), and at the C1 of neurosporene, which yields chloroxanthin (57), are both inhibited by nicotine. These reactions involve a transient carbocation. Thus, one could explain the inhibitory effect as due to the nitrogen atom of CPTA and the pyrolidinyl ring of nicotine (58), which are positively charged at physiological pH. These could compete with the transient carotenoid carbocation for the active nucleophilic amino acid residues of the epoxidase. If this assumption is valid, then irreversible blocking of the potential amino acid nucleophiles should also inhibit epoxidase activity. This was tested by using the electrophilic aziridinium (DPA) and the diazonium (DDF) cations that have been used to affinity label the active sites of antibodies (59), acetylcholinesterase (60) and acetylcholine receptor (13, 61). Preincubation of the purified epoxidase with DPA resulted in a strong inhibition of the enzymic activity (Fig. 9). As excess DPA was removed by gel filtration before determining the enzymic activity, the effect of DPA was due to alkylation of nucleophilic residues of the epoxidase. A similar observation was made with DDF. In the absence of irradiation DDF weakly inhibits the carotenoid epoxidase (Fig. 9). However, after irradiation at 410 or 295 nm, a strong inhibition was observed. These data suggest that reactive nucleophiles are involved in the enzymatic epoxidation of carotenoids. Further work is required to determine whether aromatic (Tyr, Trp, Phe) or the acidic (Glu and Asp) amino acid residues are responsible. It is noteworthy that these residues are highly conserved between the pepper and *N. Plumbaginifolia* epoxidases.

Compartmentation and Regulation of β-Cyclohexenyl Epoxidase during Plastid Development—It was shown previously that the light-harvesting pigment-protein complex of photosystem II (LHCII) is a zeaxanthin epoxidase (62). The deduced peptide sequence of pepper β-cyclohexenyl carotenoid epoxidase possesses a characteristic FAD binding domain (Fig. 2) that has also been observed in olefinic epoxidases (29, 30, 63). Since this pivotal sequence is missing in the peptide sequence of LHCII (64) and is known to be necessary for catalytic activity, one may conclude that LHCII has no epoxidase activity.

To obtain specific information on the compartmentation of β-cyclohexenyl epoxidase in plastids, polyclonal antibodies were raised against the protein expressed in *E. coli*. Antibodies specificity was verified using a histidine-tag reagent (65), which clearly indicated that the immunoreactive bands corresponded to the expressed protein as no potential contaminating proteins (66) were visualized. Additionally, the antibodies were affinity-purified (67) before probing the plastidial proteins. Our data revealed the presence of two immunoreactive bands corresponding to the predicted molecular mass (65 kDa) of the mature protein deduced from the cDNA and a lower molecular mass (58 kDa) which predominated (Fig. 10*A*). The epoxidase of pepper seedlings grown under light in the presence of carote-

noid inhibitors (Metflurazon, LS 80707, and CPTA) to induce a photooxidative stress displayed a very similar behavior. This partial proteolysis is supported by the presence of several putative PEST sequences (34) in β-cyclohexenyl, which are characteristic of rapidly degraded proteins.

Finally, it is significant that the expression pattern of the epoxidase gene in pepper seedlings treated with the different photooxidative stress-inducing herbicides, Metflurazon, Diflufenican, and LS80707, is nearly identical to that observed in ripening pepper fruits (Fig. 10, *B* and *C*). The latter process is characterized by an active formation and conversion of epoxyxanthophylls into ketocarotenoids (23), concomitantly to pepper chromoplast differentiation. It is interesting to note that abscisic acid, a product of epoxyxanthophylls, has been implicated previously (68) in chromoplast differentiation.

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