The Lycopene Cyclase CrtY from \textit{Pantoea ananatis} (Formerly \textit{Erwinia uredovora}) Catalyzes an FAD\textsubscript{red}-dependent Non-redox Reaction\textsuperscript{*§}

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The cyclization of lycopene generates provitamin A carotenoids such as \(\beta\)-carotene and paves the way toward the formation of cyclic xanthophylls playing distinct roles in photosynthesis and as precursors for regulatory molecules in plants and animals. The biochemistry of lycopene cyclization has been enigmatic, as the previously proposed acid-base catalysis conflicted with the possibility of redox catalysis as predicted by the presence of a dinucleotide binding site. We show that reduced FAD is the essential lycopene cyclase (CrtY) cofactor. Using flavin analogs, mass spectrometry, and mutagenesis, evidence was obtained based on which a catalytic mechanism relying on cryptic (net) electron transfer can be refuted. The role of reduced FAD is proposed to reside in the stabilization of a transition state carrying a (partial) positive charge or of a positively charged intermediate via a charge transfer interaction, acid-base catalysis serving as the underlying catalytic principle. Lycopene cyclase, thus, ranks among the novel class of non-redox flavoproteins, such as isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2 (IDI-2) that requires the reduced form of the cofactor.

Bicyclic carotenoids are also the source for apocarotenoids, which arise through specific cleaving reactions mediated by carotenoid cleavage dioxygenases leading to key regulatory molecules, such as abscisic acid (8) and the strigolactones (Refs. 9–11 and citations therein). Given these functions, plants defective in cyclization are expected to be nonviable.

Cyclic carotenoids and their derivatives are also biosynthesized by many microorganisms, including all photosynthetic and some heterotrophic bacteria and fungi. In contrast, animals lack such biosynthetic capacity and need to acquire these pigments from the food chain, because of their function as precursors of the vision pigment retinal and the vertebrate morphogen retinoic acid (12). Retinal is synthesized through cleavage of the central C15–C15’ double bond either directly from \(\beta\)-carotene, as shown for animals (13) and fungi (14, 15), or from longer monocyclic apocarotenols in cyanobacteria (16, 17). Retinoids can only be derived from carotenoids with at least one unsubstituted \(\beta\)-ionone ring. Thus, lycopene cyclases are the enzymes capable of providing provitamin A carotenoids. Consequently they have been employed to help alleviate vitamin A deficiency diseases (18) by enhancing the provitamin A content of crop plant tissues by genetic engineering (19–21). In some tissues, such as rice endosperm, lycopene cyclase was not required thanks to a sufficient lycopene-\(\beta\)-cyclase activity in the wild-type tissue (22, 23).

The wide occurrence of lycopene cyclization across taxa is contrasted by structural diversity. There are four families of lycopene cyclases that are only in part related to each other (24). The CrtY-type, which is the subject of this article, is found in many proteobacteria, whereas lycopene cyclases from cyanobacteria and plants belong to the CrtL family. CrtL and CrtY cyclases do not share much similarity; however, they contain conserved sequence patterns indicating evolutionary relatedness (25). Plants express two related versions of CrtL-enzymes capable of producing either \(\beta\) or \(\epsilon\)-ionone end groups, thus, defining an important branching point in carotenogenesis. The enzyme capsanthin/capsorubin synthase, known from \textit{Capsicum annuum} (26, 27), represents another member of the CrtL family. It catalyzes a ring contraction to form the so-called \(\kappa\)-ring by utilizing a mechanism thought to be very similar to that of lycopene cyclases. Additional CrtL cyclases are represented by the capsanthin/capsorubin synthase homologs from tomato and potato that were shown to mediate neoxanthin syn-

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Lycopene Cyclase Catalyzes an FAD$_{\text{red}}$-dependent Reaction

![Mechanism of lycopene cyclization](image)

**FIGURE 1. Mechanism of lycopene cyclization.** The reactions are initiated by electrophilic proton attack at the C$_1$-C$_2$ double bond. This leads to ring closure that can occur via an intermediate carbonium ion at C$_2$, and subsequently at C$_5$ (not shown) or, in a concerted reaction, via a positively charged transition state. In both cases proton abstraction results in either a β-ionone ring (H$_B$ abstraction) and/or in plants in an α-ionone ring (H$_A$ abstraction) formation. H$^+$ from solvent is inserted into position C$_2$ (adapted from Britton et al. [31]). This reaction occurs twice, at both linear ends of the symmetrical lycopene molecule.

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试验 (28, 29). However, the tentative neoxanthin synthase has been shown to represent a chromoplast-specific lycopene-β-cyclase in tomato (30).

Much of the knowledge on the diverse lycopene cyclases has been gained in vivo, such as through color complementation in lycopene-producing *Escherichia coli* cells or the analysis of mutants. Information on the enzymology of these enzymes is scarce and still insufficient to draw conclusions on the mechanism employed.

A landmark experiment was performed in vivo some decades ago with a *Flavobacterium* species that contains a CrtY-type cyclase (31). Formation of xanthophylls in deuterium oxide led to the incorporation of two $^2$H$_2$ atoms into the product. This is compatible with lycopene cyclization proceeding as outlined in Fig. 1. This model is related to isomerization reactions and agrees with the absence of net mass changes between the lycopene substrate and the β-carotene product (both have the sum formula C$_{40}$H$_{56}$). However, the likely presence of a Rossmann-fold in CrtY and CrtL-type cyclases (24) would be consistent with the presence of a dinucleotide cofactor such as FAD(H$_2$) or NAD(P)(H) and raises the possibility of a mechanism involving redox chemistry. This, however, would be in contrast with the mentioned absence of changes in the redox status between substrate and product.

A multitude of different cofactors have been tested in the past in relation to lycopene cyclase catalyzed reactions (see “Discussion”). However, no clear cut data on the nature and possible role of the cofactor involved have been obtained thus far. In view of the basic and mechanistic relevance of this biochemical step, we undertook research mainly with the CrtY-type lycopene cyclase from *Pantoea ananatis* (formerly *Erwinia uredovora*, ACC D90087). Biphasic liposomal assays were used to account for the high lipophilicity of the C$_{40}$ hydrocarbons lycopene and the cyclic reaction products. Here, we focus on the mechanistic role of the flavin, which was identified as the cofactor of CrtY in the course of this study.

**EXPERIMENTAL PROCEDURES**

**Chemicals Used**—A compilation of flavin cofactors modified at various positions has been reported elsewhere (32). It contains references to appropriate sources or synthetic methods and to methods for conversion of riboflavin analogs into the corresponding FMN and FAD derivatives as well as their redox potentials. FAD synthetase from *Corynebacterium ammoniagenes* (33, 34) was kindly provided by Dr. M. Medina (Universidad de Zaragoza). Flavin cofactor analogs were purified using HPLC$^3$ system 3, and their identity was verified by UV-visible spectroscopy and LC-MS. Neurosporene, 5,5-di-cis-lycopene, and γ-carotene were purchased from CaroteNature. Prolycopene was extracted and purified from fruits of the tangerine cultivar of tomato according to reported methods (35). $^2$H$_2$O was obtained from Euriso-top. All other fine chemicals were purchased from Sigma.

**Cloning, Protein Expression, and Complementation in E. coli**—To investigate appropriate translational fusions of the insoluble CrtY protein, we took advantage of a Gateway vector system (kindly provided by D. Busso, Département de Biologie et de Génomique Structurales, Université Louis Pasteur, Parc d’Innovation, Illkirch, France) allowing the expression of different fusion proteins (36). For this purpose, the entry vector pENTR/D-CrtY was produced by amplification of the CrtY gene from pBAD-TOPO-Thio-CrtY expressing the enzyme in fusion with thioredoxin. The amplification was performed with the primers CrtY GWF (5’-CACCGATGAGATGAAAGCCTGCCCTTTATG-3’) and CrtY-R+T (5’-TCATCCTTATCCTCTCTTTGCAAGA-3’) using 500 nM concentrations of each primer, 150 μM dNTPs, and 1 unit of *Phusion*™ High-Fidelity DNA Polymerase (Finnzymes) in the buffer provided. The resulting product was purified and cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer’s instructions. The expression plasmids pCrtY-HMGGWA, pCrtY-HGGWA, pCrtY-HNGWA, and pCrtY-HXGWA encode the fusion proteins His$_6$-MBP-EK-CrtY (termed mCrtY in the text; MBP is maltose-binding protein), His$_6$-GST-EK-CrtY (GST is glutathione S-transferase), His$_6$-NusA-EK-CrtY (Nus is N-utilizing substance A), and His$_6$-TRX-EK-CrtY (TRX is thioredoxin), respectively (see Ref. 36, DEK denotes an enterokinase cleavage site introduced through primer GWF). The plasmids were obtained by transferring the CrtY-gene from pENTR/D-CrtY into the corresponding Gateway destination vectors using the Gateway LR Clonase Enzyme Mix (Invitrogen) according to the manufacturer’s instructions.

The intron-free rice (*Oryza sativa*) lycopene β-cyclase gene OsLYCb (accessions no. AP005849) was cloned from the variety TP309 by PCR methods with the following primers: forward (5’-ATGGCCACCACCGCCCTCCTCCTCCT-3’) and reverse (5’-GAGAGATGATGAGATCCTCCTCACCTAC-3’). The PCR product was cloned into pBAD/TOPO$^3$ Thio Fusion (Invitrogen) to yield pThio-OsLYCb, and the integrity of the gene was verified by sequencing.

For site-directed mutagenesis of CrtY, the plasmid pE196A was generated using QuickChange XL site-directed mutagenesis

3 The abbreviations used are: HPLC, high performance liquid chromatography; LC, liquid chromatography; MS, mass spectroscopy; GPC, gel permeation chromatography; IMAC, immobilized metal affinity chromatography; TBA, tert-butylmethyl ether; Emid, midpoint redox potential; IDI-2, isopentenyl dipiphosphate:dimethylallyl dipiphosphate isomerase type 2; M5S, 4-morpholineethanesulfonic acid.
kit (Stratagene) to substitute Glu\textsuperscript{196} for Ala. For this purpose the plasmid pCrtY-HMGWA was amplified with the forward primer, Mut1 (5'—ACGGTCGTA TCCGGATACCGGC-3'), and the reverse primer, Mut2 (5'—TGAGCGGTA TTCGCGATCGAGCGT-3'), according to the manufacturer's protocol. The mutated CrtY was verified by sequencing.

For protein expression, the plasmids pThio-OsLYCb and pCrtY-HMGWA were transformed into BL21(DE3) E. coli cells harboring the plasmid pGro7 (Takara), which encodes the groES-groEL chaperone system under the control of an arabinose-inducible promoter. 2.5 ml of overnight cultures of transformants were then inoculated into 500 ml of 2× YT medium, grown at 37 °C to an A\textsubscript{600} of 0.5, and induced with arabinose (8 mM) and isopropyl-β-D-galactopyranoside (0.2 mM) or only with arabinose (OsLYCb-expressing cells). The induction was performed overnight at 16 °C, and cultures were harvested by centrifugation at 5000 × g for 10 min. Pellets were frozen at −20 °C for further use.

For color complementation, JM109 E. coli cells containing the plasmid pFarbeR enabling lycopene synthesis (15) were transformed with the plasmid pCrtY-HMGWA, pThio-OsLYCb, or pE196A. Bacteria were grown overnight at 28 °C, harvested, and extracted with acetone. After partition against petroleum benzenediethyl ether 2:1 (v/v) and washing with water, the organic epiphase was dried and subjected to HPLC—system 1 analysis.

**Cell Disintegration, Solubilization, and Protein Purification**

All procedures were carried out on ice. Cells from a 500-ml culture were harvested at an A\textsubscript{600} of 2.0 and resuspended in 6 ml of buffer A (100 mM Tris-HCl, pH 7.0, 5 mM MgCl\textsubscript{2}, 300 mM NaCl, and glycerol 10%) by vortexing and disintegrated by two passages through a French press cell operated at 18,000 p.s.i. A centrifugation step at 17,000 × g for 15 min was used to remove large cell debris. The supernatant was solubilized with Tween 20 at a 10 × critical micellar concentration (0.067%) final concentration.

The suspension was incubated for 30 min with occasional shaking before adding 2 ml of the IMAC resin suspension of Talon (Clontech) containing Co\textsuperscript{2+} as the metal ligand (purification method 1). The material was equilibrated with Buffer A containing 0.067% Tween 20 before use. The His-tagged mCrtY was allowed to bind for 45 min under continuous shaking at 37 rpm, and the resin was recovered by centrifugation. Three washing steps with buffer A containing 0.02% Tween 20 and 4 mM imidazole were employed to remove unspecifically bound protein. Elution of the bound protein was accomplished with buffer B, which is the same as buffer A but adjusted to a pH of 8.0 and containing 100 mM EDTA and 0.02% Tween 20. The preparation was dialyzed against buffer D (see below) before use.

Alternatively (protein purification method 2) Ni\textsuperscript{2+}-nitrilotriacetic acid–agarose (Qiagen) was employed, essentially following the procedures described above, but elution was carried out at low pH using buffer C (250 mM MES-NaOH, pH 5.0; 5 mM MgCl\textsubscript{2}, 300 mM NaCl, 10% glycerol, and 0.02% Tween 20) under gentle shaking for 20 min. The preparation was dialyzed against buffer D before freezing aliquots at −80 °C.

For gel permeation chromatography (GPC), 200-μl portions of IMAC-purified mCrtY were applied to a Superdex 200 10/300 GL column (GE Healthcare) previously equilibrated with buffer D (50 mM MES-NaOH, pH 5.8; 5 mM MgCl\textsubscript{2}, 300 mM NaCl, 10% glycerol, 0.02% Tween 20). GPC was carried out using an ÄKTA-explorer device (GE Healthcare) at a flow rate of 0.8 ml/min.

For cofactor analysis, the IMAC-purified lycopene cyclase (10 mg) was heat-denatured for 10 min, and the protein was removed by centrifugation. The supernatant was lyophilized, and the residue was redissolved in 100 μl water of which 2 μl was applied to LC-MS analysis.

SDS-PAGE was carried out according to standard procedures using 10% polyacrylamide gels. Proteins were detected using Coomassie Brilliant Blue G250 (Sigma). Protein concentrations were determined using the Bradford method.

**Approprotein Preparation and Reconstitution**—The mCrtY apoenzyme was prepared according to published procedures (37) with some modifications. In brief, 1 ml of ice-cold 3 M KBr in buffer D was added slowly to 1 ml of IMAC-purified protein (2 mg/ml; protein purification method 2). After dialysis overnight at 4 °C against buffer D containing 2 M KBr and removal of the KBr by dialysis against buffer D, eventually occurring precipitates were removed by centrifugation at 21,000 × g for 15 min. The absence of cofactors was confirmed by UV-visible spectroscopy, fluorescence measurement, and the absence of enzymatic activity. For reconstitution (see Fig. 6A), 40 μl of 6 μM apoenzyme was supplemented under anaerobic conditions with different concentrations of flavins, then buffer D was added to a total of 58 μl followed by the addition of 2 μl of freshly prepared Ti(III) citrate (see below) to reduce FAD. The mixtures were incubated at room temperature for 30 min before adding 16-μl aliquots from each mixture to the standard assay system (see below).

**Enzymatic Assays**—To prepare E. coli membranes containing lycopene, cell pellets from a 5-liter E. coli culture expressing pFarbeR and grown overnight at 28 °C in LB medium were resuspended in 60 ml of buffer A by vortexing, and cells were disintegrated by two passages through a French press cell operated at 18,000 p.s.i. Centrifugation at 17,000 × g for 15 min removed large cell debris. The crude supernatant was ultracentrifuged at 140,000 × g for 4 h. The colorless membrane-free supernatant was dialyzed against buffer A to be used in assays, as indicated. The membrane fraction was resuspended in buffer A with a Dounce homogenizer followed by another ultracentrifugation at 140,000 × g. The pellet was resuspended in buffer A and stored at −20 °C for subsequent assays.

To prepare protein-free liposomes containing carotene substrates, soybean lecithin (Sigma) was dissolved in CHCl\textsubscript{3} at a concentration of 20 mg/ml. Carotene stock solutions were prepared separately in chloroform/methanol 2:1 (v/v). Carotene concentrations were estimated spectrophotometrically (Shimadzu, UV-2501PC) using an ε\textsubscript{450 nm} = 185,230 liters mol\textsuperscript{−1} cm\textsuperscript{−1} for lycopene, ε\textsubscript{450 nm} = 161,160 liters mol\textsuperscript{−1} cm\textsuperscript{−1} for γ-carotene, and ε\textsubscript{450 nm} = 134,500 liters mol\textsuperscript{−1} cm\textsuperscript{−1} for β-carotene. From these stock solutions aliquots corresponding to 160 μg were added to 1 ml of lecithin solution. After adding 800 μl of chloroform/methanol 2:1 (v/v) and drying, the residue was taken up in 2 ml of buffer D without detergent. Liposomes were
formed by sonication for about 30 min on ice. To assess incorporation of carotenes into the lipid bilayer, an aliquot was extracted with chloroform/methanol 2:1 (v/v) and measured photometrically, as described above.

The standard lycopene cyclase assay consisted of a liposome suspension volume that resulted in a final carotene concentration of 5 μM (typically ~30 μL) when diluted to a final volume of 200 μL with buffer D, which is at the pH optimum of pH 5.8 determined for the reaction. 5 μg of cyclase protein was added, and the assay was supplemented with 20 μL of hexane. FAD was added to a 100 μM final concentration. Reducing conditions were attained with freshly prepared Ti(III)-citrate following the procedure given in Zehnder and Wurhmann (38). For this purpose 187 μL of a 10% Ti(III) chloride solution (Sigma) were added to 600 μL of an aqueous 0.42 M sodium citrate solution. The mixture was subsequently neutralized with a saturated sodium carbonate solution. 4 μL of the Ti(III) citrate solution were added to a standard incubation assay.

All solutions were equilibrated with N₂ before use, and the reactions were carried out in a glove box under an N₂ atmosphere. Incubation time was 30 min unless otherwise indicated. Enzymatic reduction of FAD was carried out using the flavin:NADH reductase PrnF from Pseudomonas fluorescens (39), kindly provided by Prof. van Pée (Technical University Dresden). For this purpose, a reaction system consisting of 200 μL of buffer D was placed in one chamber of a two-cell dialysis apparatus. The mCrtY-FAD<sub>ox</sub> holoenzyme and protein-free lycopene liposomes in 400 μL of buffer D were placed in one chamber of a two-cell dialysis apparatus. Incubation time was 30 min unless otherwise indicated. Enzymatic reduction of FAD was carried out using the flavin:NADH reductase PrnF from Pseudomonas fluorescens (39), kindly provided by Prof. van Pée (Technical University Dresden). For this purpose, a reaction system consisting of 200 μL of buffer D, which is at the pH optimum of pH 5.8 determined for the reaction. 5 μg of cyclase protein was added, and the assay was supplemented with 20 μL of hexane. FAD was added to a 100 μM final concentration. Reducing conditions were attained with freshly prepared Ti(III)-citrate following the procedure given in Zehnder and Wurhmann (38). For this purpose 187 μL of a 10% Ti(III) chloride solution (Sigma) were added to 600 μL of an aqueous 0.42 M sodium citrate solution. The mixture was subsequently neutralized with a saturated sodium carbonate solution. 4 μL of the Ti(III) citrate solution were added to a standard incubation assay.

To carry out deuteration experiments, all buffers and the lycopene-liposome suspension were prepared as described above in <sup>2</sup>H<sub>2</sub>O. The protein, Ti(III) citrate, and FAD solutions, added in very small volumes (less than 2%) were in H<sub>2</sub>O.

**Extraction and Analytical Methods—Assays** were extracted twice with 1 volume of chloroform/methanol 2:1 (v/v). The lipophilic phases were combined and dried under reduced pressure. The residue was redissolved in 50 μL of chloroform of which 20 μL were used for HPLC analysis. The HPLC device used (Waters, Alliance 2695) was equipped with a photodiode array detector and was controlled by the EMPOWER software program.

HPLC system 1 was used for the separation of carotene substrates and products employing a 3-μm C<sub>30</sub> reversed phase column (YMC-Europe) with the solvent system A (methanol/tert-butylmethyl ether (TBME)/water 5:1:1 (v/v/v)) and B (methanol/TBME 1:3 (v/v)). The gradient started at 30% A followed by a linear gradient to 0% A within 10 min at a flow rate of 1 ml/min. An isocratic segment, run for 12 min at 0% A, completed the separation program. Individual peaks resolved were integrated electronically at their individual λ<sub>max</sub> with the aid of the “maxplot” function of the software. Detector response curves for β-carotene and lycopene standard solutions were used for quantification.

HPLC system 2 was used for LC-MS applications to analyze carotenes. It consisted of a 3-μm C<sub>18</sub> reverse phase column (YMCEurope) that was developed with a gradient consisting of A (methanol, 0.01% aqueous ammonium acetate, TBME 70:25:5 (v/v/v)) and B (methanol, 0.01% aqueous ammonium acetate, TBME 73:90 (v/v/v)). The gradient was developed from 85% A to 0% A within 10 min followed by an isocratic segment at 0% A for 5 min before re-equilibration.

NADP(H), NAD(H), FAD, FMN, and flavin analogs were identified by LC-MS with HPLC system 3 consisting of a 3-μm C<sub>18</sub> reverse phase column (Hypersil Gold, Thermo-Fisher Scientific) and the solvent system A (50 mM aqueous ammonium acetate in 1% formic acid) and B (1.7 mM ammonium acetate in 70% methanol acidified with 1% formic acid). The gradient was run at a flow rate of 700 μL/min from 100% A to 50% A within 10 min with the final conditions held isocratically for 5 min.

A Surveyor HPLC system coupled to a LTQ mass spectrometer (Thermo-Fisher Scientific) was used with the HPLC systems 2 and 3. Carotenes were atmospheric pressure chemical ionization-ionized using N₂ as the reagent gas and analyzed in the positive ion mode. Further conditions were: capillary temperature, 150 °C; vaporizer temperature, 350 °C; source voltage, 6 kV; capillary voltage, 49 V; source current, 5 μA. Separated carotenes were identified by their spectra, by retention times in comparison with authentic references, and by their quasi-molecular (M<sup>+</sup>) ions.

Nucleotide cofactors were subjected to electrospray ionization using a spray voltage of 5.3 kV, the capillary voltage was maintained at 49 V, and the capillary temperature was held at 350 °C. For identification we used the chromatographic comparison with the authentic reference substances; in addition, single reaction monitoring was used to increase analytical confidence. For this purpose, the predominant MS<sup>2</sup> daughter fragments were determined using reference substances and used to filter those peaks that displayed the correct molecular ion and the expected MS<sup>2</sup> fragments. Daughter fragments were: FAD, M<sup>+</sup> 786.2 (348.1, 439.2); FMN, M<sup>+</sup> 457.1 (359.2, 439.1); NAD<sup>+</sup>, M<sup>+</sup> 664.1 (524.1, 542.1); NADP<sup>+</sup>, M<sup>+</sup> 744.1 (604.0, 622.0); NADH, M<sup>+</sup> 666.2 (348.2, 649.2); NADPH, M<sup>+</sup> 746.4 (428.1, 729.1). UV-visible spectra were taken simultaneously. Data acquisition and analyses were performed with the Xcalibur 2.0 software program.

For fluorescence spectra, samples with a concentration of ~3.6 mg of protein/ml were measured in a Cary Eclipse Spectrofluorimeter (Varian; excitation slit width, 10 nm; emission 5 nm). Potentiometric measurements were made with a Clark-type oxygen electrode using 2 mL of standard assay mixture (25 °C). For electron microscopy, the liposomes used in standard assays with and without hexane addition were negatively stained with 1% neutral phosphotungstic acid on carbon-coated copper-grid and analyzed in a Philips FEI CM 10 electron microscope at 80 kV.

**RESULTS**

**mCrtY Apoprotein Production and Purification**—Initial overexpression of CrtY in the form of N-terminal thioredoxin
fusion constructs resulted in largely insoluble protein. The cloning system described by Busso et al. (36) was used to increase the proportion of soluble active protein. Among the four different vectors, pHGGWA, pHNGWA, pHXGWA, and pHMGWA, the latter gave the best results producing ~50% protein in inclusion bodies (estimated by 13,000 × g centrifugation), whereas the remainder sedimented at 140,000 × g. This is indicative of membrane-bound mCrtY. Complementation in lycopene-producing E. coli cells using the vector pCrtY-HMGWA showed that the fusion protein was enzymatically active. It converted pink-to-yellow-colored colonies due to β-carotene formation. This was confirmed by HPLC analysis (system 1, not shown).

The membrane-bound fusion protein was solubilized and subjected to purification by metal-ion affinity chromatography (using protein purification method 1) and GPC. Tween 20 was found to be the best-suited detergent in suppressing aggregation during the purification procedures and maintaining enzymatic activity. Fig. 2 shows the purity of the protein at the stages of purification. mCrtY obtained after the GPC step did not exhibit relevant absorbance at λ > 300 nm, which is consistent with the absence of dinucleotide cofactors. Because the enzyme showed to be enzymatically active upon complementation in lycopene-accumulating E. coli, this apoprotein was considered to be in a native conformation and suitable for experiments involving binding of cofactors.

First experiments were conducted with purified membrane fractions isolated from lycopene-producing E. coli cells. This was based on the reasoning that in bacteria, CrtY (presumably binding a redox-active dinucleotide) was bound to the plasma membrane containing the lycopene substrate and, therefore, exposed to redox-active components e.g. as part of the respiratory chain.

mCrtY Apoprotein; Behavior at E. coli Membranes—Membrane preparations from lycopene-producing E. coli cells were obtained by differential centrifugation. The assays, containing purified mCrtY, lycopene-containing membranes, and various cofactors, were stopped after 30 min by the addition of CHCl₃/MeOH. No conversion of the membrane-bound lycopene into β-carotene took place in the absence of added cofactors. Among the cofactors used, NADH and NADPH were effective, whereas the oxidized forms were not (supplemental Fig. 1A). It is worth noting that the combination of NADH and FAD led to a triplication of the conversion rate of NADH. ATP was not effective under these conditions but was included because it had been used in lycopene cyclization assays with chloroplast stroma (27).

Significant further stimulation was achieved when the assays were “contaminated” by adding back supernatant from the 140,000 × g centrifugation from wild-type or lycopene-producing E. coli cells. Because this effect was abolished upon dialysis of the supernatant small cofactors could be relevant (supplemental Fig. 1B). Tests indicated that first, reduced cofactors were stimulatory. Second, ATP was stimulatory when combined with other cofactors, and third, a mixture of all cofactors was best. Seemingly a combination of diverse metabolic reactions led to a reduced membrane redox component needed for mCrtY activity. The stimulation upon removal of dioxygen pointed in the same direction.

In E. coli, 15 primary dehydrogenases are known (40) capable of oxidizing a plethora of metabolites, all reducing quinones. Consistently, the glycolysis and tricarboxylic acid cycle intermediates such as glucose, fructose 6-phosphate, and succinate were stimulatory in the presence of dialyzed supernatant, whereas 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate (not delivering reduction equivalents during glycolysis) were ineffective (supplemental Fig. 1C). In sum, all means leading to respiratory chain reduction strongly facilitated the cyclization of lycopene, initially interpreted in terms of an interaction of CrtY with elements of membrane-bound redox cofactors. This is very similar to the requirements reported for a carotene isomerization reaction catalyzed by the plant enzyme CrtISO (41). Alternatively, because the respiratory chain activities yielded anaerobic conditions very rapidly (as determined potentiometrically), a direct inhibitory effect of oxygen could not be excluded. To distinguish between these two possibilities, the redox-active cofactor bound to CrtY needed to be molecularly identified.

CrtY Is a Flavoprotein Binding FAD—Preliminary purification attempts led to the isolation of the mCrtY apoprotein, which required the addition of cofactors for activity. Purification of the holoprotein required further refinement of the procedure. The protein purification method 2 (see “Experimental Procedures”) allowed recovery of a yellowish protein fraction. The UV-visible spectra (Fig. 3) of such preparations were consistent with the presence of an oxidized flavin. The fluorescence emission spectra of untreated mCrtY, displayed in Fig. 3, showed two bands. One band had a λmax = 520 nm, which is typical of the flavin chromophore. As expected, the addition of dithionite, which generates non-fluorescent reduced flavin, eliminated the 520-nm flavin emission band. The addition of ferricyanide to a similar sample led to the disappearance of the 433 nm band, which is typical of NAD(P)H, reflecting the oxidation of the reduced nicotinamide to the nonfluorescent oxidized species.

To distinguish between FAD/FMN and NAD(H)/NADP(H), the protein was heat-denatured, and the supernatant was lyophilized, redissolved, and applied to HPLC system 3. Single reaction monitoring was employed, optimized for the specific identification of these cofactors. The results depicted in Fig. 4 reveal the presence of FAD as the main cofactor of mCrtY. The flavin was not oxidized, whereas the oxidized form was detected by the characteristic absorption at 370 nm. This result is consistent with the presence of FAD and with the catalytic properties of CrtY reported by others.

**Figure 2.** SDS-PAGE analysis of the mCrtY fusion protein induction and purification. The expected mass of the fusion protein is 87.8 kDa. Lane M, molecular mass markers; lanes 1 and 5, whole cell lysate after isopropyl 1-thio-β-δ-galactopyranoside induction; lane 2, pellet after 13,000 × g centrifugation; lane 3, pellet after 140,000 × g centrifugation; lane 4, supernatant after 140,000 × g centrifugation; lane 6, fraction after IMAC purification; lane 7, fraction after GPC purification.
component besides minute amounts of FMN, which were undetectable in the UV-visible trace. The analysis also indicated the presence of NAD at low levels in an NAD/FAD molar ratio of approximately 1:9, as determined separately by HPLC/UV-visible spectroscopic detection. No signal was observed for NADP(H) (data not shown). It could not be determined at this point whether NAD and FMN stemmed from contaminating proteins present after IMAC purification. Because GPC separation, resulting in purification to near homogeneity (Fig. 2), also led to a loss of all bound cofactors, enzymology assays needed to be employed to identify the nature of the effective CrtY cofactor.

A Reduced Flavin Cofactor Is Required for Activity—To avoid the use of redox-active biological membranes, protein-free liposomes containing lycopene were prepared. Supplementation with the mCrtY holoprotein (protein purification method 2) did not induce activity. Based on the observation of a potential inhibitory role of oxygen (see above), we developed an assay system containing reduced FAD. This was achieved in an N₂ atmosphere and in the presence of freshly prepared Ti(III) citrate (~10 mM) as a reductant. Under these conditions the reaction proceeded, albeit at a low rate. A strong stimulation was achieved by the addition of 10% hexane to the incubations. This resulted in considerable turbidity produced by structural changes of the PC-liposomes, which fused to produce rod-like structures (Fig. 5A). Under these conditions the lycopene substrate is assumed to become more accessible, which proved to be a reproducible effect as shown in Fig. 5B. Catalysis was also observed, although at lower rates, when FAD was reduced photochemically (42). The rate of β-carotene formation was further optimized by adding excess FAD, which probably leads to mCrtY saturation with reduced FAD. The reaction showed a pH optimum at pH 5.8 (data not shown).

To assess the role of NAD(H), apomCrtY was prepared by treatment with KBr as detailed under “Experimental Procedures.” Removal of all cofactors was verified by UV-visible and fluorescence spectroscopy as well as by the absence of enzymatic activity. The reconstitution of the active holo-mCrtY with increasing amounts of FAD was carried out under N₂ and in the presence of excess Ti(III)-citrate (Fig. 6A) and showed a stoichiometry equal to 1. Reconstitution attempts with NAD(P)H did not restore activity. Reduced FMN, on the other hand, was effective, yielding mCrtY with ~50% of the specific activity observed with reduced FAD (data not shown).

Reduced FAD, once protein-bound, appears to be protected from reoxidation (Fig. 6B). When the holoprotein (purification
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Formulation of fully active mCrtY was also achieved by starting from a mCrt\textsubscript{Y}-FAD\textsubscript{ox} holoenzyme and incubating anaerobically in the presence of (4 mM) NADH. It is possible that mCrt\textsubscript{Y}-bound FAD\textsubscript{ox} is reduced by NADH similarly as shown with the Type II IPP-DMAPP isomerase (43). On the other hand, the possibility of an FAD\textsubscript{ox}/FAD\textsubscript{red} exchange in mCrt\textsubscript{Y} was shown as follows; mCrt\textsubscript{Y}-FAD\textsubscript{ox} holoenzyme was placed in one chamber of a two-cell dialysis apparatus; the other chamber contained an FAD\textsubscript{red}-generating system consisting of NADH, FAD, and the flavin reductase PrnF according to Unversucht et al. (44). In an N\textsubscript{2} atmosphere, the generated FAD\textsubscript{red} was able to activate mCrt\textsubscript{Y}, although at a slow rate.

Acid-Base Catalysis Remains the Catalytic Principle—Taken at face value the requirement of reduced FAD is not easy to be reconciled with the acid-base reaction mechanism previously proposed (Ref. 31, see Fig. 1). Alternatives have been discussed (35, 45). To be reassured of the occurrence of acid-base catalysis under our experimental conditions, lycopene cyclization was studied under analogous conditions, however, in \textsuperscript{2}H\textsubscript{2}O buffer, using Ti(III) citrate as the reductant (Fig. 7). Clearly, the bicyclic \(\beta\)-carotene formed has two additional mass units, which is

Method 2) was subjected at 37 °C to anaerobic photoreduction in the presence of excess FAD but in the absence of Ti(III) citrate, the reaction proceeded as expected (compare with Fig. 4B). Under these conditions, reduced FAD is generated. Exposure to oxygen did not abolish catalysis, although the observed rate of conversion was temporarily reduced. The latter effect is attributed to the lowered incubation temperature that occurred during the manipulation. At 37 °C and in the presence of oxygen the reaction then continued at comparable conversion rates as under N\textsubscript{2}. Because all previous experiments consistently showed activity only with reduced FAD (which, in its free form, is not stable in the presence of oxygen), this experiment indicates that reduced FAD bound to mCrt\textsubscript{Y} does not react efficiently with O\textsubscript{2}. Furthermore, the flavin does not dissociate significantly from holo-mCrt\textsubscript{Y} during the \(\approx\)30 min duration of the experiment.

FIGURE 5. A, electron microscopy of the phosphatidylcholine liposomes used before (left) and after (middle) hexane addition. The bar represents 200 nm. Lycopene cyclization took place in highly turbid assays in which lycopene (red) was converted into \(\beta\)-carotene (yellow). A standard assay is shown in the absence (Con) and presence of mCrt\textsubscript{Y} after 1 h of incubation. B, shown is the time course of lycopene cyclization using 5 \(\mu\)g of holo-mCrt\textsubscript{Y} isolated according to protein purification method 2 in anaerobic standard assays in the presence of different reductants. When photoreduction was used, the addition of FAD was needed to achieve activity in a concentration-dependent manner, raising the specific activity from 0.14 pmol \(\mu\)g mCrt\textsubscript{Y}\textsuperscript{-1} min\textsuperscript{-1} (20 \(\mu\)M FAD) to 2.2 pmol \(\mu\)g of mCrt\textsubscript{Y}\textsuperscript{-1} min\textsuperscript{-1} (400 \(\mu\)M FAD). No addition of FAD was needed when Ti(III) citrate was used as the reductant to achieve an activity of 1.7 pmol \(\mu\)g of mCrt\textsubscript{Y}\textsuperscript{-1} min\textsuperscript{-1} but FAD addition (20 \(\mu\)M) was stimulatory, leading to an activity of 2.5 pmol \(\mu\)g of mCrt\textsubscript{Y}\textsuperscript{-1} min\textsuperscript{-1}. The stimulation achieved by FAD addition indicates the presence of some apo-mCrt\textsubscript{Y}. Filled symbols are for assays carried out in the absence of a reductant both in the presence or absence of FAD, resulting in complete absence of enzymatic activity.

FIGURE 6. A, reconstitution of holo-mCrt\textsubscript{Y} from the apo-form with FAD is shown, 40 \(\mu\)l of 6 \(\mu\)M apoenzyme was supplemented with different amounts of FAD to give the indicated molar ratios, then buffer D was added to arrive at 58 \(\mu\)l followed by adding 2 \(\mu\)l of freshly prepared Ti(III) citrate as the reductant. After incubation at room temperature for 30 min, 16-\(\mu\)l aliquots from each incubation mixture were applied to the standard anaerobic assay system. The activity was assayed by using HPLC system 1. B, shown is oxygen insensitivity of mCrt\textsubscript{Y}-bound FAD\textsubscript{ox}/mCrt\textsubscript{Y} (10 \(\mu\)g/200 \(\mu\)l) was irradiated for photoreduction in the presence of 400 \(\mu\)M FAD under standard anaerobic conditions. After 10 min the sample was removed from the anaerobic glove box, and FAD\textsubscript{red} was oxidized by O\textsubscript{2} (shaking with air). Reactions continued unaffected in the air and in the dark after equilibration of the samples at 37 °C. The activity plateau is due to an unavoidable transient drop in the assay temperature during sample transfer from anaerobic to aerobic conditions.
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Figure 7. Lycopene cyclization mediated by mCrtY in the presence of \(^2\text{H}_2\text{O}\). A, shown is HPLC photodiode array detection showing the conversion of lycopene (1) into \(\beta\)-carotene (2) after an incubation time of 40 min under standard anaerobic conditions. \(\mu\text{AU}\), microabsorbance units. B, shown is a corresponding mass trace detecting lycopene and \(\beta\)-carotene (both with a quasi-molecular ion at \(m/z\) (+1) 537.5) and at the expected pseudomolecular ion for the di-deuterated bicyclic \(\beta\)-carotene of one mass unit per \(\beta\)-ionone ring formed (according to the model shown in Fig. 1). The mass spectra recorded for lycopene (1) and \(\beta\)-carotene (2) are given.

Figure 8. Correlation of the rates of mCrtY activity with the redox potential of the flavin cofactor. The enzymatic activities were determined under standard conditions. They are expressed as mCrtY specific activity (pmol of \(\beta\)-carotene \(\mu\text{g}\) mCrtY\(^{-1}\) min\(^{-1}\)) and are displayed on the ordinate in the log form. \(E_m\) is the midpoint redox potential of the flavin cofactors used. The structural indications in the panel denote the modifications at the FAD position 8 (see Fig. 9 for the respective chemical structures). Note that the value for 5-dFMN has been corrected to the value shown using the ratio of activity of FAD/FMN. 5-dFMN, 5-deazaFMN<sub>red</sub>.

Figure 9. FADH<sub>2</sub> analogs used in this study.

The results obtained with reduced deazaflavins speak against a role of FAD<sub>red</sub> itself as an acid-base catalyst. However, plant lycopene cyclases and the related capsanthin/capsorubin synthase carry a conserved FLEET motif necessary for activity (27). CrtY shows a \(^{194}\text{LIEDT}\)\(^{199}\) motif at an equivalent position. Therein, the glutamate was shown to be essential.\(^5\) To assess its role, Glu\(^{196}\) was exchanged for Ala in the otherwise identical mCrtY fusion protein. The resultant E196A-mCrtY was purified and showed overall properties closely similar to those of wild-type mCrtY. Specifically it bound FAD, and the UV-visible spectra of the holoenzyme indicated that the microenvironment at the flavin site was essentially unchanged. (supplemental Fig. 2A). Moreover, the CD spectra of the wild-type and mutated mCrtY apoprotein were practically identical (supplemental Fig. 2B), indicating the absence of substantial structural perturbations caused by the one amino acid exchange. In

\(^5\) B. Camara, personal communication.

 compatible with addition/abstraction of one hydrogen per ring in accordance with a putative acid-base mechanism as the catalytic principle. The monomeric intermediate \(\gamma\)-carotene with one additional mass unit was not observed. This finding, however, does not provide information of whether catalysis by reduced FAD involves cryptic redox cycles.

The Role of Reduced Flavin in Lycopene Cyclization—Both FMN<sub>red</sub> and FAD<sub>red</sub> served as cofactors for mCrtY; however, FMN<sub>red</sub> was less effective (see above). Therefore, in the present studies involving modified flavin cofactors, both forms were used depending on availability and purity. Both of the reduced deazaflavins (used as their FMN derivatives, see “Experimental Procedures” and chemical structures in Fig. 9) supported lycopene cyclization. Compared with reduced FMN (0.7 ± 0.3 pmol of \(\beta\)-carotene \(\mu\text{g}\) of mCrtY\(^{-1}\) min\(^{-1}\)), 5-deazaFMN<sub>red</sub> showed an even better specific activity (1.4 ± 0.2 pmol of \(\beta\)-carotene \(\mu\text{g}\) of mCrtY\(^{-1}\) min\(^{-1}\)), and 1-deazaFMN<sub>red</sub> was active at 0.4 ± 0.1 pmol of \(\beta\)-carotene \(\mu\text{g}\) of mCrtY\(^{-1}\) min\(^{-1}\). Moreover, as is shown in Fig. 8, the cyclase activity is dependent on the redox potential of the reduced flavin analogs (see Fig. 9 for chemical structures), the rate increasing with decreasing \(E_m\), this altogether demonstrating a different role of FAD<sub>red</sub> (see “Discussion”).
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In contrast to this, purified E196A-mCrtY was completely inactive in the presence of FAD<sub>red</sub>. This suggests that Glu<sup>196</sup>, rather than reduced FAD, plays a role as an acid-base catalyst.

Cyclization of Carotenoids Other Than All-trans Lycopene—Apart from lycopene, another candidate substrate for cyclization is the monocyclic γ-carotene (supplemental Fig. 3), which is expected to arise as a cyclization intermediate but was hardly detectable in our assays. When used as a substrate at equimolar concentrations, it was converted into β-carotene at high velocity (7.9 pmol of β-carotene µg of mCrtY<sup>-1</sup> min<sup>-1</sup>) yielding about 100% conversion in the standard assay of 20 min where the rate of lycopene conversion was still linear. This observation is not consistent with the idea of a “half-site” recognition of the symmetrical lycopene molecule, as at equimolar concentration, lycopene provides the double concentration of cyclizing sites as compared with γ-carotene. The desaturation intermediate neurosporene is another carotene in which the polylene configuration is identical in one-half the molecule met in lycopene. It is expected to form β-zeacarotene upon cyclization. Surprisingly, conversion took place in trace amounts—only under standard conditions and at very prolonged incubation times. This may again indicate that CrtY accommodates the substrate as a whole and is capable of distinguishing the difference of one double bond. 15–15′Apolycopeanal was used to mimic the half-site of lycopene. It is in concordance with the absence of half-site substrate recognition that this substrate failed to be converted into retinal. Prolycopeanal (7,9,9′,7′-tetracis lycopene), the lycopene produced by the plant carotene desaturase system (45, 46), was not accepted as a substrate.

**DISCUSSION**

We have presented evidence that the lycopene cyclase CrtY is a flavoprotein. It employs reduced FAD as a cofactor, also effective with FMN<sub>red</sub>. Moreover, heterologously overexpressed mCrtY retains FAD when purified under mild conditions, suggesting that this flavin form is the likely cofactor.

The nature of the cofactor that binds to the dinucleotide binding site in CrtY or to the related CrtL-type plant cyclases has long been enigmatic. Several cofactors have been used to account for the predicted requirement, such as NAD(P)H with CrtY (47, 48), NADP<sup>+</sup>, NADPH, and ATP with both the C. anuum lycopene cyclase and the related capsanthin/capsorubin synthase (27). NAD(P)H was found to be essential to drive a cis to trans isomerization plus a cyclization reaction in Narcissus pseudonarcissus chromoplast homogenates, which was attributed to the isomerization partial reaction (35). The carotene isomerase CrtISO (49, 50) had not been identified at the time. An earlier report (51) showed FAD to be essential in one of the protein fractions obtained from spinach, whereas NADPH had a stimulatory effect. In our assays, NAD(P)H was effective in the presence of membranes, strongly stimulated by additional FAD (supplemental Fig. 1A) and/or by cytoplasmic proteins (supplemental Fig. 1, B and C). Under those conditions a multitude of cofactors and primary catabolites stimulated lycopene cyclization. This stimulation is, as we show, due to anaerobic and reducing conditions in the assays, attained by increased respiratory chain activity. In conclusion, much of the existing confusion about cofactors used by CrtY and plant cyclases is probably due to the complexity of the systems employed. In this context it is worth noting that chloroplast membranes, such as from *N. pseudonarcissus*, possess an alternative redox chain that utilizes oxygen as a terminal electron acceptor, leading to anaerobic conditions at the expense of NAD(P)H (52). This suggests that the time has come to revisit the role of NAD(P)H requirement in prolycopeanal cyclization.

Anerobic conditions driving cyclization have been reported previously but were misinterpreted. The cyclization of prolycopeanal (7,9,9′,7′-tetracis lycopene) was found to be possible only under anaerobic conditions (35, 45), which was interpreted in terms of a redirection of electrons toward the cis/trans isomerase/cyclase system to drive cryptic redox cycles instead of having an electron flux toward oxygen. Kushwaha *et al.* (51) noted that their soluble lycopene cyclase preparation from spinach was significantly more active in a nitrogen atmosphere. This was interpreted in terms of sulfhydryl protection from oxidation.

As we show here, the function of anaerobic conditions *in vitro* is to establish the conditions needed to allow the formation of the mCrtY-FAD<sub>red</sub> complex required for cyclization activity. Overexpressed CrtL-type lycopene cyclase from *OsLycB* was shown to require the same reaction conditions for activity as CrtY, indicating that the underlying mechanisms are very similar (data not shown).

Any mechanistic consideration for CrtY must be confronted with the unquestionable recognition that (i) the flavin is necessary for catalysis, (ii) it must be in its reduced form, (iii) it does not catalyze a redox reaction, and (iv) the general mechanism relies on acid-base catalysis. The latter is in agreement with the fact that there is no change in the redox state between lycopene and β-carotene.

In recent years a new mechanistic function has emerged for flavoenzymes that utilize reduced flavins but do not catalyze a net redox reaction (53). Isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2 (IDI-2), for instance, requires reduced FMN for catalysis (43). ApoIDI-2 reconstituted with 5-deazaFMN resulted in an inactive enzyme, whereas 1-deazaFMN-IDI-2 was active. It has been proposed (55–57) that IDI-2 employed reduced FMN as an acid-base catalyst. The flavin positions N(1) and, more likely N(5), were proposed to act as the acid/base functional groups (58). This contrasts with the present finding that mCrtY is catalytically active with both reduced 5- and 1-deazaflavins. From this, a role of the positions N(5) and N(1) and, thus, a role of the flavin as an acid/base catalyst appears very improbable. The substitution of N(5) with C(5)-Hm as in 5-deazaflavinism introduces severe restraints in the capacity of the reduced form to carry out acid-base reactions. The same holds for 1-deazaflavins (59). These two deazaflavins in their reduced state can, thus, be used to assess the roles of these specific positions in acid-base catalysis.

The role of an acid-base catalyst is proposed to be played in CrtY by Glu<sup>196</sup> alone or in conjunction with an additional still unidentified group. Mutation of this conserved amino acid residue (His<sup>147</sup>, Asn<sup>149</sup>, Gln<sup>152</sup>, and Glu<sup>159</sup>) in close proximity to the FMN bind-
ing site in the mechanistically related IDI-2. In CrtY an analogous group could represent the second acid/base required.

As a further mechanistic variant, cryptic net one electron transfer, previously suggested for IDI-2 (43) and later revoked (56), is also unlikely in CrtY. This deduction is based on the present finding that reduced 5-deazaflavin is at least as good a catalyst as normal reduced flavin (Fig. 8). However, for thermodynamic reasons, 5-deazaflavins are not prone to form radical species. They are hindered in redox catalysis due to the kinetic stability of the C(5)-H2 function and are, thus, considered to be “half-dead” redox cofactors for several types of catalysis (61–63).

The interpretation and contraposition of data from IDI-2 and CrtY, thus, present a dilemma; Are the underlying mechanisms different despite the apparent similarities with respect to requirement of reduced flavin and absence of redox changes? To investigate, we took advantage of the fact that the introduction of substitutions with specific properties into the isoalloxazine system affects its redox potential (32, 64), thus enabling the use in the determination of linear free energy relationships (32). Because the introduction of modifications can have steric and chemical consequences, experience suggests modifications at a single position in the isoalloxazine ring system. The flavin position C(8) has proven to be the less critical (Fig. 9) and most sensitive as it is placed in para position to the site of redox catalysis, N(5)-C(4a). The experiment shown in Fig. 8 is based on the long established linear free energy relationship concept, according to which the rate of a reaction proceeding via a charged transient state will correlate with the electron donating/accepting properties of the involved molecules. A linear correlation between the 1e− oxidation potential of the reduced flavin and the $E_m$ has previously been demonstrated for a series of flavins carrying different substitutions at position C(8) (64). The results of Fig. 8 show that the rate of the lycopene cyclization increases with increasing “electron donating properties” (i.e. with decreasing $E_m$) of the reduced flavin analogs. This is compatible with “transfer of negative charge” from the reduced flavin in the transition state. In view of this, we concur in a mechanistic interpretation with the basic concept that was formulated by Laupitz et al. (60) for the IDI-2 reaction, “… the cofactor might act as a dipole stabilizing a cationic intermediate or transition state of the reaction.” However, it is necessary to extend this concept to read, “… the (anionic) reduced flavin cofactor might stabilize a cationic intermediate or transition state of the reaction.” The mechanism of Arabidopsis lycopene cyclases has been speculated to be similar (65).

We, thus, think that the present data can be interpreted in terms of Scheme 1. The reaction is initiated by formation of a π-complex between the reduced flavin in its anionized form and lycopene. Two variants can be envisaged. In one, shown on top, an acidic group (−B1H+) carrying a solvent-borne hydrogen (HS) interacts with the lycopene C(1)A C(2) double bond, whereas concomitantly, a base initiates a nucleophilic attack on HC. In the ensuing transition state the orbitals of the (partially) positively charged lycopene overlap with those of the negatively charged reduced flavin in a charge transfer complex. In the second variant (bottom structures) the reaction proceeds via a definite intermediate in which a positive charge is located either at the lycopene position C(1) or C(5) as in the original formulation by Britton et al. (31). The stereochemistry of orbital over-

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**Scheme 1**

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Lycopene Cyclase Catalyzes an FADred-dependent Reaction

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lap is formulated in analogy to that proposed by Arigoni et al. (54) for ring formation in the biosynthesis of lutein. Note that cyclization goes along with the formal transfer of a $\text{H}^+$ from base $B_1$ to base $B_2$. One of these could be Glu196. Although it is assumed that the species involved interact face to face via a $\pi$-complex, the further orientation of the molecules is arbitrary, and only the flavin orbitals that carry the largest negative charge density are shown. To further validate the model, attempts to crystallize the mCrtY-FAD-lycopene complex are currently under way.
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