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# Biochemical aspects of carotene desaturation and cyclization in chromoplast membranes from *Narcissus pseudonarcissus*

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Abstract- In *Narcissus* chromoplast membranes, phytoene is converted into coloured carotenes by two desaturases which differ in stereospecificity and which can be distinguished by herbicidal compounds. The desaturases represent flavoproteins, which gain FAD in an assisted folding pathway after being imported into the organelle. Electrons liberated in the desaturation are transferred to oxygen via an intermediate quinone carrier. The possibility of a co-reduction mechanism is discussed. The redox-state of quinones is regulated by an NADPH: quinone oxidoreductase which is membrane-peripherally localized. The end product of the desaturation, 7,9,7',9'-tetra-*cis*-lycopene (prolycopene), is the preferred substrate for cyclization, which is accompanied by an NADPH-dependent isomerization yielding *trans* cyclic products.

## INTRODUCTION

In plastids the carotenogenic pathway is topologically segregated to two distinct sites. The formation of phytoene is mediated by a membrane-peripheral complex consisting of three enzymes (isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase and phytoene synthase). Phytoene, as a very lipophilic product, is delivered to membrane enzymes which obey the operational criteria for integral membrane proteins. Within the membranes, an ordered succession of desaturation reactions leads to the formation of lycopene, which is then cyclized to form α- or ß- end groups.

The biochemistry of these enzymatic activities acting between phytoene and α/ß-carotene will be reviewed here, with reference mainly to investigations made with a system from isolated chromoplasts of *Narcissus pseudonarcissus*.

## TWO CAROTENE DESATURASES IN PLANTS

In contrast to bacteria and fungi, where only one desaturase is required to introduce the complete set of double bonds, two individual desaturation systems, each introducing two double bonds, operate in sequence in plastids. In *Narcissus* chromoplast membranes they can be distinguished biochemically by their different stereospecificity (Fig. 1). Whereas the first one introduces *trans* double bonds in the 11 and 11' positions, the second exhibits specificity for introducing *cis* double bonds in the 7 and 7' positions. *In vitro*, with [$^{14}$C]15-*cis*-phytoene as substrate, the bipartition of the desaturation sequence is also evident from the fact that the reaction is halted at the 15-*cis*-ζ-carotene stage. It has been found that the reaction proceeds beyond

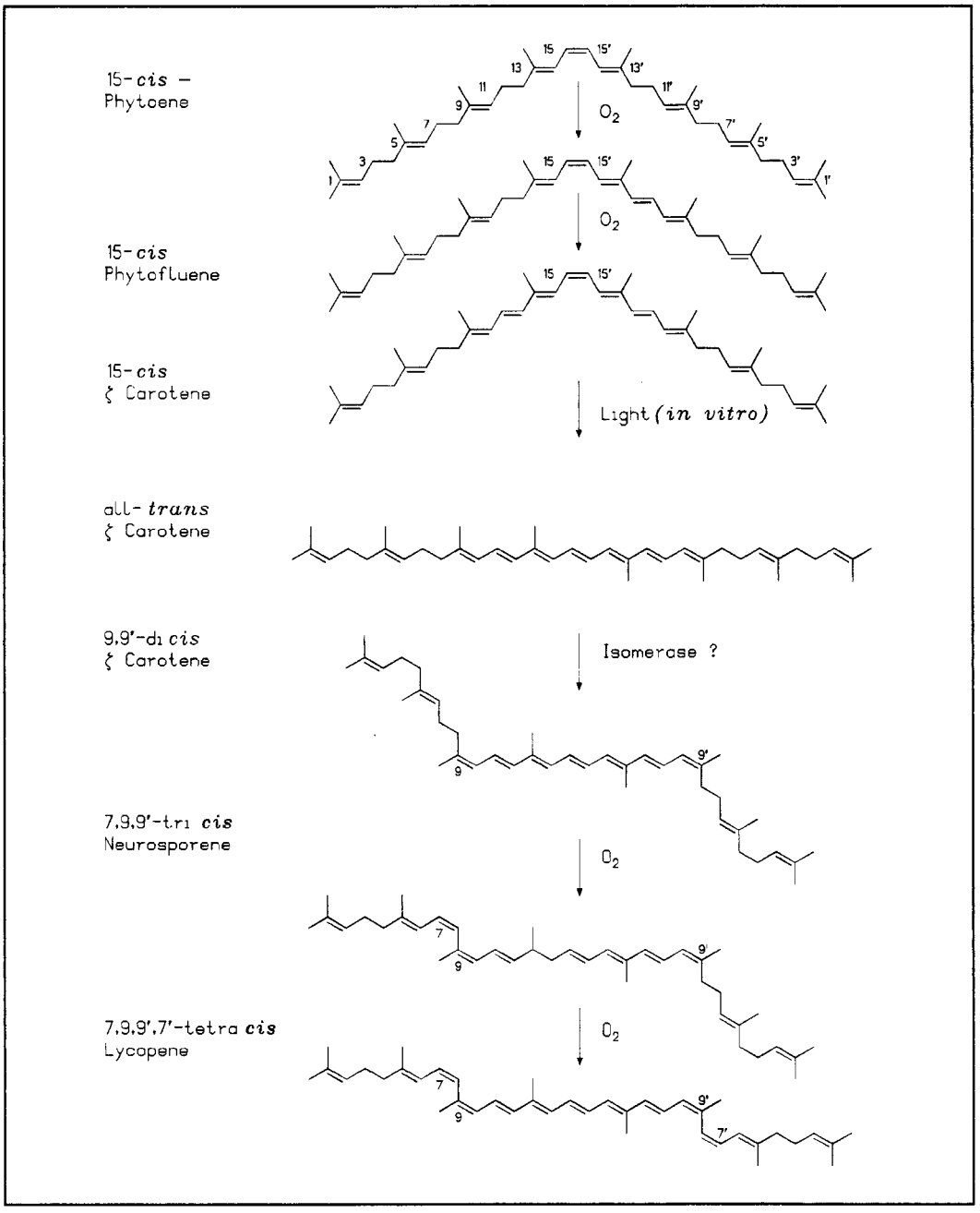


Fig. 1 Desaturation reactions in chromoplast membranes *in vitro*

this point when the central *cis* double bond in ζ-carotene is photoisomerized to *trans*. Then the stereochemical requirements of the second desaturase are fulfilled and the system proceeds towards lycopene. Prolycopene (7,9,7',9'-tetra-*cis*-lycopene) is the final product; daffodil desaturases perform a poly-*cis* pathway (ref. 1). A complication arises with respect to the double bonds at the 9 and 9' positions, which are not newly formed, but are present in phytoene. Because these do not have the *cis* configuration in phytoene, phytofluene and ζ-carotene, an isomerase must be assumed to act between the two

desaturases, converting these two double bonds from *trans* to *cis*. A definite answer to this problem is presently not available.

Two individual desaturases can also be distinguished by the mode of action of inhibitors, herbicidal compounds which in most cases exert bleaching effects on treated plants (ref. 2). Two examples are given in Table 1. The $I_{50}$ values show that Norflurazon is effective on the first desaturase exclusively, whereas $J_{334}$ is capable of affecting both desaturases, but is about twice as effective on the second one.

Unequivocal evidence for two different desaturases in plants has also been obtained by means of molecular biology, with the demonstration, by heterologous complementation, of the functional equivalence of cDNA-derived gene products from two different organisms. These gene products lead to the accumulation of ζ-carotene in transformed bacteria and thus represent in both cases the first desaturase (refs. 3-5). The second desaturase has now also been found in cyanobacteria (G.Sandmann, pers. communication).

TABLE 1. Inhibition of desaturation reactions

| Reaction | $I_{50}$ ($\mu$mol $l^{-1}$) | |
|---|---|---|
| | Norflurazone | $J_{334}$ |
| Phytoene to ζ-carotene | 0.4 | 16.7 |
| ζ-Carotene to lycopene | no inhibition | 7.3 |

## CONSIDERATIONS OF THE MECHANISMS OF THE DESATURASE REACTIONS

Desaturases require electron acceptors for the two electrons that are eliminated for each double bond being formed. We have reported previously in this series that molecular oxygen plays this role (ref. 6). However, unlike the case of fatty acid desaturation, for example, a direct interaction of oxygen with the desaturase cannot be claimed, since oxygen can be replaced by quinones. Thus, as can be seen in Fig. 2, phytoene desaturation in purified chromoplast membranes is possible also under anaerobic conditions in the presence of an oxidized quinone. Since a competition between oxygen and a quinone for the active center of the desaturase is unlikely, a plastid quinone appears to be an intermediate electron carrier between the desaturase and oxygen (ref. 7).

Further support for the relevance of quinones comes from recent work in which the mode of action of a herbicidal compound (SC-0051), which causes bleaching in plants, has been elucidated (ref. 8). Other bleaching herbicides typically affect the phytoene desaturase *per se*. In contrast, this compound, although it also leads to massive phytoene accumulation *in vivo*, acts as an inhibitor of the enzyme *p*-hydroxyphenylpyruvate dioxygenase (E.C. 1.13.11.27), which catalyses an intermediate step in the formation of plastoquinone and tocopherol in plants. Therefore, phytoene accumulation caused by this compound and the concomitant bleaching in treated tissues must be regarded as indirect desaturase inhibition, affected by the withdrawal of an essential quinone and/or tocopherol cofactor.

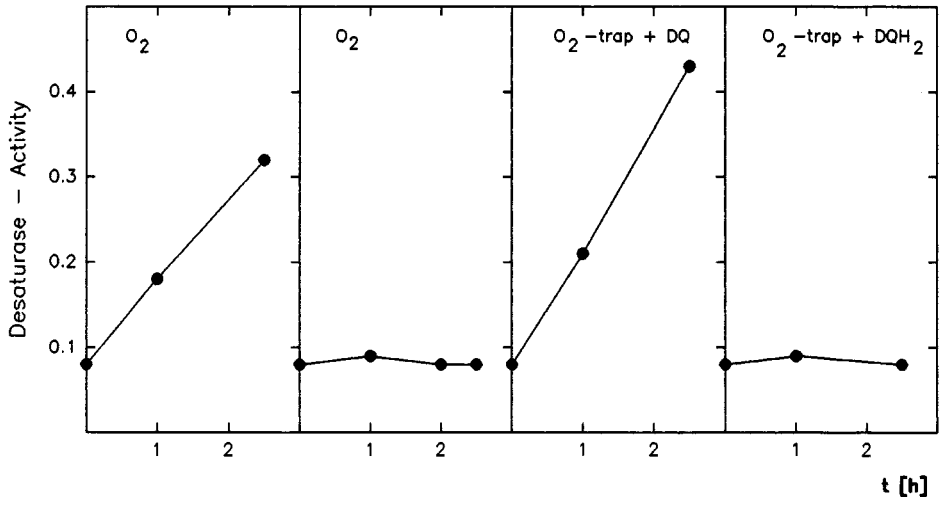


Fig. 2. Reactivation of ζ-carotene formation under anaerobic conditions. The substrate, unlabelled 15-*cis*-phytoene was supplied from an acetone stock solution to give a final concentration of 6 μM. Duroquinone (DQ) was supplied at a 80 μM final concentration. Desaturase activity is in nmol·ml$^{-1}$ ζ-carotene formed.

Since desaturation reactions in general represent oxidation reactions, given the involvement of quinones and oxygen in carotene desaturation, the implication is that the redox state of the micro-surroundings of the desaturase is central for the reactions to proceed. All phytoene desaturases so far examined possess in their sequence a highly conserved domain which is common for many FAD-containing enzymes, presumably an FAD binding site. A number of such FAD sites, including the one from a *Narcissus* carotene desaturase are given in Fig 3.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| I | 102 | IVIA- | GAG | LA | GL | STAKYLADAGHKPI | 126 |
| II | 113 | EIVIA | GAG | LA | GL | STAKYLADAGHKPI | 138 |
| III | 3 | RVAIA | GAG | LA | GL | SCAKYLADAGHTPI | 25 |
| IV | 3 | KTVVI | GAG | FG | GL | ALAIRLQAAGIP-T | 25 |
| V | 10 | RAVVI | GAG | LG | GL | AAAMRLGAKGYK-V | 32 |
| **Narcissus** | | EVVVV | GAG | LA | GL | STAKYLADAGHKPI | |

Fig. 3. Amino acid sequence alignment of FAD-binding sites in carotene desaturases. (I) *Glycine max*, (II) *Lycopersicon esculentum*, (III) *Synechococcus*, (IV) *Erwinia herbicola*, (V) *Rhodobacter capsulatus*.

A purified phytoene desaturase from *Capsicum* chromoplasts was shown to have the spectrum of a yellow substance (ref. 4) and the desaturase from *Erwinia uredovora*, purified from a recombinant source as inclusion bodies, showed a partial FAD-requirement for activity (ref. 9). In

contrast, in our system we do not find any requirement for exogenously added FAD by phytoene desaturase. Phytoene desaturase in purified membranes or after reconstitution into liposomal membranes works more actively in the absence of all cofactors than in their presence. We conclude that FAD is tightly bound to the enzyme and, as a consequence, desaturation stringently requires the quinone-oxygen redox pathway in the plane of the lipid bilayer.

Thus, it must be assumed that a mechanism exists which, at least once in a lifetime of the protein, assembles FAD into the polypeptide chain.

In plants the carotenogenic enzymes are nuclear encoded. As has been shown in recent years for chloroplasts, nuclear-encoded proteins are synthesized in the form of pre-proteins on cytoplasmic ribosomes and then transported to the plastid envelope for translocation. Cytoplasmic factors are required to maintain translocation competence, basically by maintaining the protein in a loosely folded state. Once translocated into the plastid, similar stromal factors, molecular chaperonins, play a major part in the assembly of soluble oligomeric protein complexes and in the integration of proteins into membrane structures (ref. 10). Similarly, at least the membrane-bound components of the carotenogenic path, which we believe to be structurally highly ordered, could assemble and fold into the membrane in an assisted way, thereby gaining the FAD. This seems indeed to be the case in chromoplasts, since it is possible to enrich a large hetero-oligomeric particle from the membrane-free supernatant, which contains phytoene desaturase. Phytoene desaturase can be released *in vitro* into protein-free liposomes, which we then have purified by several washings, including high-salt treatments.

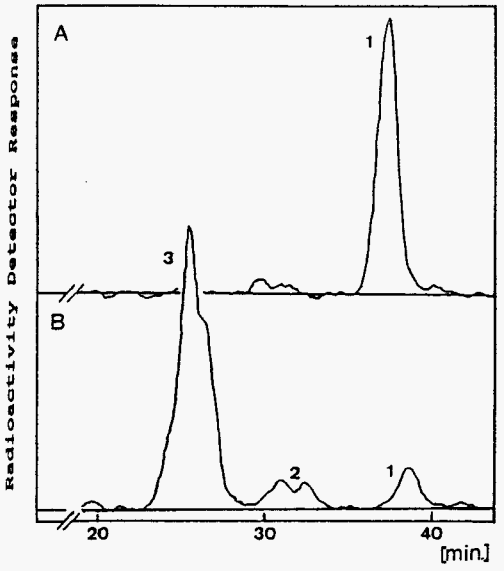


Fig. 4. Incorporation of radioactive phytoene into carotenoid products in liposomes after folding in the absence (A) and presence (B) of FAD. 1, phytoene; 2, phytofluene; 3, ζ-carotene. Substrate: 15-*cis*[$^{14}$C]phytoene, 70 000 dpm.

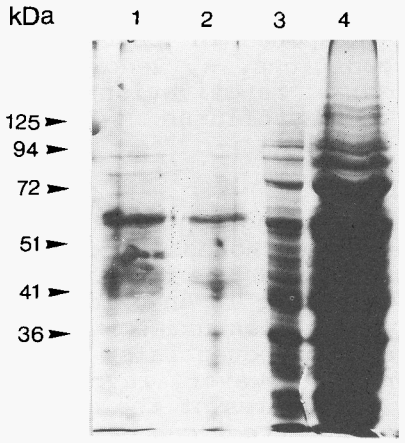


Fig. 5. SDS-PAGE of washed liposomes after folding in the absence (1) and presence (2) of FAD. Lane 4 represents, in a quantitative comparison, the total stromal proteins from which the proteins in lanes 1 and 2 in Fig. 4 are derived.

When FAD, as a cofactor, is included in the membrane folding/assembly experiments, this results in a desaturase-active liposome. In the absence of FAD no desaturase activity is obtained, although the protein also assembles in the liposomal membrane under these conditions (Fig. 4). This can be shown by SDS-PAGE (Fig. 5) in which in both cases essentially only one band is observed under stringent folding conditions. Interestingly and also consistent with the idea of tightly-bound FAD, these inactive liposomes cannot be activated by subsequent addition of the cofactor. Thus, phytoene desaturase gains its cofactor during the membrane assembly. We are currently investigating two chaperonin proteins, members of the hsp 60 and hsp 70 families, which we believe are involved in this folding. Both are up-regulated during the cloroplast to chromoplast differentiation.

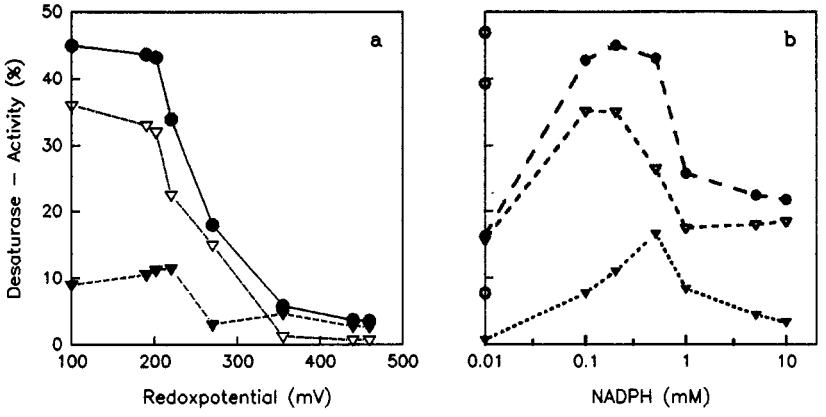


Fig. 6. Response of phytoene desaturase towards externally applied redox conditions. a, Redox-titration of purified membranes with ferricyanide; b, reactivation of the ferricyanide-inactivated system with NADPH. ▲- phytofluene, △- ζ-carotene, ●- phytofluene plus ζ-carotene.

Given the findings outlined above, it must be concluded that the redox state of the quinones plays a major part in the reaction mechanism, and one would expect the desaturase to work with maximal velocity in membranes which are in an oxidized state. The converse, however, is true. When phytoene desaturase is transferred to environments of increasingly positive redox potentials, its activity decreases (Fig. 6a). This inactivation is, however, reversible upon the addition of NADPH, and the initial velocities of the desaturase then show an optimum (Fig. 6b); a very similar effect can be obtained by using hydroquinones. A numerical value for the redox potential at the optimum (at less positive redox potentials, Fig. 6a) cannot be given since aerobic conditions are required for desaturase to function. It may be speculated that reduced as well as oxidized states of quinones must be present. The presence of reduced species would be stringently required if phytoene desaturase represents a homodimer, each monomer introducing the symmetrical double bond in the form of a half-site recognition of the phytoene or ζ-carotene substrate as was proposed earlier. Under these conditions each individual double bond in the product would be related to oxygen, so a co-reduction of a second substrate could become necessary in order to gain stoichiometric balance. The cytochrome $b_5$-dependent fatty acid desaturation system may be taken as an analogous example. These possible interrelations are currently being investigated.

If quinones do indeed play an important redox role, it would be necessary to postulate an enzymatic activity that resides peripherally to the membrane, and thus is capable of regulating the redox states of quinones. Photosystems, which exert this function in chloroplast, are not present in chromoplasts, and the above mentioned reactivation of phytoene desaturation in oxidized membranes points to NADPH as the electron donor. We have purified a protein which exhibits these postulated properties and has a molecular mass of 44 kDa as judged by SDS PAGE and gel filtration. The investigation of the specificity of the electron acceptor for this oxidoreductase revealed that the enzyme is capable of reducing quinoid compounds at the expense of NADPH ($K_m$ 123 $\mu$M) and NADH ($K_m$ 897 mM) (ref. 11). Partial sequences so far obtained do not show homology with other oxidoreductases.

## CYCLASE

Oxygen plays a dual role in the pathway from phytoene to ß-carotene, inasmuch as it is required for the desaturation reactions, whereas the cyclization reactions proceed only in its absence, as was pointed out earlier (ref. 1). Cyclization requires a cofactor, NADPH, which is surprising, because lycopene and $\alpha$/ß-carotene possess an equal number of electrons (ref. 12). Such a cofactor requirement may become explicable if NADPH is involved in an additional isomerization reaction: only tetra-*cis*-lycopene and related *cis* structures are accepted as substrates in the cyclization reaction *in vitro* in *Narcissus* chromoplast membranes (Table 3), whereas the $\alpha$/ß-carotene that is formed is predominantly all-*trans*. This means that the lycopene cyclization comprises two partial reactions. First, isomerization of the tetra-*cis*

TABLE 3. Conversion of unlabelled, chemically synthesized lycopene isomers into products in chromoplast membranes.

| Lycopene isomers (Substrates 2.5 $\mu$M) | % Conversion | Products | | | |
|---|---|---|---|---|---|
| | | $\delta,\gamma$-car. | $\alpha$-car. | ß-car. | other lyc. isomers |
| 7,9,7'9'-tetra-*cis* | 35 | 3 | 2 | 17 | 13 |
| 7,7'-di-*cis* | 37 | trace | 1 | 17 | 19 |
| 5,5'-di-*cis* | 0 | - | - | - | - |
| all-*trans* | 0 | - | - | - | - |

substrate into *trans* and, second, the cyclization reaction *per se*. That isomerization reactions take place at the stage of lycopene can be recognized from the analysis of a cylization reaction as given in Fig. 7. The isomerization of lycopene proceeds separately in each half of the symmetrical molecule, since 7,9-di-*cis* and all-*trans*-lycopene appear as main products. It cannot be distinguished at present whether a separate isomerase exists or whether the conversion of the tetra-*cis* substrate into (mainly) all-*trans* bicyclic products is an intrinsic property of the cyclase itself, but we have designed experimental conditions under which the isomerization reaction can be investigated independently. By using the well-known inhibitor CPTA at low concentrations (20 $\mu$M) a complete inhibition of prolycopene cyclization is observed, whereas the isomerization reaction remains active; it is inhibited at much higher CPTA-concentrations (800 $\mu$M) (Table 4).

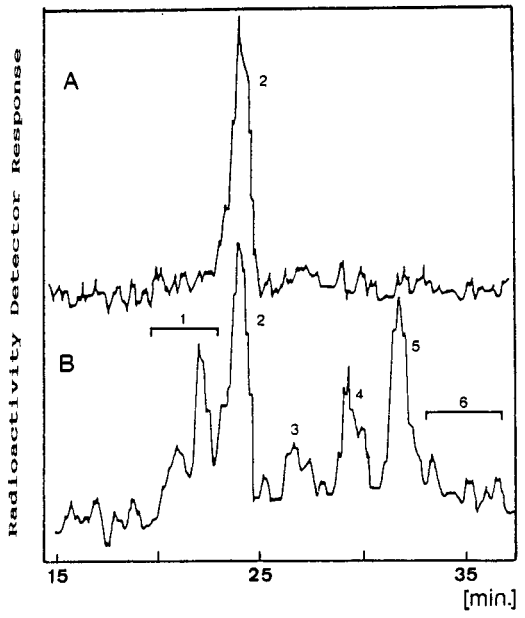


Fig. 7. HPLC separation of substrate and products in a cyclization assay. A, control, prolycopene, coincubated in the presence of organic solvents. B, enzymatically active assay. 1, lycopene isomers other than 2; 2, prolycopene; 3, monocyclic carotenes; 4, α-carotene; 5, *trans*-ß-carotene; 6, other ß-carotene isomers.

It has been found that the decoupled isomerization reaction is as dependent on NADPH as is the overall cyclization reaction, so that it may be an intramolecular redox reaction involving the sequence: reduction of the *cis* double bond, rotation, reoxidation and thereby *trans* double bond formation. The coenzyme NADPH may participate in a catalytic way (being regenerated after the reaction cycle) or its two electrons may be transferred to other reactions. The latter seems to be the case, since at saturating substrate concentrations (prolycopene, 2 μM; NADPH, 1 mM) and under the anaerobic incubation regime used, a stereochemically correct reversal of the preceding desaturation is observed, and prolycopene is reduced to proneurosporene (7,9,9'tri-*cis*-neurosporene; data not shown).

TABLE 4. Differential Inhibition of prolycopene isomerase and cyclase activity by CPTA. Radioactivity recovered after incubation with [$^{14}$C]prolycopene.

| CPTA (μM) | [14C]Prolycopene (%) | Other [14C] lycopene isomers (%) | Cyclic carotenes (%) |
|---|---|---|---|
| Control | 100 | 0 | 0 |
| 0 | 29.8 | 20.0 | 50.2 |
| 20 | 56.7 | 43.3 | 0 |
| 800 | 100.0 | 0 | 0 |

## OVERVIEW

The scheme in Fig. 8 is an attempt to unify the findings made with purified chromoplast membranes into a concept for further investigations, in which pure proteins from recombinant sources will play an important role. As depicted, two desaturases, differing in stereospecificity work sequentially, releasing 7,9,7',9'-tetra-*cis*-lycopene (prolycopene). The arrest at the ζ-carotene stage *in vitro* is, in our in-

terpretation, due to a pertubation of the topological order of the complex, caused by the experimental manipulations. This is why photoisomerization of 15-*cis*-ζ-carotene to *trans* is required for the formation of the additional two double bonds (although light plays no role in the desaturation reactions *in vivo*). For the isomerization of the 9 and 9′ double bonds from *trans* to *cis* (see Fig. 1), an isomerase must be postulated; however, the reaction could also be a property of the second desaturase itself. Similarly, prolycopene represents an additional arresting point in *vitro*; it is not accumulated *in vivo*, and only trace amounts can be detected in membranes. This point can be overcome

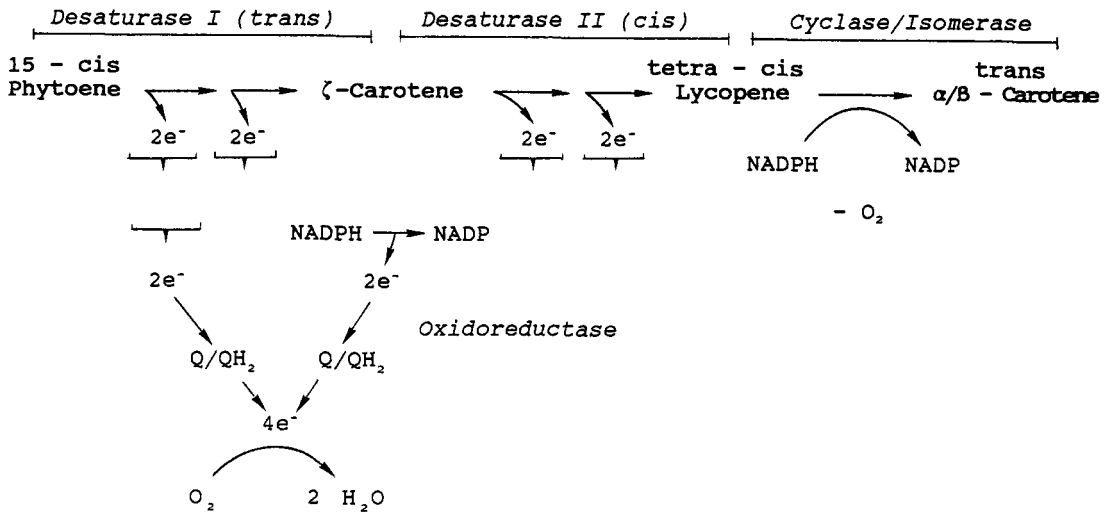


Fig. 8. Let's have a hypothesis. For explanations, see text.

experimentally by using anaerobic conditions under which the cyclases are active, although anaerobiosis is unlikely *in vivo*. Again, microtopology may provide an explanation, inasmuch as the oxygen-consuming desaturation reactions could become structurally and functionally coupled, rendering the active center of the cyclase oxygen-free. Indeed, it was proposed earlier that the chemical mechanism of the cyclase is an anaerobic one (ref. 13). Cyclization comprises two partial reactions, isomerization from *cis* to *trans* and cyclization itself; the isomerization is NADPH-dependent.

In the redox mechanism of the desaturases, an electron transfer to oxygen via quinones takes place. The electron-donating branch accounts for the fact that fully oxidized membranes (all quinones oxidized) are desaturation-inactive, but can be reactivated by NADPH (via the NADPH:-quinone oxidoreductase) or reduced quinone compounds. The mechanism (co-reduction, similar to fatty acid desaturation mechanisms) could provide a stoichiometric balance in oxygen reduction, as discussed above. A co-reduction mechanism could become essential, if one desaturase is present in a homodimeric assembly, each monomer being responsible for the formation of a single double bond, but this remains to be shown.

Two further questions should be briefly considered: Which enzyme reduces oxygen to water and what energy drives the desaturation pathway? We have not yet investigated the hydroquinone-dependent oxygen reducing activity, but it is interesting to note that just such an

activity is involved in the phenomenon of chlororespiration (ref. 14). The related question arises as to how a plant-type phytoene desaturase, is capable of exerting its function in *E. coli* membranes (ref.15) where the plastid redox systems do not exist. Since phytoene desaturase from daffodil is quite non-specific with respect to quinones (e.g. accepting duroquinone, see above), the enzyme may well take advantage of the pre-existing respiratory redox chain, using its ubiquinone pool. The desaturation reactions depicted in the horizontal direction in Fig. 8 proceed energetically uphill. They could be driven by the redox potential difference between the NADPH/NADP and $H_2O/O_2$ redox-couples. These still speculative proposals are being investigated further using the folding pathway of desaturases with the aid of properly designed liposomes.

ACKNOWLEDGEMENTS

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