FAD is a further essential cofactor of the NAD(P)H and O_2-dependent zeaxanthin-epoxidase

Karen Büch, Harald Stransky, Achim Hager*

Botanisches Institut, Allgemeine Botanik und Pflanzenphysiologie, Universität Tübingen, Auf der Morgenstelle 1, D-72076 Tübingen, Germany

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Abstract In chloroplasts of plants the xanthophyll cycle is suggested to function as a protection mechanism against photodamage. Two enzymes catalyze this cycle. One of them, violaxanthin de-epoxidase, transforms violaxanthin (Vio) to zeaxanthin (Zea) via antheraxanthin (Anth) and is bound to the lumenal surface of the thylakoid vesicles, when being in its active state. The other enzyme, Zea-epoxidase, is responsible for the backward reaction (Zea → Anth → Vio) and is active at the stromal side of the thylakoid. For the epoxidation of Zea this enzyme requires NAD(P)H and O_2 as cosubstrates. Using isolated thylakoid membranes we found that FAD enhances the epoxidase activity (decrease of apparent K_m for NAD(P)H and two-fold increase of V_{max}). The flavin functions as a third cofactor which is partially lost during the isolation procedure of thylakoids. Other flavins, such as FMN or riboflavin are without effect. The involvement of FAD in the enzymatic reaction is also demonstrated by the inhibitory action of diphenyleneiodoniumchloride (DPI) (IC_{50} = 2.3 μM), a compound that blocks the reoxidation of reduced flavins within enzymes. The Zea-epoxidase is a multicomponent enzyme system which can be classified as FAD-containing, NAD(P)H- and O_2-dependent monooxygenase that is able to epoxidize 3-hydroxy β-ionone rings of xanthophylls in the 5,6 position.

Key words: Diphenyleneiodoniumchloride; Epoxidation; FAD; Flavin-containing monooxygenase (FMO); *Spinacia*; Xanthophyll cycle

1. Introduction

The conversion of Vio to Zea in thylakoids of green plants upon strong illumination is triggered by a surplus of energy [1–9] that cannot be used for the photosynthetic process due to different reasons, e.g. shortage of CO_2, of water, of intermediate products, etc. In this case, the enzyme Vio de-epoxidase, normally mobile within the thylakoid lumen, is activated via a lowered luminal pH [10,11] and becomes tightly bound to the membrane, thus gaining access to its substrate Vio [12] and transforming it to Zea. Under low-light conditions or in the dark another enzyme is responsible for the backward reaction, the conversion of Zea to Vio. This enzyme is a mixed-function oxygenase [13] using O_2 [14,15] and NAD(P)H [16–18] as cosubstrates. In vitro, the activity of this enzyme and its affinity for the substrate NADH is relatively low [17]. Since NADPH cannot move through the thylakoid vesicle membrane, the epoxidase was suggested to be associated with the stromal side of this membrane [16]. Recently, Zea epoxidation activity was attributed to isolated LHCsII [19,20].

In this study we demonstrate that a further cofactor, flavin adenine dinucleotide (FAD), is involved in the NAD(P)H- and O_2-dependent epoxidation of Zea. Presumable, one part of the protein-associated flavin moiety is lost during the isolation procedure of the thylakoid membranes and consequently the epoxidase activity is attenuated. Addition of FAD to the thylakoid suspensions restores the enzyme activity. A further indication for the importance of this flavin during the Zea epoxidation is the strong effect of DPI, an inhibitor of flavin-containing enzymes [21,22].

2. Materials and methods

2.1. Plant material

Spinach plants (*Spinacia oleracea* L. cv. Butterfly) were cultivated in a growth chamber (10 h light, 23°C, 55% rel. humidity; 14 h dark, 15°C, 70% rel. humidity). The illumination period was started and terminated by a dawn and dusk period of 1 h. For illumination HQI-R 250 W bulbs (Osram) were used (140 μmol photons · $m^{-2} \cdot s^{-1}$). All experiments were performed with 6-week-old leaves.

2.2. Thylakoid isolation

Thirty leaves were illuminated (2800 μmol · $m^{-2} \cdot s^{-1}$) for 30 min under N_2-gas to accumulate Zea. Subsequently, leaves were homogenized in 100 ml of isolation buffer (pH 7.5) containing 400 mM sucrose, 50 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 1% (w/v) bovine serum albumin (BSA) and 20% glycerol. Cell debris was removed by centrifugation at 310 × *g* for 5 min. The supernatant was recentrifuged at 3000 × *g* for 5 min. The dark-green pellet was resuspended (80 ml isolation buffer) and recentrifuged as above. The chloroplasts in the pellet were lysed by addition of 12 ml $MgCl_2$ (6 mM in destilled water). After 3 min an equal amount of twofold concentrated isolation buffer was added. The thylakoids were pelleted at 10000 × *g* for 5 min. The pellet was resuspended in 40 ml isolation buffer and adjusted to a concentration of 300 μg chlorophyll · ml^{-1}. For epoxidation experiments (25°C) this suspension was diluted 1:2 with test solution (100 mM Tris-HCl, 2 mM NADH, pH 7.5).

2.3. Epoxidation and extraction of pigments

Epoxidation reactions were terminated by rapid cooling and centrifugation of the thylakoid suspensions at 2°C (3900 × *g* for 10 min). Thereafter 10 ml of acetone (100%, 0°C) and 0.2 g $NaHCO_3$ were added to the pellet. The green suspensions were passed through a filter funnel (porosity 4; Schott AG, Mainz, Germany), the filtrates were evaporated to dryness and redissolved in 2 ml of acetone. To obtain a particle-free solution of pigments for HPLC analysis, a centrifugation at 20,000 × *g* was performed.

2.4. HPLC of thylakoid pigments

Pigment extracts were analysed using an ODS-Hypersil column (4.6 × 60 mm, 3 μm particle size) in combination with a guard column (4.0 × 20 mm, 5 μm particle size). A mobile phase gradient consisting of increasing portions of acetone in aqueous buffer (1 mM $NaHCO_3$) was run at 30°C with a flow rate of 1.2 ml · min^{-1} [23].

*Corresponding author. Fax: (49) (7071) 293287.

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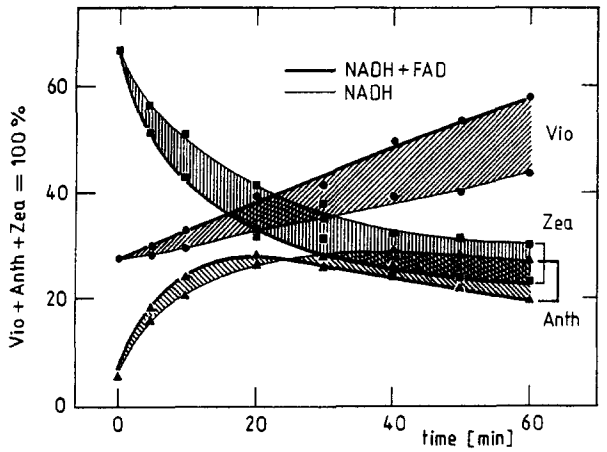

Fig. 1. Influence of FAD on the kinetic of the NADH- and O_2-dependent epoxidation of Zea to Vio via Anth. Suspensions of thylakoids (150 μg Chl·ml^{-1}) were incubated at pH 7.5 and 25°C with or without FAD (10 μM) in the presence of NADH (2 mM). Pigments were extracted by acetone and separated by HPLC. Results are mean values ± S.D. of three independent experiments (n = 3).

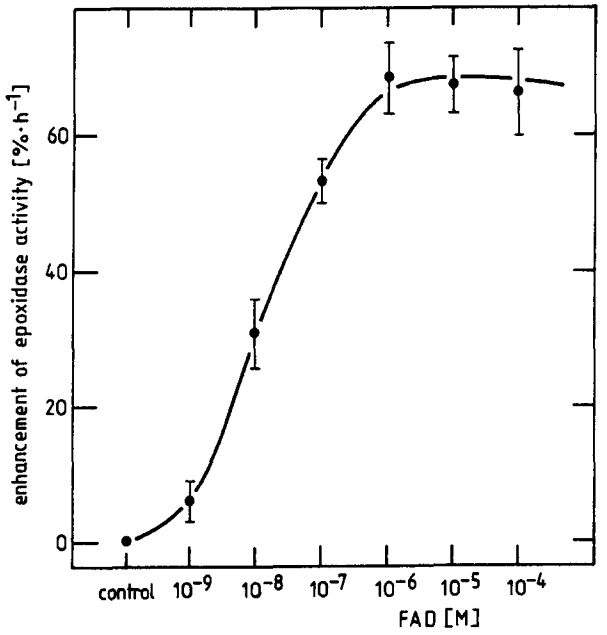

Fig. 2. Stimulation of the NADH- and O_2-dependent epoxidase activity by FAD. Conditions as described in Fig. 1 and section 2 (n = 3).

3. Results

3.1. Stimulation of Zea-epoxidase by FAD

The time course of the NAD(P)H- and O_2-dependent transformation of Zea to Vio in isolated thylakoid membranes is demonstrated in Fig. 1. In the presence of FAD the decrease of Zea and an appropriate increase of Vio are augmented. Only 10 μmol·l^{-1} FAD are sufficient to raise the epoxidase activity by 31% (Fig. 2). As displayed by the Lineweaver-Burk plot FAD increases V_{max} and decreases the apparent K_m of the NADH-dependent epoxidation of Zea (Fig. 3). The action of FAD is specific; other flavins, such as FMN or riboflavin (Fig. 4) have no or only a small inhibitory effect presumably caused by the oxidation of NADH via flavin-catalyzed side reactions. The stimulation of the epoxidation by FAD occurs without an appreciable lag phase indicating a fast binding of FAD to the reaction center (results not shown).

3.2. Inhibition of Zea-epoxidase by DPI

The mode of action of DPI, a special inhibitor for flavin-containing enzymes [21,22], is probable attributed to the formation of an adduct between reduced flavin and the inhibitor preventing a reoxidation of the flavin. If the Zea-epoxidase is a flavin-containing monooxygenase, DPI should be able to inhibit the activity of this enzyme. The action of DPI (dissolved in DMSO) on Zea epoxidase activity in thylakoid suspensions (150 μg Chl·ml^{-1}) was tested at different concentrations (0.8–10 μM) (Fig. 5). Already 2.3 μM DPI cause an inhibition of 50%. In order to check whether NADH is sufficiently present in the reduced state during the entire reaction time, its amount was measured at 340 nm in the thylakoid supernatant without interference by DPI (absorption around 274 nm). It was found that 90% of the added NADH (2 mM) was still present at the end of the experiment (data not shown). Also, in a control test the inhibitor itself did not change the redox state of NADH. The inhibition of Zea-epoxidase by DPI is a further proof of the participation of a flavin in this reaction. In contrast, DPI is without effect on the activity of the other xanthophyll-cycle

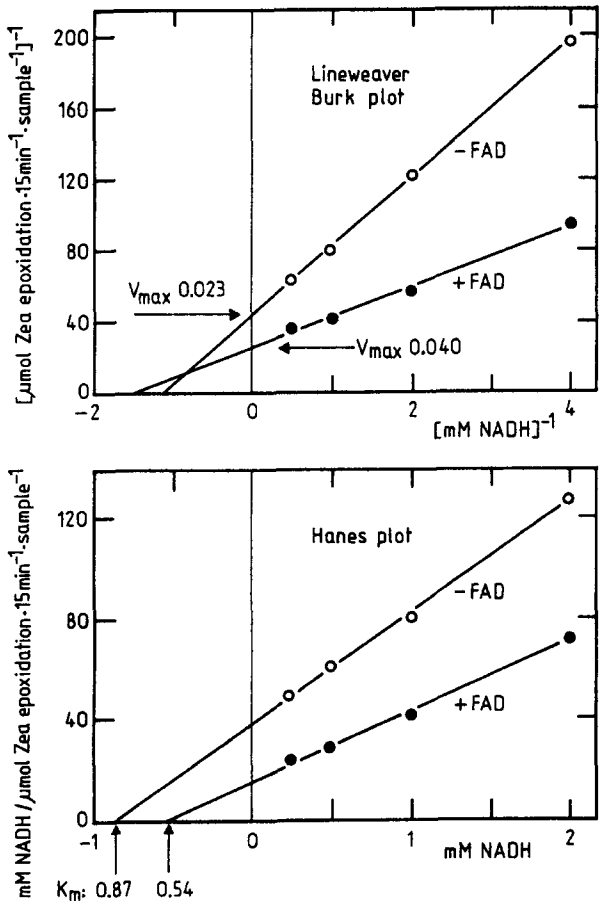

Fig. 3. Lineweaver-Burk and Hanes plot for the NADH-dependent reactions catalyzed by Zea-epoxidase in the presence and absence of FAD at pH 7.5, 25°C.

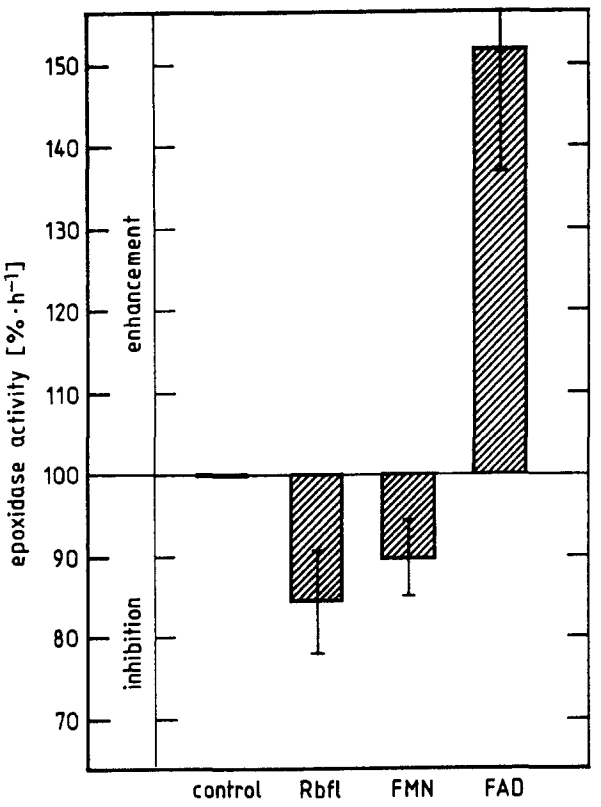

Fig. 4. Influence of flavins (10 μM) on the NADH- and O_2-dependent Zea-epoxidation. Conditions see Fig. 1 and section 2. Only FAD enhances the reaction (n = 3).

enzyme, the ascorbate-dependent Vio de-epoxidase (results not shown). However, this latter enzyme can strongly be inhibited by DTT [24], a compound that does not influence the epoxidase activity. Therefore, for each of the two xanthophyll-cycle enzymes a specific inhibitor exists.

4. Discussion

Monooxygenases incorporate one atom of oxygen into its substrate while the other atom of oxygen is reduced to water. These enzymes use external reducing agents (NAD(P)H; ascorbate; H_4-pterin) to provide electrons for the reduction of oxygen to the level of hydrogen peroxide, which then is responsible for the insertion of oxygen into the substrate [25]. This fixation of oxygen into the reaction products is an energy-consuming process.

All oxygenases contain a non-protein oxygen-reactive group, such as heme, flavin, pterin, and transition metal ions (Fe, Cu). Flavin-containing oxygenases seem to be limited to those types of oxidations that are possible with alkylperoxides or oxygen in the absence of a metal ion [25]. FAD is the most important coenzyme of those flavin-dependent monooxygenases [26]. However, until now only a few flavin-containing monooxygenases have been described that epoxidize an alkene as substrate, e.g. the squalene epoxidase from pig liver [27] and the alkene monooxygenase from *Mykobacterium* [28].

The most thoroughly studied member of a group of flavoproteins is *p*-hydroxybenzoate hydroxylase (pHBH), an enzyme that carries out hydroxylation of activated aromatic compounds [29]. In the first reaction cycle of the enzyme the exogenous reducing agent NADPH rapidly interacts with FAD and the substrate to form a ternary complex [30]. This complex is competent for rapid reduction of FAD. The central dihydropyrazine ring of $FADH_2$ can easily be oxidized by the removal of one or two electrons. The semiquinone intermediate is the basis for the fast reaction of $FADH_2$ with O_2 forming a flavin-C4a-hydroperoxide [31,32]. In an intermolecular reaction the cleavage of the O–O bond of this hydroperoxide within the active center of the enzyme leads to the monooxygenation of the substrate. The residual pseudo-base, 4a-hydroxyflavin, is finally retransformed to FAD through the detachment of H_2O, an intramolecular generated molecule [26,25].

Interestingly many flavoenzymes can reversibly be converted to their corresponding apoproteins under relatively mild conditions [33], and subsequently be reconstituted by incubation with an excess of FAD [34]. This property may be important for the interpretation of experiments of our studies describing the epoxidation of xanthophylls in the thylakoid membrane of spinach. The enzymic reaction is light independent and occurs at the stromal side of the thylakoids using the substrates Zea or Anth, which are localized within the thylakoid membrane either in the lipid bilayer adjacent to (enzymic) proteins [12] or in pigment protein complexes, e.g. the inner antenna complexes [35] or the LHCs of PSI and II [36] or in ELIPs (early light-induced proteins) [37]. The co-substrates of the reaction were reported to be NAD(P)H and O_2 [16–18]. However, we found that the epoxidase activity can be strongly enhanced by FAD. A portion of these flavin molecules of the epoxidases is apparantly lost during the isolation of thylakoids. Consequently, the addition of FAD to the membrane suspension enhances the enzyme activity again (Figs. 1 and 2). Other flavin species, such as riboflavin or FMN are without effect (Fig. 4).

The question arose whether the reductant NADH, which is able to reduce FAD, can be substituted by the reduced cosubstrate $FADH_2$. The reduction of FAD was performed in the dark by Cu(I)-EDTA or dithionite, or by illumination of thy-

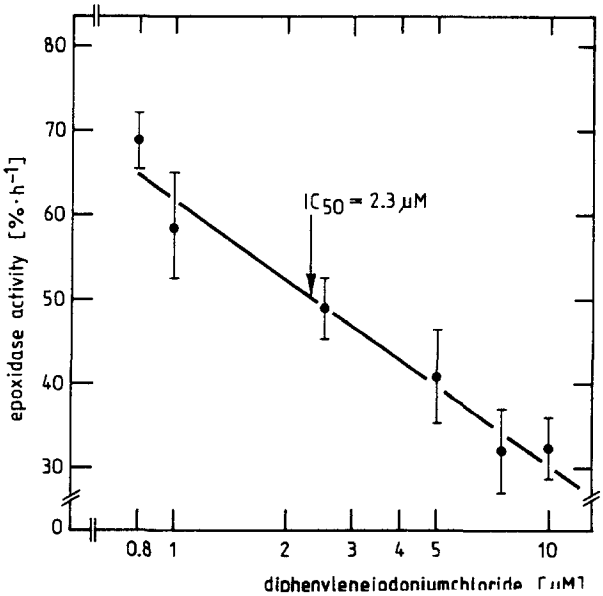

Fig. 5. Inhibition of the NADH- and O_2-dependent epoxidation of Zea to Vio by the flavin-specific inhibitor DPI (dissolved in DMSO, final concentration 0.2%). Conditions, see Fig. 1 (n = 3).

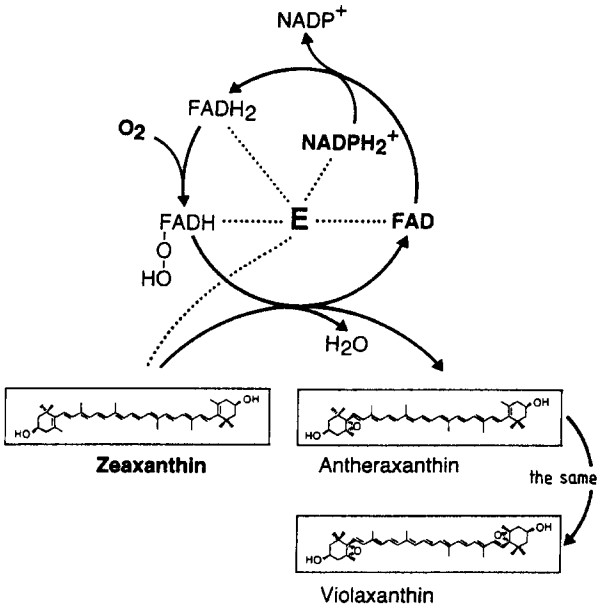

Fig. 6. Model of the flavin-catalyzed epoxidation of Zea to Vio via Anth. Epoxidation is presumed to occur in a ternary complex of $NADPH_2$, FAD and the substrate Zea (or Anth) at the enzyme (E); in the active center, exogenous $NADPH_2$ reduces FAD to $FADH_2$ which is subsequently transformed with O_2 to a 4a-hydroperoxide; this peroxide mojety is used for the monooxygenation of Zea (or Anth) and the detachment of the other O atom as H_2O.

lakoids in the presence of electron donators, such as ferredoxin, EDTA or DTT. It was found that the epoxidation of Zea could neither be triggered by adding reduced FAD nor by reducing the intrinsic FAD (data not shown).

We therefore suggest that during Zea-epoxidation a sequential mechanism occurs which is comparable with that catalyzed by pHBH (see above). This enzyme rapidly forms a ternary complex with NADPH, oxidized FAD and the substrate *p*-hydroxybenzoate [30,38]. The consumption of NADPH is strictly controlled at this point. A binary complex without the substrate *p*-hydroxybenzoate reduces FAD 10^5-fold slower [39]. This property of the enzyme is advantageous, because the reaction of reduced FAD with oxygen in the absence of the substrate would wastefully produce H_2O_2.

The kinetic behaviour of the Zea-epoxidase in respect to the substrate NADH ± FAD as shown by the Lineweaver-Burk and the Hanes plots (Fig. 3) can be interpreted as a partially non-competitive effect of FAD on the reaction. The formation of a complex of the diverse substrates with the enzyme simultaneously with a conformational change of the enzyme is likely. Fig. 6 illustrates the presumed ordered sequential mechanism between NAD(P)H, FAD, O_2 and Zea resulting in the monooxygenation of Zea or Anth.

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