FEBS Letters 350 (1994) 271–274

FEBS 14397

Epoxidation of zeaxanthin and antheraxanthin reverses non-photochemical quenching of photosystem II chlorophyll *a* fluorescence in the presence of trans-thylakoid ΔpH

Adam M. Gilmore**, Narendranath Mohanty, Harry Y. Yamamoto*

University of Hawaii, Department of Plant Molecular Physiology, St. John 503, 3190 Maile Way, Honolulu, Hawaii 96822, USA

Received 28 June 1994

Abstract

The xanthophyll cycle apparently aids the photoprotection of photosystem II by regulating the nonradiative dissipation of excess absorbed light energy as heat. However, it is a controversial question whether the resulting nonphotochemical quenching is soley dependent on xanthophyll cycle activity or not. The xanthophyll cycle consists of two enzymic reactions, namely deepoxidation of the diepoxide violaxanthin to the epoxide-free zeaxanthin and the much slower, reverse process of epoxidation. While deepoxidation requires a transthylakoid pH gradient (ΔpH), epoxidation can proceed irrespective of a ΔpH. Herein, we compared the extent and kinetics of deepoxidation and epoxidation to the changes in fluorescence in the presence of a light-induced thylakoid ΔpH. We show that epoxidation reverses fluorescence quenching without affecting thylakoid ΔpH. These results suggest that epoxidase activity reverses quenching by removing deepoxidized xanthophyll cycle pigments from quenching complexes and converting them to a nonquenching form. The transmembrane organization of the xanthophyll cycle influences the localization and the availability of deepoxidized xanthophylls is to support nonphotochemical quenching capacity. The results confirm the view that rapidly reversible nonphotochemical quenching is dependent on deepoxidized xanthophyll.

Key words: Nonradiative energy dissipation; Xanthophyll-cycle function; Violaxanthin deepoxidation; Xanthophyll-cycle localization; Carotenoid function

1. Introduction

Characterization of the photoprotective mechanisms by which higher plants dissipate excess absorbed light energy is currently a major concern in photosynthesis research [1]. One molecular mechanism that protects photosystems against excess light during stresses that restrict carbon assimilation involves xanthophyll. This exists in all higher plants surveyed so far. This protective mechanism is stimulated under excess light conditions that energize the chloroplast thylakoid membrane [2,3]. Protection of the photosynthetic apparatus is achieved through the harmless and radiationless dissipation of excess light energy, through release as heat. This is accompanied by a decline in chlorophyll (Chl) *a* fluorescence yield which can be monitored as nonphotochemical quenching (NPQ) of photosystem II fluorescence [1–3]. It is generally accepted that NPQ depends on the activity of the xanthophyll cycle and the presence of a thylakoid ΔpH. The linkage between the acidification of the thylakoid lumenal space and the biophysical mechanism of Chl fluorescence quenching, however, is complex and much of it remains to be understood [1]. The most concise picture indicates that NPQ correlates linearly with the product of the lumen proton concentration and the combined concentrations of the monoepoxide antheraxanthin (Anth) and epoxide-free zeaxanthin (Zeax) [4]. The biochemistry of the interconversions of Violaxanthin (Viol), Anth and Zeax in the xanthophyll cycle has been extensively characterized using isolated chloroplast systems [5] and is outlined schematically as follows:

$$\text{Viol} \underset{\text{(pH} \approx 7\text{, NADPH, O}_2\text{)}\ \text{Epoxidation}}{\overset{\text{Deepoxidation (pH} \approx 5\text{, Ascorbate)}}{\rightleftharpoons}} \text{Zeax } \textit{via} \text{ Anth}$$

The Viol deepoxidation reaction depends on ascorbate and an acidic lumen pH for activation of the deepoxidase enzyme. The sulfhydryl reagent dithiothreitol (DTT) inhibits deepoxidation without affecting light-induced or ATP hydrolysis-induced proton pumping [6,7,8]. Deepoxidation at ambient temperatures usually takes less than 10 min to reach completion. In contrast, the reverse epoxidation reaction is mediated by a separate DTT-insensitive enzyme and is much slower than deepoxidation, taking up to one hour for complete rever-

*Corresponding address: HITAHR, 3050 Maile Way, Gilmore 202B, Honolulu, HI 96822, USA. Fax: (1) (808) 956 9105.

***Current address:* A.M. Gilmore, University of Illinois at Urbana-Champaign, Dept. of Plant Biology, 265 Morrill Hall, 505 S Goodwin, Urbana, IL 61801, USA. Fax: (1) (217) 244 7246.

Abbreviations: Anth, antheraxanthin; Chl, chlorophyll; DTT, dithiothreitol; F_o, minimum fluorescence under de-energized state with PSII traps fully open; F_M and F'_M, maximum fluorescence at de-energized and energized states, respectively; F_V, variable fluorescence ($F_M - F_O$); NPQ, non-photochemical quenching of chlorophyll *a* fluorescence; SV_N, Stern-Volmer calculation of NPQ; Viol, violaxanthin; Zeax, zeaxanthin.

SSDI 0014-5793(94)00784-5

sal [9]. Epoxidation requires NADPH, molecular oxygen, and enzyme activity is optimal at a neutral pH and strongly inhibited below pH 6. Thus it appears that the epoxidase exists in a place in the thylakoid membrane that remains at a neutral pH during transthylakoid proton pumping. Epoxidation can be activated during light-induced lumen acidification after inhibiting the deepoxidase with DTT. It has not yet been determined how the epoxidation reaction influences the yield of NPQ given that the light-driven ΔpH is maintained. One expects that if epoxidation reverses NPQ, this would be direct evidence that the quenching depended on the deepoxidized xanthophylls. In this paper we have simultaneously compared the kinetics of both the deepoxidation and epoxidation reactions to the NPQ yield. We report that, after inhibiting deepoxidation in the light and without uncoupling, epoxidation of Zeax and Anth effectively reverses NPQ.

2. Materials and methods

Chlorophyll *a* fluorescence quenching and $\Delta A_{505-540}$ that measures changes in the deepoxidation state of the xanthophyll cycle [10,11], were measured simultaneously with a PAM 101 chlorophyll fluorometer (Heinz-Walz, Effeltrich, Germany) and a DW-2000 UV-Vis spectrophotometer (SLM-Aminco), respectively, as described previously [12]. Nonphotochemical fluorescence quenching is expressed as Stern–Volmer type and calculated as $SV_N = (F_M/F'_M)-1$, where F_M is the maximal fluorescence quenching under nonenergized conditions and F'_M is the maximal fluorescence under energized conditions, according to Gilmore and Yamamoto [12]. Chloroplasts were isolated from *Lactuca sativa* L. cv Romaine and reaction preparation was as described previously [8] except the reaction mixture was adjusted to pH 7.4 and essentially fatty-acid free bovine serum albumin (Sigma) was added to yield a final albumin:Chl *a+b* ratio of 30:1 (w/w). The Chl concentration was 30 μg Chl *a+b* ml^{-1} of suspension. The reaction temperature was 26°C and the actinic light intensity was 200 μmol photons m^{-2}·s^{-1} of red light (Corning CS2–58 and Corning CS1–75). Pigment HPLC analyses were according to Gilmore and Yamamoto [10]. Light-induced lumen acidification was measured from Neutral red uptake-induced absorbance changes at 520 nm as per Gilmore and Yamamoto [4]. The ΔA_{520} readings were the difference between the light-induced A_{520} due to Neutral red (5 μM) uptake and the light-induced scattering changes at A_{520} without Neutral red.

3. Results and discussion

Fig. 1A shows the effects of NADPH on the kinetics and extent of NPQ, expressed as SV_N [12]. Fig. 1B shows simultaneous recordings of the absorbance changes at 505 *minus* 540 nm ($\Delta A_{505-540}$). DTT abruptly stops any further changes in $\Delta A_{505-540}$ in the reaction without NADPH, whereas in the reaction with NADPH, there is a clear and steady decrease in $\Delta A_{505-540}$ indicative of epoxidation [9]. Kinetic analysis (not shown) of the signal with NADPH showed that the epoxidation was a first order reaction. Fig. 1A shows that in contrast to $\Delta A_{505-540}$, SV_N continues to develop after adding DTT in both reactions. SV_N in the reaction without NADPH

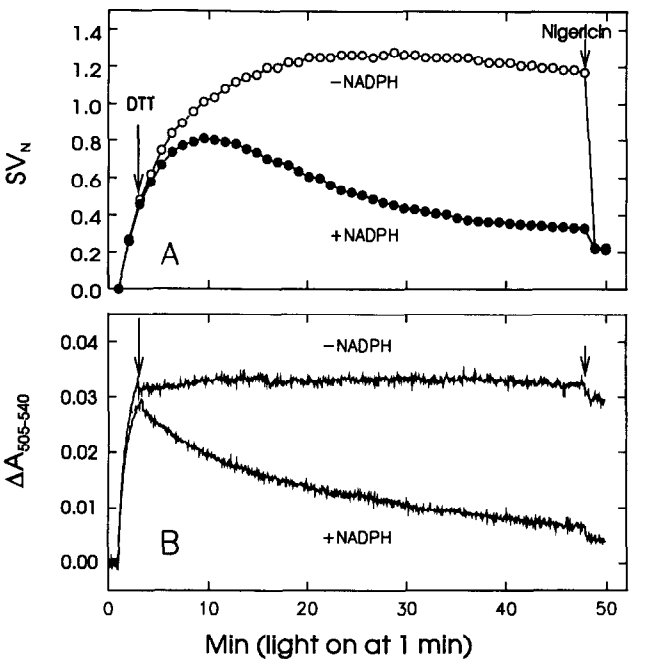

Fig. 1. Effects of NADPH-dependent epoxidation on simultaneous recordings of (A) SV_N and (B) $\Delta A_{505-540}$. Na-ascorbate (30 mM) was added to activate deepoxidation and to induce electron flow via the Mehler ascorbate-peroxidase reaction [22]. When present, NADPH (0.75 mM) was added prior to illumination. When added as indicated by the arrows in the Fig. 1A, the dithiothreitol (DTT) concentration was 2.25 mM and nigericin was 2 μM. SV_N was calculated using the maximal fluorescence level obtained immediately prior to illumination; the ratio of variable (F_V) to maximal fluorescence, F_V/F_M, was 0.81 ± 0.01 prior to illumination in both reactions shown in Fig. 1.

levels off after about 15 min, however, in the reaction containing NADPH, after about 10 min the SV_N reversed and steadily decreased for the remaining time course. The lag between the initial deepoxidation and the maximal expression of fluorescence quenching (−NADPH thylakoids) (Fig. 1) is similar to earlier reports [4,8,12] and shows that other changes which are slower than lumen acidification and deepoxidation are required for active quenching. The uncoupler insensitive quenching, which is low (about 0.2 SV_N) in both reactions, apparently is due to photodamage (Fig. 1). This is normal for prolonged light treatments of chloroplasts and apparently caused by photodamage [12].

Table 1 compares the levels of the xanthophyll-cycle pigments and lumen acidity in relation to the uncoupler-sensitive SV_N. Epoxidation in the reaction with NADPH is evident by the decrease in Zeax and the increases in Viol and Anth (Table 1A). The measured level of (de)epoxidation and calculated differences (not shown) from the final levels of $\Delta A_{505-540}$, in the two reactions, were in close agreement; thus it was clear that the absorbance changes accurately reflected the pigment interconversion. Notably, by the end of the reaction, the rate of epoxidation had slowed significantly although a consid-

erable amount of antheraxanthin still remained to be epoxidized to violaxanthin. The accumulation of antheraxanthin can be explained as due to the first epoxidation from Zeax to Anth apparently being faster than the second epoxidation from Anth to Zeax. A similar type of differential effect has been observed for deepoxidation of the first and second epoxide group [14] where deepoxidation of violaxanthin to antheraxanthin is slower than antheraxanthin to zeaxanthin. Thus, during deepoxidation, antheraxanthin does not usually accumulate to high levels.

Just prior to adding nigericin, the final ratio of [Zeax + Anth] to SV_N was much higher with epoxidation (+NADPH) than without (−NADPH) (Table 1B). This suggests that the stoichiometric relationship between [Zeax + Anth] and SV_N was not strictly linear under the epoxidation conditions and that a considerable amount of [Zeax + Anth] appeared less actively involved in the quenching than when epoxidation was inhibited. The ratios of Zeax to SV_N and Anth to SV_N suggest that Anth in particular may be less effective as a quencher or possibly non-quenching when formed during epoxidation. This lower effectiveness could be related to the slow kinetics of the conversion of Anth to Viol whereby significant substrate levels of Anth may be bound to the epoxidase or sequestered in a pool of pigments during the epoxidation reaction and thus unavailable for quenching. The latter highlights the possible functional significance of the transmembrane organization of the xanthophyll cycle. Consequently, it appears that Anth formed on the lumenal side via Viol deepoxidation leads to quenching [4] whereas when formed on the stromal side by Zeax epoxidation leads to a reversal of quenching.

Table 1
State of the xanthophyll cycle pigment pool and lumen acidity in relation to nonphotochemical fluorescence quenching (SV_N) after prolonged actinic-illumination with and without NADPH-dependent epoxidation in the presence of DTT

	−NADPH	+ NADPH	Δ
A. Xanthophylls*			
Viol	74.75	97.88	+ 23.13
Anth	16.10	44.11	+ 28.01
Zeax	58.72	7.58	− 51.14
Zeax + Anth	74.82	51.69	− 23.13
B. Quenching			
SV_N	0.777	0.101	− 0.676
[Zeax + Anth]: SV_N	96.30	511.80	–
[Zeax]: SV_N	75.60	75.05	–
[Anth]: SV_N	20.72	436.73	–
C. Lumen acidity**	10.13	9.77	0.36

*All xanthophyll-cycle pigments are expressed as mmol xanthophyll (mol Chl a)$^{-1}$. Epoxidation was stopped 2 min after uncoupling with nigericin by deep-freezing the reaction mixture (−80°C). The pigments were extracted and analyzed by HPLC rapidly after thawing, being careful not to allow the temperature to rise above 0°C prior to extraction. SV_N was calculated after 45 min illumination using the F_M level obtained after uncoupling.

**Lumen acidity was shown as the ratio $\Delta A_{520} \times 10^4)/\mu g$ Chl. All other conditions as mentioned in the legend to Fig. 1.

The combined effects of NADPH and DTT on the lumen pH were examined in separate experiments by measuring Neutral red uptake induced absobance changes at 520 nm (Table 1C). Light-induced lumen acidification was not affected by DTT in the presence of NADPH (Table 1). The lumen acidity in reactions with NADPH was reduced by only 4% compared to that in the reactions without NADPH. Therefore, we conclude that the SV_N reversal with NADPH was due to epoxidation and not due to a decrease in ΔpH. Lack of NADPH and DTT effects on the thylakoid proton gradient was expected. NADPH did not inhibit the initial development of SV_N or deepoxidation in the first few minutes (Fig. 1), thus indicating that NADPH does not affect the lumen pH. As reported previously, DTT does not affect ΔpH [7] and does not reverse the preformed SV_N in absence of NADPH [8,12]. Our results, aside from showing that NPQ depends on epoxidation-deepoxidation cycle activity, have other important and interesting implication. They show that the quenching mechanism and the epoxidation reaction compete for the same substrate, namely the deepoxidized endgroups of Zeax and Anth; similar substrate specificity is implicit because no other pigments take any measurable part in either NPQ or epoxidation.

Gruszecki and Krupa [15] have reported that the isolated and purified light-harvesting pigment-protein complex of photosystem II, LHCII, catalyzes NADPH-dependent xanthophyll epoxidation. This result, however, has not been confirmed. Peters and Thornber [16], and Dainese et al. [17] reported that the xanthophyll cycle pigments are associated with the minor LHCII complexes whereas Thayer and Björkman [18] reported that the xanthophyll pigments are more widely distributed among all the light-harvesting proteins of photosystem II and photosystem I. It is generally believed that xanthophyll-dependent NPQ occurs within the light-harvesting antennae complexes of photosystem II [2,3] and possibly involves LHCII aggregation [19]. The localization of newly re-epoxidized xanthophylls in the thylakoids, however, has not been established.

The present results confirm earlier reports that activation of the quenching by the deepoxidized pigments requires other lumen-pH dependent changes that are slower than deepoxidation and lumen acidification. It appears that these rate-limiting changes partly involve the diffusion-limited and stereospecific binding of the deepoxidized endgroups to key pH-activated binding sites in the light-harvesting pigment–protein complexes within the lipid-core of the thylakoid membrane. Xanthophyll binding to quenching sites is apparently sufficiently reversible for the substrate to be available to the epoxidase. Apparently, the lifetime of the binding at the

quenching site exceeds that required for deexcitation of the excited chlorophyll and quenching of the fluorescence. Although these data are consistent with a direct role for Zeax and Anth in quenching, they do not directly confirm or exclude that the xanthophylls themselves quench the singlet excited state of chlorophyll as suggested by Owens et al. [20] and Frank et al. [21]. Overall, we conclude that xanthophyll epoxidation inhibits and reverses NPQ by converting the active deepoxidized quenching species into an inactive epoxidized form and that pigment localization as determined by the transmembrane organization of the cycle apparently influences pigment quenching capacity.

Acknowledgements: This research was supported by a U.S. Department of Agriculture National Research Initiative Competitive Research Grant 93–37100–9062 to H.Y. A.G. acknowledges support by a U.S. Department of Energy Postdoctoral Fellowship in Integrative Photosynthesis research award to the Dept. of Plant Biology UIUC during the preparation of this manuscript. The authors also thank Govindjee for thoughtful discussion and critical comments about the manuscript.

References

[1] Krause, G.H. and Weis, E. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349.
[2] Demmig-Adams, B. and Adams III W.W. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 599–626.
[3] Björkman, O. and Demmig-Adams, B. (1993) in: Ecological Studies (Schulze, E.-D. and Caldwell, M. Eds.) Vol. 100, Springer, pp. 14–47.
[4] Gilmore, A.M. and Yamamoto, H.Y. (1993) Photosynth. Res. 35, 67–78.
[5] Yamamoto, H.Y. (1979) Pure Appl. Chem. 51, 639–648.
[6] Yamamoto, H.Y. and Kamite, L. (1972) Biochim. Biophys. Acta 267, 538–543.
[7] Sokolove, P.M. and Marsho, T.V. (1976) Biochim. Biophys. Acta 430, 321–326.
[8] Gilmore, A.M. and Yamamoto, H.Y. (1992) Proc. Natl. Acad. Sci. USA 89, 1899–1903.
[9] Siefermann, D. and Yamamoto, H.Y. (1975) Biochem. Biophys. Res. Commun. 62, 456–461.
[10] Yamamoto, H.Y., Kamite, L. and Wang, Y.Y. (1972) Plant Physiol. 49, 224–228.
[11] Siefermann, D. and Yamamoto, H.Y. (1975) Arch. Biochem. Biophys. 171, 70–77.
[12] Gilmore, A.M. and Yamamoto, H.Y. (1991) Plant Physiol. 96, 635–643.
[13] Gilmore, A.M. and Yamamoto, H.Y. (1991) J. Chromatogr. 543, 137–145.
[14] Yamamoto, H.Y. and Higashi, R.M. (1978) Arch. Biochem. Biophys. 190, 514–522.
[15] Gruszecki, W.I. and Krupa, Z.J. (1993) Biochim. Biophys. Acta 1144, 97–101.
[16] Peter, G.F. and Thornber, J.P. (1991) J. Biol. Chem. 266, 16745–16754.
[17] Bassi, R., Pineau, B., Dainese, P. and Marquardt, J. (1993) Eur. J. Biochem. 212, 297–303.
[18] Thayer, S. and Björkman, O. (1992) Photosynth. Res. 33, 213–225.
[19] Horton, P., Ruban, A.V., Rees, D., Pascal, A.A., Noctor, G. and Young, A.J. (1991) FEBS Lett. 292, 1–4.
[20] Owens, T.G., Shreve, A.P. and Albrecht, A.C. (1992) in: Research in Photosynthesis, Vol. I (Murata, N. Ed.), pp. 179–186, Kluwer, The Netherlands.
[21] Frank, H.A., Cua, A., Young, A., Gosztolz, D. and Wasielewski, K., Photosynth. Res., in press
[22] Neubauer, C. and Yamamoto, H.Y. (1992) Plant Physiol. 99, 1354–1361.