The Influence of the Proton Gradient on the Activation of Ferredoxin-NADP$^+$-oxidoreductase by Light

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Ferredoxin-NADP$^+$-oxidoreductase (FNR, EC 1.18.1.2) has been shown to be activated by light within a few seconds during dark-light transitions and inactivated in the dark. In previous papers this could be pointed out by the correlation of cytochrome f induction kinetics to the rate of NADP-photoreduction and the variable fluorescence. The present study deals with the role of the proton gradient during the activation process. The transition from an inactive to an active form is followed continuously in an *in situ* system. The steady-state rate of NADP-photoreduction is affected only by ionophores which inhibit a formation of the proton gradient, but not by inhibitors of the electric field. It correlates to the 9-aminoacridine fluorescence quench and the light scattering signals at 535 nm. The perception of the pH-gradient through the enzyme is still a matter of discussion.

Introduction

The reduction of NADP as the terminal step of the photosynthetic electron transport chain in higher plant thylakoids is catalyzed by the redox enzyme ferredoxin-NADP$^+$-oxidoreductase (FNR, EC 1.18.1.2), a nuclear coded protein with a molecular weight of about 33 kDa [1−3]. A 17.5 kDa binding protein is anchored to the non-appressed regions of the thylakoids which binds this small form (FNR$_s$, 33 kDa) *via* a "connectein"-protein [4−7]. Previous studies concerning this enzyme complex and its regulation have pointed out an activation of the FNR within a few seconds during dark-light transitions [8−11].

An *in vivo* activation has been confirmed by the correlating results of the following experiments.

1) The rate of NADP-photoreduction as used in this study represents a direct way of measuring the FNR-activation.

2) The progress of cytochrome f redox-kinetics reflects an indicator of changing activities of photosystem I and II [9]. An induction of these kinetics takes place within a few seconds, which could be correlated to an increasing activation of the FNR by light until a steady-state is reached.

3) The parallel decline of the variable fluorescence gives us further informations in that an increased photosystem I activity leads to an oxidation of redox enzymes between both photosystems [9, 12, 13]. Also the induction effects of photoacoustic and photosystem I signals do agree with this point of view [13−15].

4) The effects of inhibitors and rebinding studies provide further arguments for an activation process [8, 16]. However, some kind of conformational changes have to take place [16−18] during this process.

The present investigation attempts to tackle the question, how light is used to activate the enzyme.

Materials and Methods

Plants of *Spinacia oleracea* were grown in a Heraeus-Vötsch growth chamber with a light-dark schedule of 11 h : 13 h, temperatures of 18 °C : 15 °C, and a relative humidity of about 60% : 80%. The light intensity was 50 Wm^{-2}. About 5−6 weeks old *Spinacia* leaves were taken for the measurement and isolation procedures.

Intact spinach chloroplasts were isolated according to [19] at 0 °C with a 40% percoll gradient underlying the medium. A second centrifugation (60 s at $1100 \times g$) with 40% percoll was used to purify the intact chloroplasts. The intactness of the class A

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chloroplasts was about 90% as determined by the assay of [20] with a Clark-type oxygen electrode. Class B chloroplasts were revealed as described in [9]. The reaction medium for the intact chloroplasts consists of:

0.33 M sorbitol, 2 mM EDTA, 0.5 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaCl, 1 mM 3-phosphoglycerate, 1 mM oxaloacetate, 10 mM $NaHCO_3$, and 40 mM HEPES, pH 7.6 (NaOH) (*cf.* [19]).

Further additions were done as indicated in the text.

The ionophores valinomycin, gramicidin, nigericin, and Ca^{2+}-ionophor # C 9275 were obtained from SIGMA.

The kinetical measurements of cytochrome *f*, light-scattering at 535 nm, and the electrochromic shift at 520 nm have been described previously [9]. The photomultipliers of the used spectrophotometers − AMINCO DW-2 or SIGMA ZWS 11 − were shielded against actinic light with a 5 mm BG 39 filter (Schott, F.R.G.).

The 9-aminoacridine fluorescence quench was measured with the SIGMA ZWS 11 using the difference of the photomultiplier signal at 404 and 453 nm excitation.

Kinetics of NADP-photoreduction were recorded using the dual-wavelength mode at 366−390 nm with the SIGMA ZWS 11 under similar conditions as the other kinetics.

Dark-light transitions were revealed by a 25 Wm^{-2} illumination in the range of 15 s to 3 min through a 4 mm RG 645 filter with a following dark period of 60 s−90 s. The obtained signals were recorded and averaged (*cf.* [9]).

Chlorophyll concentration was determined according to [21].

Results

Fig. 1a depicts the cytochrome *f* kinetics after simultaneous excitation of photosystem I and II. The signal shows an induction effect consisting of three phases. During phase 1 cytochrome *f* becomes oxidized as indicated by an increase of the signal. It is reduced subsequently in a second phase. A third oxidative phase leads to a steady-state level. The reduction after turning off the light shall not further be discussed within this context.

The third phase reflects an increasing activity of photosystem I [8, 9], while the first two phases demonstrate the transient accumulation of electrons at the acceptor side of photosystem I. 1 µM nigericin prevents the induction effect, and cytochrome *f* remains mainly in the reduced state.

Fig. 1b exhibits that the reduction of NADP does not immediately start with the onset of light. The rate of NADP-photoreduction is low at the beginning and increases parallel to phase 3 of the cytochrome *f* signal. A steady-state is reached after ca. 5 s like that of the cytochrome *f* kinetics. The uncoupler of the proton-gradient inhibits the NADP-reduction completely. A variation of the Mg^{2+}-concentration in the medium does not influence the acti-

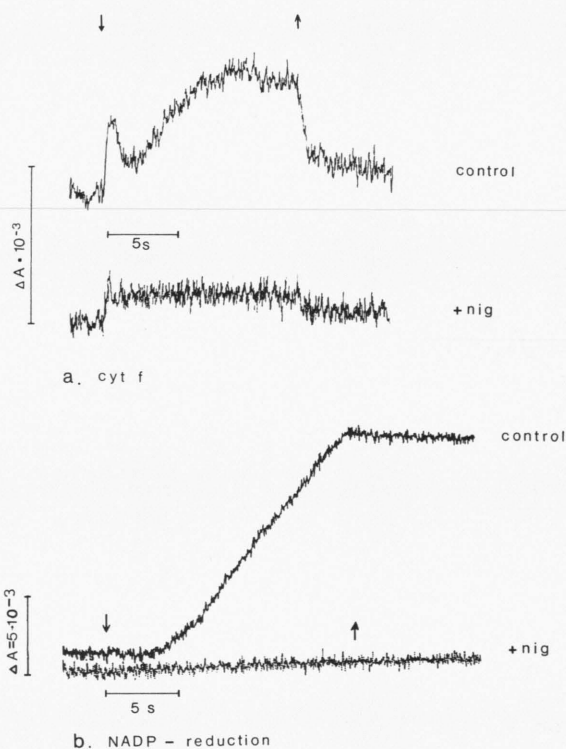
Fig. 1. a. Cytochrome *f* kinetics of intact class B spinach chloroplasts as measured by absorbance changes at 560−554 nm. Addition of 1 µM nigericin compared to the control causes a loss of the induction. Averages of 10 measurements with 25 Wm^{-2} RG 645 light in a light-dark schedule of 15 s light to 60 s dark. b. Kinetics of NADP-photoreduction with addition of 1 µM nigericin compared to the control chloroplasts. The start of the illumination is marked by the left arrow. It is an average curve of 4 measurements.

vation. High concentrations of more than 20 mM Mg^{2+} even slow down the NADP-reduction rate (data not shown).

The light-scattering signal at 535 nm develops parallel to phase 3 (Fig. 2), and it is also prevented by addition of nigericin.

The same behaviour is found for the quench of 9 AA-fluorescence, which is an additional method to monitor the proton gradient. Fig. 3 describes the dependence of the 9 AA-fluorescence quench on the concentrations of different ionophores. The sigmoid shape of the curve for nigericin and gramicidin, which are known as uncouplers of the pH-gradient, leads to an almost complete inhibition at a concentration of 1 μM. The same concentration influences the 9 AA-fluorescence quench only about 30% with valinomycin and the Ca^{2+}-ionophore C9275. An equivalent effect can be shown for the slow phase of the light-scattering signals.

The steady-state rates of NADP-photoreduction are affected by the ionophores in a similar way (Fig. 4). For higher ionophore concentrations it was necessary to illuminate up to 2 min in order to reach a steady-state rate of NADP-reduction.

These steady-state rates correlate very well to the quench of the 9 AA-fluorescence with a mean corre-

lation coefficient of $r = 0.990$ (Fig. 5) and to the amplitude of the light-scattering signals ($r = 0.967$, data not shown).
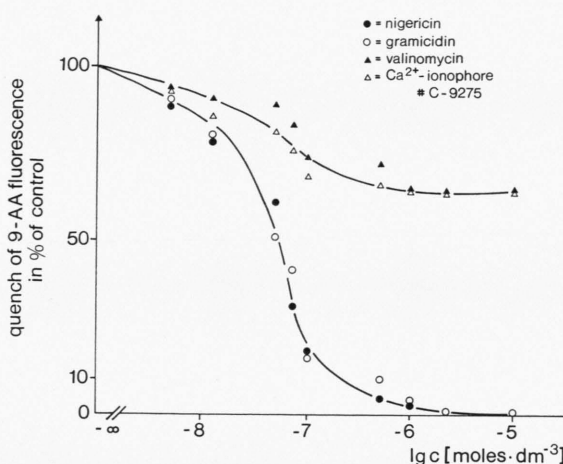

Fig. 3. Quench of the 9 AA-fluorescence in % of control *versus* the logarithmic concentration of several ionophores (*cf.* Fig. 4).
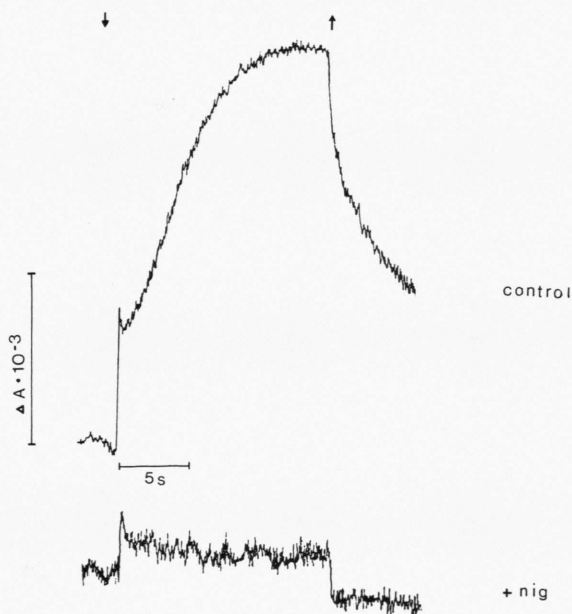

Fig. 2. Depiction of light-scattering kinetics at 535 nm measured under the same conditions as stated in Fig. 1a.
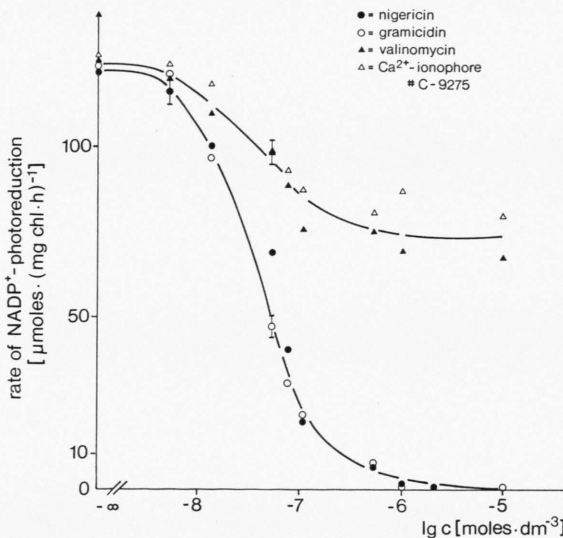

Fig. 4. Rate of NADP-photoreduction calculated from the slope of the kinetics in the steady state *versus* the logarithmic concentrations of several ionophores. Mean values of 4 measurements. A steady-state was reached within 2 min of illumination in each case.
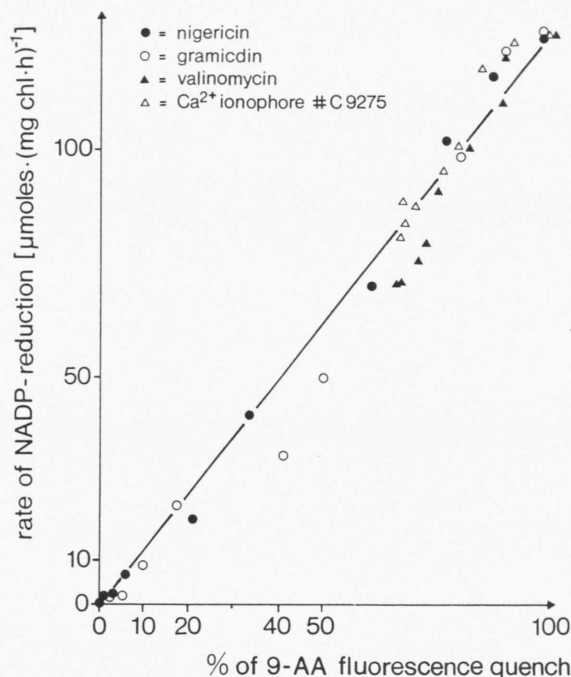
Fig. 5. Correlation of the results depicted in Fig. 3 and 4 ($r = 0.990$).

Discussion

Evidence for an activation of the FNR

The results of the experiments described in Fig. 1 clearly demonstrate that there is an activation of electron transport at the acceptor side of photosystem I. There is a parallel increase of the NADP-photoreduction rate and the oxidation of cytochrome *f* during "phase 3". Experiments with Hill-reagents and phenyl-mercuric acetate as a specific inhibitor of the FNR have pointed out that this enzyme is regulated [8–10, 16]. It was possible to demonstrate active and inactive forms of the enzyme *in vitro* using its diaphorase activity [10, 11]. Thus the shape of the cytochrome *f* induction curve can well be explained by this transient "block" for electrons during phase 1 and 2, and by an increasing flow to NADP during phase 3. This is also consistant with the simultaneous decline of the variable fluorescence [9, 12], and furthermore it is in agreement to induction effects found in photoacoustic signals or photosystem I measurements [13–15].

Possible activation mechanisms

The activation can be achieved by red light whereas the chromophores of the FNR absorb only in the blue region. Therefore, a direct role of light *via* an absorption by chromophores is improbable for the activation mechanism.

A further mechanism for the activation of an enzyme can be redox-controlled through the thioredoxin system [22, 23]. Studies on the function of the enzyme's SH-groups do not provide evidence for this mechanism [24, 25].

A possible influence of the proton gradient on this protein was discussed previously [5, 16].

As the pH-gradient is accompanied by the efflux of Mg^{2+} there may be also the possibility of an ion-effect on the enzyme. However, the variation of the external Mg^{2+}-concentration was not able to simulate such an influence.

The activation depends on the existence of a proton gradient because it can be prevented by appropriate ionophores. The signals of the light-scattering at 535 nm and the quench of 9 AA-fluorescence are considered to reflect the pH-gradient [26]. In Fig. 2 and Fig. 3 it is demonstrated that nigericin and gramicidin are effective uncouplers of the proton gradient. Gramicidin additionally inhibits the electric field across the thylakoid membrane, which was indicated by the electrochromic shift at 520 nm (data not shown). Valinomycin and the Ca^{2+}-ionophore mainly inhibit the electric field and affect the pH-gradient only slightly.

The proton gradient and the rate of NADP-photoreduction exhibit a similar dependence on the concentration of the ionophores. The slight decrease of the NADP-reduction rate under the influence of valinomycin and the Ca^{2+}-ionophore can well be explained by their little effect on the proton gradient.

So a good correlation is found between the FNR-activity and the proton gradient. A significant contribution of the electric field to the activation process can be ruled out.

Interaction of the pH-gradient with the FNR

As stressed above an energetization may explain the source of the activation process which seems to look as follows.

According to Fig. 6 (*cf.* [27]) FNR is anchored to the membrane surface with a trimer of a 17.5 kDa
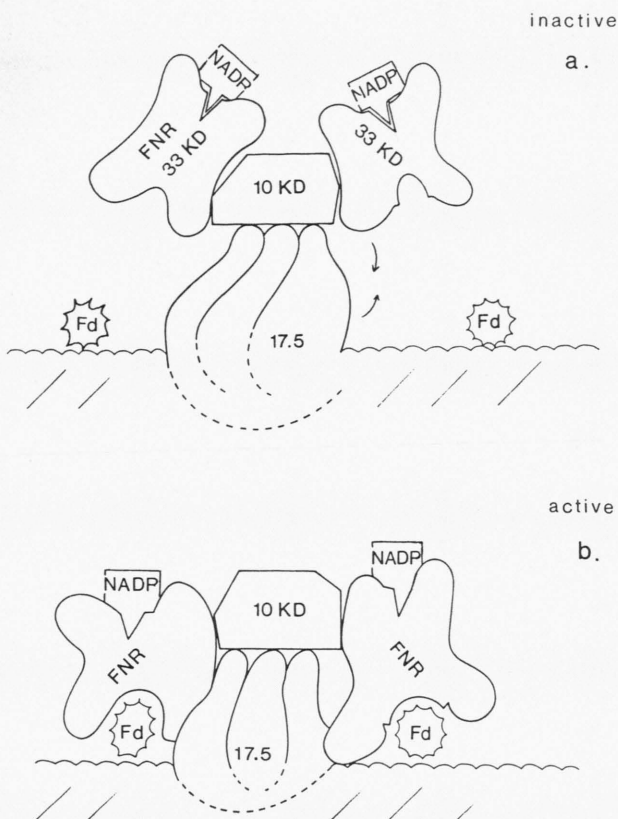
inactive

a.

active

b.

Fig. 6. Model of the FNR bound to the thylakoid membrane in the inactive and active state [27].

binding protein [7, 28, 29]. This view is supported by cross-link experiments [30–32].

Nozaki and Shin [4, 33] found an additional protein factor, connectein, which connects the FNR to the binding protein in a 2:1 stoichiometry resulting from the determined molecular weights.

Matthijs and coworkers [30] reported about a tightly and a loosely bound FNR. The latter one represents the inactive form, and the active form is expressed in the tighter binding not only to connectein but also to the 17.5 kDa protein (cf. Fig. 6). The activated form possibly derives from a conformational change of the 17.5 kDa protein sensing the light-driven proton gradient (see also [7]). The accessibili-

ty of the 17.5 kDa protein for antibodies differed according to the pH-gradient [29]. This demonstrates that the binding protein senses the proton gradient and thereby may change its conformation.

The proposed chemical complexation of ferredoxin with FNR [34, 35] forming covalent compounds between carboxyl groups of ferredoxin and defined amino groups of the FNR [25, 36] requires this conformational change as a prerequisite. Thus the neighbourhood of both substrate molecules is achieved. This is necessary for the complexation and the electron transfer per se [27, 37].

Such conformational changes may occur through the light-driven pH-gradient but not through the electric field alone.

Physiological relevance

In addition, other regulatory processes are important and should be investigated in their interrelation. Ferredoxin represents a pool of electron distribution [36, 38, 39]. An inactivation of the FNR in the dark is convenient to prevent a reverse electron flow which would consume NADPH [10, 27]. Additionally, the accumulation of electrons at ferredoxin during a dark-light transition may be necessary for an effective stimulation of the thioredoxin system, and thus for the activation of Calvin cycle enzymes. Alternatively these electrons may promote a transient cyclic electron flow, which is said to proceed in two ways, one via cytochrome b_6 and the other via FNR [40, 41]. The cytochrome b_6 pathway may represent a transient source for ATP until the FNR is activated.

In summary, we propose that the light-driven proton gradient, and not the electric field, plays a crucial role in the in vivo regulation of the enzyme via conformational changes. They may enable the complexation and the electron transfer between the substrates.

Acknowledgements

[1] N. Carrillo, Eur. J. Biochem. **150**, 469−474 (1985).
[2] A. Lax, K. Vaughn, V. Sisson, and G. Templeton, Photosynth. Res. **6**, 113−120 (1985).
[3] N. Carrillo and R. H. Vallejos, Biochim. Biophys. Acta **742**, 285−294 (1983).
[4] Y. Nozaki, M. Tamaki, and M. Shin, Physiol. Veg. **23**, 627−633 (1985).
[5] N. Carrillo, J. L. Arana, and R. H. Vallejos, J. Biol. Chem. **256**, 6823−6828 (1981).
[6] N. Carrillo and R. H. Vallejos, Plant Physiol. **69**, 210−213 (1982).
[7] R. H. Vallejos, E. Ceccarelli, and R. Chan, J. Biol. Chem. **13**, 8048−8051 (1984).
[8] K. Satoh, Plant Physiol. **70**, 1413−1416 (1982).
[9] W. Rühle, R. Pschorn, and A. Wild, Photosynth. Res. **11**, 161−171 (1987).
[10] N. Carrillo and R. H. Vallejos, Trends Biochem. Sci. **8**, 52−56 (1983).
[11] N. Carrillo, H. A. Lucero, and R. H. Vallejos, Plant Physiol. **65**, 495−498 (1980).
[12] K. Satoh, Biochim. Biophys. Acta **638**, 327−333 (1981).
[13] S. Katoh and A. Yamagishi, Biochim. Biophys. Acta **767**, 185−191 (1984).
[14] S. Malkin, Planta **171**, 65−72 (1987).
[15] J. Harbinson and F. Woodward, Plant Cell Environment **10**, 131−140 (1987).
[16] N. Carrillo, H. A. Lucero, and R. H. Vallejos, J. Biol. Chem. **256**, 1058−1059 (1981).
[17] M. K. Nikolaeva and O. P. Osipova, Doklady Academii Nauk SSSR **286** (2), 494−497 (1985).
[18] R. Wagner, N. Carrillo, W. Junge, and R. H. Vallejos, Biochim. Biophys. Acta **680**, 317−330 (1982).
[19] B. Barenyi and G. H. Krause, Planta **163**, 218−226 (1985).
[20] U. Heber and K. A. Santarius, Z. Naturforsch. **25b**, 718−728 (1970).
[21] R. Ziegler and K. Egle, Beitr. Biol. Pfl. **41**, 11−37 (1965).
[22] B. Buchanan, Ann. Rev. Plant Physiol. **31**, 341−374 (1980).
[23] M. Droux, J. P. Jacquot, M. Miginac-Maslow, P. Gadal, J. C. Huet, N. A. Crawford, B. C. Yee, and B. B. Buchanan, Arch. Biochem. Biophys. **252**, 426−439 (1987).
[24] E. M. Valle, N. Carrillo, and R. H. Vallejos, Biochim. Biophys. Acta **681**, 412−418 (1982).
[25] Y. Yao, K. Wada, Y. Takahashi , S. Katoh, and H. Matsubara, J. Biochem. **98**, 1079−1082 (1985).
[26] S. Köster and U. Heber, Biochim. Biophys. Acta **680**, 88−94 (1982).
[27] R. Pschorn, W. Rühle, and A. Wild, Photosynthesis Research, submitted (1987).
[28] E. Ceccarelli, R. Chan, and R. H. Vallejos, FEBS Lett. **190**, 165−168 (1985).
[29] R. Chan, E. Ceccarelli, and R. H. Vallejos, Arch. Biochem. Biophys. **253** (1), 56−61 (1987).
[30] H. C. Matthijs, S. Coughlan, and G. Hind, J. Biol. Chem. **261** (26), 12154−12158 (1986).
[31] G. Merati and G. Zanetti, FEBS Lett. **215**, 37−40 (1987).
[32] G. Zanetti, A. Aliverti, and B. Curti, J. Biol. Chem. **259**, 6153−6157 (1984).
[33] M. Shin, H. Ishida, and Y. Nozaki, Plant Cell Physiol. **26** (3), 559−563 (1986).
[34] C. Batie and H. Kamin, J. Biol. Chem. **259**, 11976−11985 (1984).
[35] C. Batie and H. Kamin, J. Biol. Chem. **261** (24), 11214−11223 (1986).
[36] B. Vieira and D. Davis, Arch. Biochem. Biophys. **247**, 140−146 (1986).
[37] C. Batie and H. Kamin, J. Biol. Chem. **259**, 8832−8839 (1984).
[38] M. Hirasawa, J. M. Boyer, K. A. Gray, D. J. Davies, and D. B. Knaff, Biochim. Biophys. Acta **851**, 23−28 (1986).
[39] G. Forti and P. Grubas, FEBS Lett. **186**, 149−152 (1985).
[40] J. P. Hosler and C. F. Yocum, Biochim. Biophys. Acta **808**, 21−31 (1985).
[41] J. P. Hosler and C. F. Yocum, Plant Physiol. **83**, 965−969 (1987).