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Kinetics of NADPH-induced non-photochemical reduction of the plastoquinone pool in spinach chloroplasts

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Abstract

Kinetics of non-photochemical reduction of the photosynthetic intersystem electron transport chain by exogenous NADPH was examined in osmotically lysed spinach chloroplasts by chlorophyll (Chl) fluorescence measurements under anaerobic condition. Upon the addition of NADPH, the apparent F_0 increased sigmoidally, and the value of the maximal slope was calculated to give the reduction rate of plastoquinone (PQ) pool. Application of 5 μM antimycin A lowered significantly both the ceiling and the rate of the NADPH-induced Chl fluorescence increase, while the suppressive effect of 10 μM rotenone was slighter. This indicated that dark reduction of the PQ pool by NADPH in spinach chloroplasts under O_2-limitation condition could be attributed mainly to the pathway catalysed sequentially by ferredoxin-$NADP^+$ oxidoreductase (FNR) and ferredoxin-plastoquinone reductase (FQR), rather than that mediated by NAD(P)H dehydrogenase (NDH).

Additional key words: actinomycin A; chlorophyll fluorescence; rotenone; *Spinacia oleracea*.

Introduction

Cyclic electron transport plays a crucial role in optimising the ratio between ATP and NADPH *in vivo* for photosynthetic organisms to adapt to variable environments. Studies on cyclic electron transport focus mainly on the return of electrons from the reducing side of PS1 to the PQ pool (Bendall and Manasse 1995). In addition, chlororespiration, which has been reported in a variety of photosynthetic organisms (Bennoun 1982, 2001, Garab *et al.* 1979, Mi *et al.* 1992, Feild *et al.* 1998) and is supposed to be a common feature of all photosynthetic cells (Scherer 1990), also involves electron transfer from stromal reductants to the PQ pool. There is good evidence for the involvement of at least two pathways in this process, one is mediated by NDH, and the other sequentially catalysed by FNR and FQR (Endo *et al.* 1998, Joët *et al.* 2001). However, since the molecular identity of NDH as well as that of FQR has not yet been clearly established, there is still extensive controversy in literature over the details of the mechanism.

NADPH-induced increase in level of apparent F_0 in broken chloroplasts has been used as an indicator of non-photochemical reduction of intersystem electron carriers (Mills *et al.* 1979, Endo *et al.* 1997, 1998, Corneille *et al.* 1998). Kinetic analysis of the NADPH-induced Chl fluorescence increase can provide information about the molecular mechanism of electron transfer from NADPH to the PQ pool. However, the evaluation of the Chl fluorescence increase is complicated by an initial lag presumably originating from the reaction system. In the present work, the maximal rate of NADPH-induced apparent F_0 increase in osmotically ruptured spinach chloroplasts under anaerobic condition was calculated precisely to represent the rate of non-photochemical reduction of the PQ pool. The relative contributions of the NDH- and the FNR-FQR-mediated pathways to the electron transfer from NADPH to the PQ pool were assessed by comparing the effects of different specific inhibitors on the kinetic properties of the NADPH-induced Chl fluorescence increase.

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Abbreviations: Chl – chlorophyll; CK – check, curve of NADPH-induced apparent F_0 increase in osmotically lysed spinach chloroplasts measured under anaerobic condition in the absence of inhibitors (Fig. 2) or its maximal slope (Fig. 3); F_0 – chlorophyll fluorescence yield observed under non-actinic measuring radiation; FNR – ferredoxin-$NADP^+$ oxidoreductase; FQR – ferredoxin-plastoquinone reductase; NDH – NAD(P)H dehydrogenase; PQ – plastoquinone; PS2 – photosystem 2.
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Materials and methods

Isolation of chloroplasts: Spinach seedlings were purchased at a local market (Shanghai). Intact chloroplasts were obtained by purification with *Percol* (*Pharmacia*, Sweden) gradient centrifugation according to Asada *et al* (1990). Chloroplasts were osmotically lysed by re-suspension in HEPES buffer (pH 7.6) containing 30 mM $MgCl_2$. The broken chloroplasts were re-suspended in an assay medium adapted from Corneille *et al.* (1998), which contains 30 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 30 mM KCl, 0.25 mM KH_2PO_4, and 50 mM HEPES (pH 7.6). The sample was diluted to a Chl concentration of 51.6 g m^{-3} for measurement.

Apparent F_0 was measured at 35 °C using a pulse-modulated amplitude fluorimeter (*PAM 101* with *101 US* emitter-detector unit and reaction chamber system; *Walz*, Effeltrich, Germany) with a modulated measuring beam (1 μs-duration, 1.6 kHz, 0.05 μmol(photon) $m^{-2}s^{-1}$, peak emission at 650 nm). NADPH was added by injection. Anaerobiosis was achieved by addition of glucose and glucose oxidase 5 min before measurement, to a final concentration of 1.5 mM and 12 unit cm^{-3}, respectively. A magnetic rod stirred the reaction mixture. An analogue-digital converter attached to a computer digitised the outputs. The sampling frequency was 10 Hz.

Data process: As illustrated by Fig. 2, a 30-s section of the Chl fluorescence curve after the addition of NADPH was truncated and smoothed by the following model

$$F(t) = a_0 + \frac{a_1}{[1 + a_2 \exp(a_3 t)]^{a_4}} \quad (1)$$

The maximal rate of the Chl fluorescence increase (V_m) was defined as the first derivative of Eq. 1 at the inflexion point, and was obtained numerically. The changes in V_m with increasing exogenous NADPH concentration were approximated by dual Michaelis-Menten equation. All fittings were carried out by home-designed software based on non-linear Levenberg-Marquardt algorithm as described in Jin *et al.* (2001).

Results and discussion

The kinetics of PQ reduction by exogenous NADPH was followed directly by measuring the change in Chl fluorescence in the dark (apparent F_0). The addition of NADPH to osmotically lysed spinach chloroplasts in the dark caused a sigmoidal increase in Chl fluorescence, and the section around the inflexion point fitted well to Eq. 1, from which the maximal slope of the Chl fluorescence curve was calculated (Fig. 1).

The level of Chl fluorescence is generally determined by the ΔpH across thylakoid membranes as well as the redox state of the PQ pool. Recent examination of mitochondria-chloroplast interactions revealed that the contribution of chlororespiration to the membrane potential is negligible (Bennoun 1994, Rappaport *et al.* 1999). Thus, the NADPH-induced increase in apparent F_0 observed in our experiment was caused exclusively by the reduction of the PQ pool.

The initial rate of changes in substrates or products has often been taken in enzymatic kinetics as the reaction rate. Similarly, the initial slope of the Chl fluorescence increase in the dark upon the addition of exogenous NADH or NADPH into broken chloroplasts mixture has been employed by Corneille *et al.* (1998) as an indicator of the rate of NAD(P)H-induced PQ reduction. However, the precision of this evaluation was challenged by the fact that the behaviour of the Chl fluorescence curve upon the addition of NADPH was not convex at the initial stage, and the whole assumed the form of sigmoid curve. Although we cannot exclude the possibility of a physiological origin, it is more likely that the initial lag of the

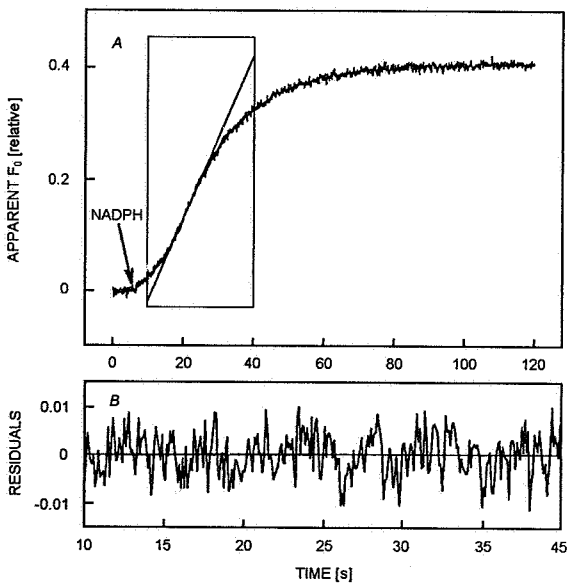

Fig. 1. Trace of NADPH-induced Chl fluorescence increase in osmotically ruptured spinach chloroplasts under anaerobic condition and the evaluation of the maximal ascending rate. The concentration of lysed chloroplasts was 51.6 g(Chl) m^{-3}. NADPH was added by injection to a final concentration of 123.5 μM. Anaerobic condition was achieved by the addition of glucose (1.5 mM) and glucose oxidase (12 unit cm^{-3}) 5 min before the measurement. The encircled section in *A* was truncated and smoothed by Eq. 1 to calculate the maximal increase rate as indicated by the maximal slope. *B* showed the residual plot of smoothing.

Chl fluorescence increase was caused by the buffering effect of mixing NADPH with the reaction medium. The maximal ascending slope of the Chl fluorescence curve is thus the most appropriate indicator that can be calculated precisely to represent the reduction rate of the PQ pool.

Fig. 2 demonstrates the effects of oxygen and specific inhibitors on the NADPH-induced Chl fluorescence increase. Under aerobic condition, reduction of plastoquinone in the dark was partially counteracted by its oxidation by molecular oxygen through the Mehler reaction (Hosein and Palmer 1983) or the pathway mediated by plastoquinol:oxygen oxidoreductase (Casano *et al.* 2000), resulting in a suppression of the NADPH-induced Chl fluorescence increase. Anaerobiosis, which prevents the leakage of electrons from the intersystem electron transport chain to molecular oxygen, is thus necessary for

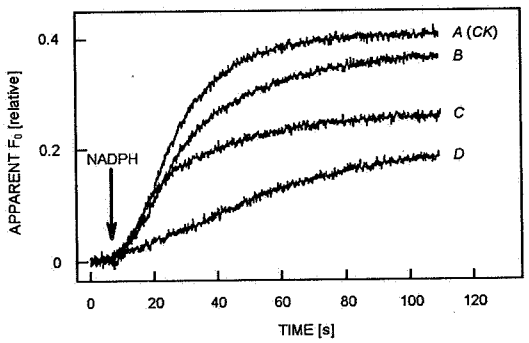

Fig. 2. Effects of oxygen, antimycin A (5 μM) and rotenone (10 μM) on the NADPH-induced Chl fluorescence increase in osmotically ruptured spinach chloroplasts. The final concentration of NADPH was 123.5 μM. *A(CK)*: anaerobiosis, without inhibitor; *B*: anaerobiosis, + 10 μM rotenone; *C*: aerobiosis, without inhibitor; *D*: anaerobiosis, + 5 μM antimycin A.

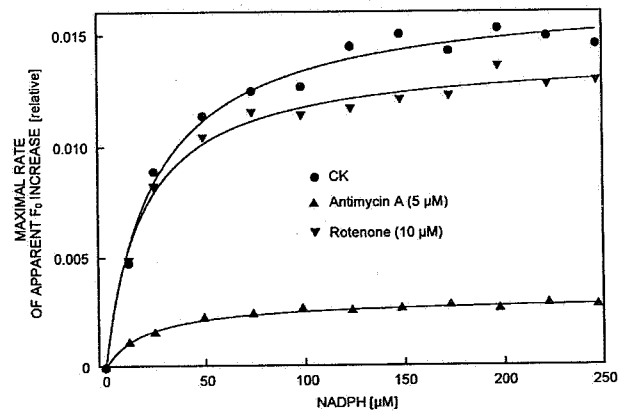

Fig. 3. Effect of NADPH concentration on the maximal rate of the NADPH-induced Chl fluorescence increase (V_m) and its inhibition by specific inhibitors measured in osmotically ruptured spinach chloroplasts under anaerobic condition.

the evaluation of kinetic properties of PQ dark reduction. Antimycin A, a specific inhibitor of FQR that possibly binds at the quinone reduction site (Moss and Bendall 1984, Cleland and Bendall 1992), strongly lowered the ceiling of the NADPH-induced Chl fluorescence increase. Compared with antimycin A, the suppressive effect of rotenone, a specific inhibitor of NDH, was small.

The rate of reduction of the intersystem chain by NADPH under anaerobic condition, as indicated by V_m, was further investigated as a function of the dose of exogenous NADPH (Fig. 3). The V_m increased convexly with NADPH concentration and approached to a maximum. The kinetic properties of the NADPH-induced Chl fluorescence increase reflected a convolution of the behaviour of the NDH-mediated pathway and of a rate-limiting process in the consecutive reactions catalysed sequentially by FNR and FQR, hence a dual Michaelis-Menten model was employed to approximate the change in V_m with NADPH concentration. However, the extensive multiplicity of the least-square solution (values not shown) caused by the divergence of experimental results does not allow a meaningful analysis of the parameters. Fig. 3 also showed that antimycin A and rotenone suppressed the maximal value of V_m by 82 % and 16 %, respectively. The dosage of inhibitors used in the present experiments was sufficient to effect an almost full suppression on antimycin A- or rotenone-sensitive pathway (cf. Endo *et al.* 1998). These results, together with those of Fig. 2, lead to the conclusion that in spinach chloroplasts the FNR-FQR pathway contributes mainly to electron transfer from NADPH to the PQ pool in the dark. This conclusion is also supported by the results observed in osmotically lysed spinach chloroplasts under aerobic condition (values not shown).

Chl fluorescence is emitted predominantly from the antenna system of PS2 (Krause and Weis 1991), which is thermodynamically equilibrated with the PQ pool. Most components of the electron transport from NADPH to PQ involved in the present investigation are extrinsic or intrinsic polypeptides, except ferredoxin, which carries electrons from FNR to FQR by diffusion. It seems that the rate of electron transfer in the antimycin A-sensitive pathway is controlled by the diffusion rate of ferredoxin. However, since NADPH is not normally considered to be a carrier in the cyclic electron transfer sequence, the rate-limiting step might well be the energetically less effective transfer of electrons from NADPH to FNR (Mills *et al.* 1979) or NDH. There is growing evidence for the association of FNR with some sub-units of the chloroplast NDH complex, of which FNR has been proposed to be an integral part that functions as a NADPH-binding site (Guedeney *et al.* 1996, Mi *et al.* 1998, Quiles and Cuello 1998).

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