Kinetic Studies of Protochlorophyllide Reduction *in vitro* in the Greening Mutant C-2A′ of the Unicellular Green Alga *Scenedesmus obliquus*

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The NADPH-protochlorophyllide oxidoreductase, an enzyme catalysing the light-driven conversion of protochlorophyllide to chlorophyllide, was studied in the greening mutant C-2A′ of the unicellular green alga *Scenedesmus obliquus*. Studies of the enzyme activity *in vitro* showed strong dependence on the presence of glycerol and the detergent Triton X-100. Prerequisite for the formation of a photoactive enzyme complex is a sufficient preincubation time with the substrates PChlide and NADPH. A continuous assay system, reading the absorbance increase at the wavelength of chlorophyllide, was used to determine the kinetic constants. The K_m value for NADPH is 4.2 μM, the V_{max} is 5.9 pmol · s^{-1}. The K_m and V_{max} for protochlorophyllide are 0.19 μM and 6.5 pmol · s^{-1}, respectively. The pH-dependence of the reaction exhibits a broad maximum between pH 7–8.5 typically for an enzyme active during chloroplast development, when pH-changes might be expected.

The obtained kinetic data outline that the light dependent formation of chlorophyll *in vivo* is not limited by the substrates PChlide and NADPH, indicating that only light is the triggering factor in the very early greening process.

Introduction

The light-dependent conversion of protochlorophyllide to chlorophyllide in higher plants is the triggering step in the formation of chlorophyll, which is catalysed by the enzyme NADPH:protochlorophyllide oxidoreductase (PChlide reductase, [E.C. 1.3.1.33]). The enzyme, especially its function during chloroplast formation and the light regulation of its expression has been the object of intense studies over the last decades (reviewed in Schulz and Senger, 1993). The photoconversion of protochlorophyllide and the occurrence of several intermediates during the formation of chlorophyllide have been studied in great detail (Dobek *et al.*, 1981; Griffiths, 1974; Ryberg and Sundqvist, 1982; Shibata, 1957; Thorne, 1971a, 1971b). However, only few data about the enzyme *in vitro* have been reported in the literature so far. Griffiths (1975) developed an assay system sufficiently sensitive to monitor continuous absorbance of chlorophyllide increase after the application of light flashes to a solution containing photoactive complexes. With this technique it was possible to characterize an angiosperm PChlide reductase by its kinetic properties (Griffiths, 1978).

Less is known about the reduction of PChlide in green algae. Under normal conditions, green algae are able to form chlorophyll in the dark, when grown on a carbon source. However, there are several algal mutants, which are able to form chlorophyll only in the light (reviewed in Senger, 1987), like the yellow-in-the-dark mutant C-2A′ of *Scenedesmus obliquus* (Bishop, 1971; Bishop and Senger, 1971; Brinkmann and Senger, 1978).

The present paper is the first report on an extensive characterization of kinetic properties of an algal PChlide reductase from the light dependent greening mutant C-2A′ of *Scenedesmus obliquus*.

Abbreviations: ALA, 5-Aminolevulinic acid; Chlide, chlorophyllide; PChlide, protochlorophyllide; PChlide reductase, NADPH: protochlorophyllide reductase; TX-100, Triton X-100.

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Materials and Methods

Organism and growth

All experiments described here were carried out with 3-day-old cultures of the yellow-in-the-dark mutant C-2A′ of *Scenedesmus obliquus* (Bishop, 1971). Cells were cultivated in 500 ml Erlenmeyer flasks in total darkness at 33°C in a gyratory shaker. The inorganic medium (Bishop and Senger, 1971) was supplemented with glucose and yeast extract as external carbon source (Bishop and Wong, 1971).

Preparation of cell-free crude homogenates

Cells were harvested by centrifugation for 5 min at 1,400 x*g*. The cells were resuspended in 20 mM Tricine, 10 mM Hepes, pH 8.0, 1 mM DTT, 1 mM EDTA, 1 mM Triton X-100, 20 % (w/v) glycerol (unless stated otherwise) and broken in a Vibrogen cell (Bühler, Tübingen, FRG) mill for 10 min. After incubation for 30 min on ice to solubilize the PChlide reductase the homogenate was clarified by centrifugation for 90 min at 250,000 x*g* at 2°C.

Prior to kinetic measurements crude homogenates were desalted by chromatography on DL-10 columns according to the manufacturer's recommendation (BioRad, München, FRG) using the buffer as described above.

Isolation of protochlorophyllide

Protochlorophyllide (PChlide) was prepared by methanolic extraction of total pigments from ALA-grown cells of mutant C-2A′ of *Scenedesmus obliquus* followed by separation of PChlide from the crude pigment extract via ion exchange chromatography as described in Knaust *et al.* (1993).

Quantitative assay of PChlide reduction

PChlide reductase activity was assayed by continuous measurement of the absorbance at 672 nm with a reference wavelength at 730 nm with an Aminco DW-2 spectrophotometer (American Instruments Company, Silver Spring, Maryland, USA). Photoconversion was induced by successive blue light-pulses (broad band blue light filter, BG12, Schott, Mainz, FRG) of 4 s each followed by 26 s-dark phases. The activity of the PChlide reductase was expressed as pMol chlorophyllide formed in 1 s at 20°C. The amount of Chlide formed was calculated using the molar extinction coefficient for an aqueous solution of 91.2 $mM^{-1}cm^{-1}$ (Griffiths, 1978).

PChlide was provided as cholate complex as described in Griffiths (1978).

Results and Discussion

Factors influencing the enzyme activity

Preliminary experiments showed a strong dependence of the enzyme activity of the PChlide reductase on the presence of detergents or chaotropic substances.

Next to the effect that the addition of TX-100 stabilizes the cholate complex (Griffiths, 1978), the addition of detergents seems to be essential for the measurement of enzyme activity *in vitro*. For the PChlide reductase of barley 1 mM TX-100 seems to be most effective for highest enzyme activity (Apel *et al.*, 1980). For *Scenedesmus* the optimal test conditions are attained at TX-100 concentrations of 1 mM, too (Fig. 1). The addition of detergent seems to be necessary to promote the binding and release of the relatively hydrophobic pigments, while higher detergent concentrations have a negative effect on the enzyme activity.

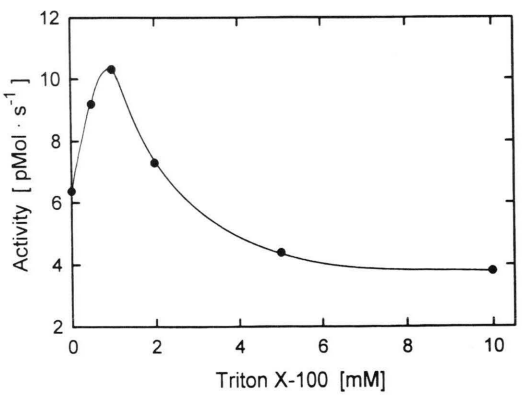

Fig. 1. PChlide reductase activity as a function of the TX-100 concentration. Cells of *Scenedesmus* (mutant C-2A') were harvested by centrifugation and broken in a buffer without TX-100 but with 30% (w/v) glycerol (Urbig *et al.* 1995). Cell-free crude homogenates were supplemented with different concentrations of TX-100. Activity measurements were done in extraction buffer in the presence of saturating amounts of NADPH and PChlide (as cholate-complex, see *Materials and Methods*) of 1.1 mM and 2 µM, respectively. Enzyme concentration and final volume were kept constant in all measurements.

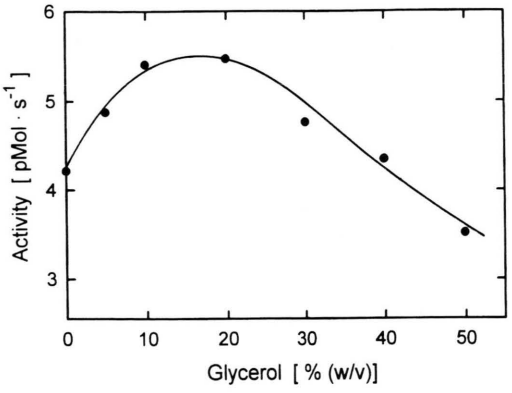

Fig. 2. Effect of glycerol concentration on the activity of PChlide reductase. Cells were broken in the presence of 20 mM Tricine, 10 mM Hepes, pH 8.0, 1 mM DTT, 1 mM EDTA, and 1 mM TX-100. Enzyme activity was assayed in the presence of varying glycerol concentrations in extraction buffer. Conditions of measurement as described in Fig. 1.

The addition of glycerol to the enzyme assay also affects the activity. Highest yields in photoconversion could be observed at glycerol concentrations of 15 to 20%, whereas higher concentrations decreased the enzyme activity (Fig. 2). This could also be observed with PChlide reductases from angiosperms (Apel *et al.*, 1980; Richards *et al.*, 1987). 90% glycerol totally prevents the release of the chlorophyllide from the enzyme complex, indicated by a missing absorption shift (C. Sundqvist, pers. commun.).

The most time-consuming process in the regenerative reaction cycle *in vitro* seems to be the formation of the photoactive complex of PChlide, NADPH, and the enzyme. Preincubation of the reaction mixture is hence necessary, as shown by Fig. 3.

The intensity of the actinic light has also a remarkable effect on the Chlide synthesis, as shown in Fig. 4. Photon flux rates were calculated for 630±10 nm. The illustration shows a saturation curve. Light saturation was not reached below a photon flux rate of $500 \cdot 10^3$ mol m^{-2} s^{-1}.

As consequence of these results all measurements of kinetic constants were done under the optimal conditions described here: The buffer used in all further experiments was 20 mM Tricine, 10 mM Hepes, pH 8.0, 1 mM DTT, 1 mM EDTA, 1 mM TX-100, and 20 % (w/v) glycerol. Illumination was done with light pulses of $500 \cdot 10^3$ mol m^{-2} s^{-1} of blue light and reaction mixtures were preincubated for 5 min.

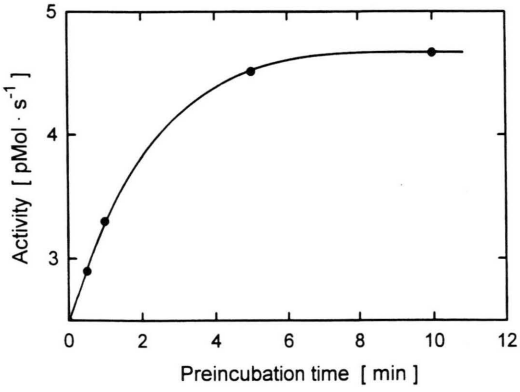

Fig. 3. Formation of photoactive PChlide reductase. Enzyme assays were performed as described in Fig. 1. Activity measurements were performed after different time intervals following mixing of the enzyme assay. Preincubation was started by addition of the cell-free crude homogenate, prepared as described in *Materials and Methods*. Measurements of enzyme activity were done in extraction buffer, containing 20 mM Tricine, 10 mM Hepes, pH 8.0, 1 mM DTT, 1 mM EDTA, 1 mM TX-100, and 20% (w/v) glycerol.

Studies on the initial reaction velocity of PChlide reductase

Light dependent Chlide formation was measured as described in *Materials and Methods* over a wide range of concentrations both of NADPH

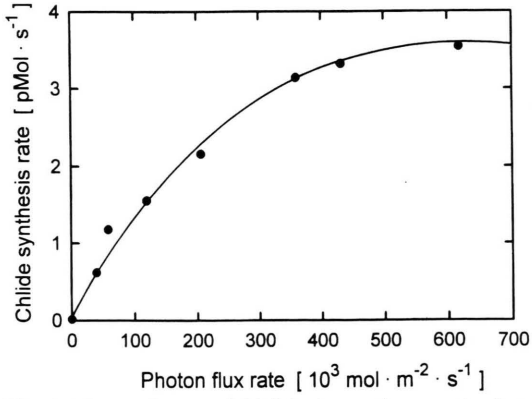

Fig. 4. Dependence of Chlide formation on the intensity of blue-light (actinic light). Rate measurements as described in Fig. 3. Intensities of blue light pulses were varied by changing the projector voltage. Measurements of light intensity were done with a thermopile and calculated for 630±10 nm in respect to the used BG12-filter.

and PChlide. Kinetic constants were calculated by direct fitting of the substrate concentrations and the corresponding initial reaction velocities using a digital computer.

A prerequisite for the measurement of kinetic data is a linear relationship between protein concentration and reaction velocity. In the concentration range up to 1.8 mg ml^{-1} final assay volume, the rate of Chlide synthesis increases linearly with the applied protein concentration under substate saturation conditions (data not shown).

Prior to the measurement, all samples were depleted of low molecular weight substances by size exclusion chromatography. In addition, photoactive complexes of the enzyme were depleted of endogenous substrates by successive light flashes. For the measurement of NADPH-dependence, preillumination was done in presence of an excess of PChlide. Complete absence of endogenous NADPH was checked by addition of PChlide and repeated flashing (Fig. 5a). No enzyme activity could be measured upon illumination until the second substrate NADPH was added. Analogously, assay mixtures were depleted of PChlide by the addition of excess NADPH and repeated flash illumination until no further absorption increase could be observed. Enzyme activity could restored only by addition of PChlide (Fig. 5b).

Relations between the rate of Chlide synthesis and the substrate concentration for both PChlide and NADPH followed hyperbolic saturation curves (Fig. 6a and b). Apparent K_m and V_{max} values for NADPH are 4.2±0.6 μM and 5.9±0.2 pmol · s^{-1}, respectively. For PChlide the K_m was estimated to be 0.19±0.03 μM, the V_{max} was 6.5±0.3 pmol · s^{-1}.

The magnitude of the K_m for PChlide of the *Scenedesmus* PChlide reductase is close to the K_m value reported for the barley enzyme. The approximate PChlide concentration in *Scenedesmus* is about 6 μM, as calculated for the total cell volume (Knaust, 1994). Provided that this represents roughly the concentration of free pigment, this would indicate that a saturating concentration of PChlide for the PChlide reductase is present in the etiochloroplast of the greening mutant C-2A′, making available a sufficient amount of chlorophyllide for the initial setup of photosynthetic units. The K_m for NADPH of the *Scenedesmus* PChlide reductase is only one tenth of that of the barley enzyme (Griffiths, 1978). Griffiths (1978) concluded that the barley K_m value may never be attained within the etioplast membrane system, thus representing a possible regulatory step in the light-dependent formation of chlorophyll. Nevertheless, in *Scenedesmus* the endogenous concentration of NADPH seems to be high enough which would exclude NADPH as rate limiting factor in chlorophyll synthesis (In non-desalted *Scenedes-*

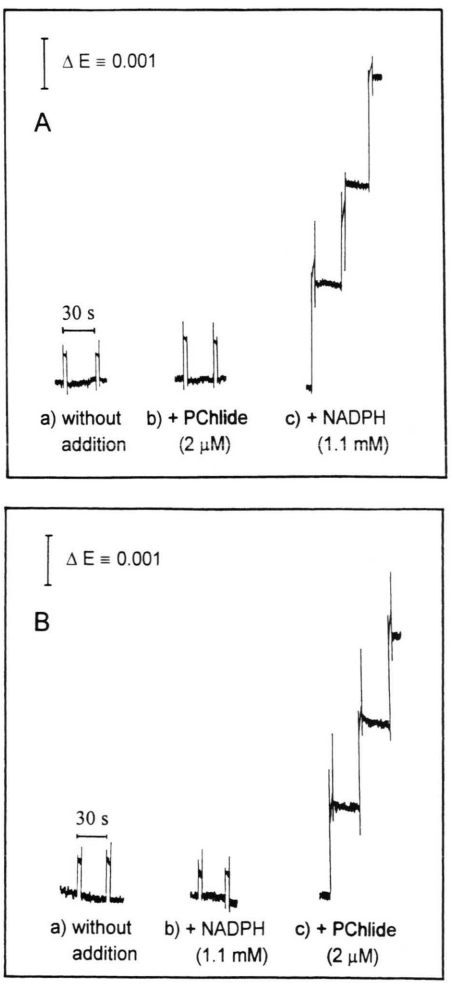

Fig. 5. Test on the absence of endogenous substrates in the reaction assay of the PChlide reductase. (A) No Chlide synthesis was detected upon illumination of the assay mixture depleted of NADPH (a). Enzyme acitivity was not restored with PChlide (b), but after addition of NADPH (c). (B) Reaction assay depleted of PChlide. Absorption increase upon illumination without any addition (a), with NADPH (b) and with PChlide (c), indicating the absence of PChlide in the assay mixture. Further experimental conditions as described in the text.

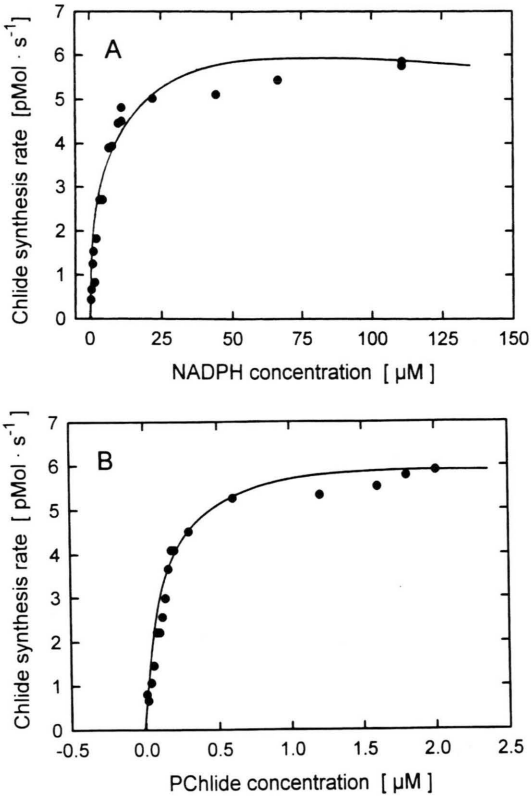

Fig. 6. Plot of the rate of Chlide synthesis *versus* (A) NADPH and (B) PChlide concentration, respectively. Cell-free crude homogenates were prepared as descibed in *Materials and Methods*, desalted and substrate depleted as described in the text. For measurements of the dependence of the initial reaction velocity on PChlide, the cholate concentration was kept constant during all measurements by addition of cholate in buffer without PChlide to rule out any effects of varying detergent concentrations.

mus cell-free crude extracts addition of exogenous PChlide and NADPH does not enhance photoconversion rates (data not shown), indicating of no limitation by endogeneous substrates). Hence, the only regulatory factor limiting the formation of chlorophyll in *Scenedesmus* mutant C-2A′ may possibly be the light applied to the algal system.

Effect of pH on the activity of the PChlide reductase

The dependence on pH of light-dependent Chlide formation was determined in a complex

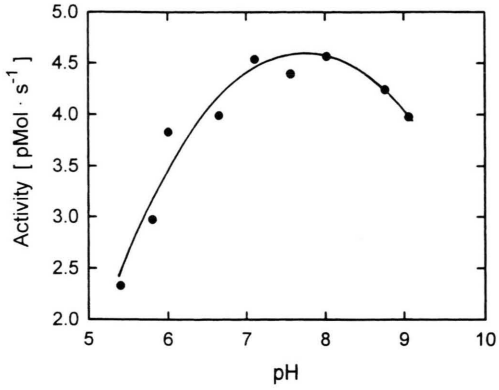

Fig. 7. Effect of pH on the activity of PChlide reductase. Experimental conditions as described in the text. Cells were broken as described in *Materials and Methods*. Aliquots of the cell-free extracts were adjusted to the various pH with high-molar buffers consisting 100 mM Bistris, 100 mM Hepes, and 100 mM Tricine, titrated to pH between 4 and 10, resulting in pH values in the test mixture between pH 5 and 9. Measurements were done in the presence of surplus NADPH and PChlide, as described in Fig. 3.

mixture of buffers to rule out effects due to changing ionic strength over the pH range from 5 to 9 (Stevens, 1992).

Aliquots of cell-free crude homogenates, prepared as described in *Materials and Methods*, were titrated with a buffer containing each 100 mM Bistris, 100 mM Hepes, and 100 mM Tricine, adjusted to various pH values between 4 and 10 to yield different pH values in the homogenate. The relation of enzyme activity of PChlide reductase to pH value is shown in Fig. 7. The curve exhibits a broad maximum between pH 7 and 8.5. This correlates to the behaviour of angiosperm PChlide reductases (Griffiths, 1978; Ikeuchi and Murakami, 1982) and may be necessary for an enzyme which is subject of intense pH changes caused by ATPases during greening (Ikeuchi and Murakami, 1983; Ryberg and Sundqvist, 1982; Wellburn, 1977).

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