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 \(\mu\)mol, the \(V_{max}\) is 5.9 pmol \cdot s\(^{-1}\). The \(K_m\) and \(V_{max}\) for protochlorophyllide are 0.19 \(\mu\)mol and 6.5 pmol \cdot 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.

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 xg. 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 xg 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.

![Activity vs. Triton X-100 concentration](image)
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 500·10^3 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 500·10^3 mol m^-2 s^-1 of blue light and reaction mixtures were preincubated for 5 min.

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
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 substrate saturation conditions (data not shown).

Prior to the measurement, all samples were depleted of low molecular weight substances by size exclusion chromatography. In addition, photoreactive 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 etioplast membrane 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. 6. Plot of the rate of Chlide synthesis versus (A) NADPH and (B) PChlide concentration, respectively. Cell-free crude homogenates were prepared as described 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.

**Effect of pH on the activity of the PChlide reductase**

The dependence on pH of light-dependent Chlide formation was determined in a complex 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 AT-Pases during greening (Ikeuchi and Murakami, 1983; Ryberg and Sundqvist, 1982; Wellburn, 1977).

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