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Activation volume and activation energy of the protochlorophyllide phototransformation and the Shibata shift determined with high pressure fluorescence spectroscopy

Katalin Solymosi[1], László Smeller[2], Judit Fidy[2], Béla Böddi[1]*

[1]Department of Plant Anatomy, Eötvös University, Budapest, Hungary, [2]Institute of Biophysics and Radiation Biology, Semmelweis University, Budapest, Hungary

ABSTRACT The kinetics of protochlorophyllide phototransformation and of the subsequent blue shift were studied in homogenates prepared from etiolated wheat leaves using fluorescence spectroscopy at high pressures. The pressure dependence of the processes was studied between 0.1 and 400 MPa. The activation volumes were calculated from the pressure dependence of the kinetical curves of the processes and were compared at 20ºC and at 40ºC. The temperature dependence of the processes was analyzed at atmospheric pressure (0.1 MPa) between 10ºC and 40ºC. The activation energy of the processes at atmospheric pressure was calculated using Arrhenius plot. The simultaneous variation/change of temperature and pressure provides information about the molecular events occuring during the activation of the processes.
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The transformation of protochlorophyllide (pchlide) into chlorophyllide (chlide) is a light requiring step in the chlorophyll biosynthesis of higher plants. The reaction is driven by the light-dependent NADPH: protochlorophyllide oxidoreductase (POR), and was studied in detail in the leaves of etiolated seedlings (see for review Sundqvist and Dahlin 1997). The enzyme forms ternary complexes with pchlide and the cosubstrate NADPH (Griffiths 1978) and its subunits form aggregates in the etioplast inner membranes (Wiktorsson et al. 1992). Phototransformation has unusual kinetic properties, *i.e.* it completes on msec time scale (Belyaeva et al. 1988), has maximal rate at –14ºC and the enzyme has full activity at –70ºC (Sironval and Brouers 1970). These properties suggest that the process is photophysical or photochemical rather than biochemical. The large difference in the fluorescence maxima of the substrate (pchlide) and the product (chlide) allows the clear analysis of the reaction process. Pressure influences all processes that are accompanied by volume changes. The pressure dependence of the reaction kinetics allows the determination of the activation volume (Balny et al. 1989), which is the volume difference between the activated and the ground state.

Subsequent to the phototransformation the fluorescence emission maximum of the newly formed chlide shifts from 690 nm towards the blue resulting in a chlide form emitting at 680 nm. This blue shift, referred in the literature as Shibata shift in case of intact leaves, completes in 15-30 min (Shibata 1957) at 20ºC and is almost fully inhibited at 0ºC (Treffry 1970). The molecular background of this phenomenon is very complex and is still not very well understood. The spectra show the desaggregation of the pigments, which can occur via conformational changes of POR (Wiktorsson et al. 1993) or via disaggregation of the POR subunits (Akoyunoglou and Michalopoulos 1971). In parallel with Shibata shift, the disruption of the highly regular membrane system of the etioplasts (the prolamellar bodies) was observed (Artus et al. 1992) as well as esterification of chlide (Sironval et al. 1965). All the above mentioned processes may occur at the same time. Besides the conventional spectroscopic methods, other tools should be used to get satisfactory explanation for the Shibata shift. The use of high pressure can provide information about the importance and the nature of the pigment-pigment, protein-pigment, protein-protein, protein-lipid and lipid-lipid interactions during this process (see for reviews Robinson and Sligar 1995; Scarlata 1996; Heremans 1997; Heremans and Smeller 1998). The activation volume calculated from the pressure dependence of the processes, the temperature dependence of the activation volumes together with the activation energy calculated at atmospheric pressure can complete earlier models describing the molecular interactions in the process, and give new insight into the molecular events.

*Corresponding author. E-mail: bbfotos@ludens.elte.hu

Materials and Methods

From the middle section of the leaves of 14-day old dark-grown wheat (*Triticum aestivum*, L., cult. MV17) seedlings a homogenate was prepared in a phosphate buffer (0.05 M Na_2HPO_4/KH_2PO_4, pH 7), containing 20% (w/v) sucrose and 50% (v/v) glycerol. The spectral properties of the homogenates was similar to those of leaves, and was checked before each experiment with 77 K fluorescence spectroscopy. The fluorescence intensity changes were recorded using a FS900CD Luminometer (Edinburgh Analytical Instruments, UK) combined with a thermostatted high pressure optical cell (Unipress, Poland). The pressure was generated by a manually driven pump (Nova, Switzerland). The cuvette containing the sample was positioned in the high pressure sample compartment and pressurized to 400 MPa in steps of 50 MPa. The etiolated samples were irradiated with the excitation

beam of the luminometer (the intensity was appr. 6 µmol $s^{-1}m^{-2}$) at 440 nm, with 0.1 s integration time. The excitation and emission slits were 18 and 3.6 nm, respectively. In order to follow the kinetics of phototransformation the fluorescence intensitiy was recorded at 690 nm [14] during the first 60 seconds of illumination (for details see Solymosi et al. in press).

In order to study the Shibata shift, a series of spectra was recorded at different intervals after phototransformation. The excitation wavelength was 440 nm, the integration time was 0.5 s, the bandwidths of the excitation and emission slits were 9.0 and 3.6 nm, respectively. Measurements were performed at 10, 20, 30 and 40ºC at atmospheric pressure and at 20 and 40ºC the pressure dependence of the processes was studied at 6 different pressure values between 0.1 and 400 MPa.

To study the reversibility of the pressure treatment, the pressure was released to 5 MPa at certain stages of the shift and another set of spectra was measured. (5 MPa was used instead of atmospheric pressure in order to avoid bubble formation in the samples.)

The spectra were analysed with the Spserv V. 3. 14 program (copyright Csaba Bagyinka, Biophysical Institute, Biological Research Centre, Szeged - Hungary). The emission maximum position of each measured spectra was determined (software: Smeller 1998) in order to describe and quantify the kinetics of the blue shift.

Results and Discussion

The kinetics of phototransformation was fitted with two exponentials, because the monoexponential fit did not give satisfactory agreement with the experimental data. The parameters of the exponentials showed strong pressure dependence at 20 and at 40ºC, from which the activation volume could be calculated for both temperatures using the equation: $\Delta V^{\#}=-RT\partial\{\ln(k/k_0)\}/\partial p$ (Balny et al. 1989) where k is the reaction rate at a given pressure (p), and k_0 the reaction rate at atmospheric pressure. At 20ºC the activation volumes corresponding to the two exponential processes were 1.8±1.2 mL mol^{-1} and 2.9±0.5 mL mol^{-1}. The detailed analyses of phototransformation (*i.e.* the recording of the fluorescence intensity decrease and increase at 655 and 690 nm, respectively) at 20 ºC showed, that an activation volume corresponding to 3 $Å^3$ volume change per molecule (1.8 mL mol^{-1}) can be attributed to phototransformation (for details see Solymosi et al., in press). This volume change corresponds to the volume change occuring during the formation of one H-bond per molecule, suggesting the role of H-bonds between the substrate and the enzyme or between NADPH and the enzyme, which is in agreement with earlier models (Van der Cammen and Goedheer 1984). At 40ºC the obtained activation volumes were 6.41± 3.35 mL mol^{-1} and 5.74±1.48 mL mol^{-1} but were not analyzed in details. The increase in the activation volume with increasing temperature indicates greater thermal expansion of the transition state compared to the initial state.

Temperature had no remarkable effect on the rate of phototransformation within the examined temperature range on atmospheric pressure, thus the activation energy could not be obtained with enough precision. It is well known that temperature influences only slightly this process in the 10-40ºC temperature range (Sironval and Brouers 1970), but it was important to check this, in order to distinguish between the effect of temperature and pressure.

In order to study the blue spectral shift (Shibata shift) the peak position of the emission spectra recorded at different time intervals after phototransformation were determined. The shift of the peak position showed exponential kinetics and was fitted with one exponential (the error of the fit was within the range of the experimental data scattering). At 20ºC the process slowed down remarkably at 100 MPa and almost stopped at 400 MPa. The activation volume of the process was obtained the same way as above. Using high pressure fluorescence spectroscopy two different components could be distinguished in the Shibata shift, one was almost pressure independent, *i.e.* it proceeded at high pressures and had zero activation volume, the other component had a strong pressure dependence and was almost stopped at 100 MPa (activation volume: 43±11 mL mol^{-1}). The pressure independent component can be attributed to small changes within the structure, while the second component can be related to conformational changes of POR and depends strongly on the lipid-protein or lipid-lipid interactions within the membrane structure. At 40ºC the pressure dependent phase was faster and the pressure independent phase could not be observed within the range of our measurements. At this temperature, the shift proceeded even at pressures higher than 100 MPa, but its kinetics showed strong pressure dependence. The activation volume obtained for the pressure dependent component was 34.9±1.5 mL mol^{-1}. This means, that the activation volume does not change, or even decreases with increasing temperature. This could be explained by the loosened structure of the lipid layer at 40ºC already in the initial state of the Shibata shift. Therefore the transition state is closer to the initial one in terms of volume at 40ºC than at 20ºC.

At atmospheric pressure, the kinetics of Shibata shift showed strong temperature dependence, *i.e.* it slowed down remarkably at 10ºC and was fast at 40ºC. From the temperature dependence of the reaction rate the activation energy was calculated using Arrhenius plot. The activation energy of Shibata shift at atmospheric pressure was 110359±21887 J/mol, which refers to strong molecular interactions (falls in the range of a covalent bond formation).

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