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Reversible heat-inactivation and photo-reactivation of oxygen evolving center (OEC) of spinach chloroplasts

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Introduction

Heating of chloroplasts at 50 °C for 2 min produced severe inhibition at the OEC and the inhibited chloroplasts could hardly restore the activity of OEC, however, it was also noticed that previous treatment of chloroplasts with reduced DCPIP (DCPIP-treated chloroplasts) stimulated both of the heat-inhibition and the recovery of a small OEC activity by the photo-reactivation (Yamashita and Tomita 1975). The present investigation improved the heat-inactivation condition to be mild enough as to restore the high rate of OEC activity by photo-reactivation. As this will be the first case to have restored the vigorous activity in heat-inactivated chloroplasts the conditions of heat-inactivation and the factors of photo-reactivation are inquired in detail, such as the most effective inactivation temperature and heating time, the requirement and effective concentration of Mn^{2+}, Ca^{2+} and ascorbate, the light intensity and time course of photo-reactivation , and the possibility to generate energy on the thylakoid membrane or to accumulate H^+ into the thylakoid by coupling to the electron transport which was indirectly proved by the stimulation of electron transport by adding an uncoupler or H^+-carrier NH_4Cl (Krogmann, Jagendorf and Avron 1959, and Whitehouse, Ludwig and Walker 1971). And also the degeneration and recovery of PS specific characters, such as the light-induced chlorophyll fluorescence rise (Ebina and Yamashita 1996) and the high and low potential sift of cytochrome-b_{559} (Mizusawa, Ebina and Yamashita 1995) were investigated as the specific symptom of inactivation and reactivation. To identify the specificity of heat-inactivated chloroplasts the best conditions for the photo-reactivation of heat-inactivated chloroplasts and Tris-inactivated and DCPIP-treated chloroplasts was also compared

Materials and methods

Chloroplasts were prepared by grinding 20g spinach leaves in 140 ml ice cold SHN solution, which contained 0.4 M sucrose, 0.01 M NaCl and 0.05 M Tris-HCl (pH 8.0), further added with 10 mM ascorbate, passing the homogenate through two sheets of muslin, and fractional centrifugation (300*g* × 10 min – 600*g* × 10 min chloroplast precipitate was washed by suspending them in 25 ml of S(0.2)HN (sucrose concentration of SHN was decreased to 0.2 M) and by centrifuging at 1000*g* for 10 min. The chloroplast washing was

repeated again and suspended in 25 ml of S(0.2)HN added with 0.01 mM DCPIP and 2 mM ascorbate, kept for 30 min at 0 °C at 1000*g* for 10 min. This treatment with reduced DCPIP (DCPIP-treatment) is essential for the following reversible heat-inactivation. The chloroplast precipitate was suspended in S(0.2)HN to be 2 mg chlorophyll per ml and kept in ice until the use. The heat-inactivation was performed by heating 0.5 ml chloroplast suspension in a small test tube (10 mm × 75 mm) wrapped tightly with aluminum foil and shaking very vigorously in a water bath at. The heat-inactivated chloroplasts were kept in ice and used for the photo-reactivation. In the photo-reactivation the heat-inactivated chloroplasts were suspended to be 50 μg Chl⁄ml in 2 ml reacti-vation mixture which contained 50 mM HEPES-NaOH (pH 7.8), 20 mM NaCl,1 white incandescent light at 240 W light-induced chlorophyll fluorescence rise mM $Ca(CH_3COO)_2$, 20 μM $Mn(CH_3COO)_2$ and 1 mM ascorbate and incubated for 20 min in a small test tube (13 × 100 mm) kept in a water bath white light at 3.4 Ereactivated chloroplasts were directly introduced in the cell equipped with an oxygen electrode to measured the photosynthetic O_2-evolution, or diluted 50 times with S(0.2)HN solution for the measurement of blue actinic light-induced red chlorophyll fluorescence rise. Some of the photo-reactivated chloroplasts were centrifuged, suspended in S(0.2)HN and used to measure the restoration of high-potential cytochrome-b_{559}. Chlorophyll of chloroplasts was extracted with ethyl alcohol and determined the concentration by the method of Wintermans and Mots (1965). Oxygen-evolving activity was measured with a oxygen electrode in a cell of 1 ml containing 50 mM HEPES-NaOH (pH 7.8), 20 mM NaCl, 5 mM $K_3[Fe(CN)_6]$, 2 mM NH_4Cl, 0.05% bovine serum albumin (BSA) and chloroplasts contained 50 μg chlorophyll at the cell with was measured with a hand-made fluorometer by illuminating chloroplast suspensions (1 μg and red) was detected with a photodiode (Ebina and Yamashita 1996). The red/ox spectra differences of high- and low-potential cytochrome-b_{559} were measured with a computer equipped JASCO spectrophotometer V550 by adding 2 mM hydroquinone or ascorbate as a reductant in a sample cell and 0.5 mM $K_3[Fe(CN)_6]$ as an oxidant in a difference cell as reported previously (Mizusawa, Ebina and Yamashita 1995).

Results

The most essential process in the reversible heat-inactivation is the reduced DCPIP-treatment (incubation of chloroplast with reduced DCPIP in the last step preparation, see Materials and Methods. Omission of DCPIP-treatment produced chloroplasts less sensitive to the heat-inactivation at sec. Ascorbate (2 mM) –treatment and oxidized DCPIP-treatment could hardly be substituted for the reduced DCPIP-treatment. Heating 30 sec severely injured the activity of chloroplast OEC but little injured another property of thylakoid membrane, the H^+-uptake driven by the electron transport, as its evidence the photosynthetic electron transport between $DCPIPH_2$ and methyl viologen (O_2) of heat-inactivated chloroplasts was stimulated 2.1 times by adding 2 mM NH_4Cl, an uncoupler or H^+-carrier through the thylakoid membrane. The photosynthetic O_2-evolution of photo-reactivated chloroplasts was also stimulated 2.7 times by adding NH_4Cl. The heat-inactivated chloroplasts kept 10-20% of original O_2-evolving activity and restored 50-70% activity by photo-re-activation. When the heat-inactivation was not enough, the inactivation time may be extended to 40 sec, but the inactivation at higher temperature was avoided because it severely injured the chloroplast ability of photo-reactivation.

Table 1: Effect of heat-inactivation and photo-reactivation on OEC of chloroplasts

Conditions of Chloroplasts	O_2-evolving activity (μmol/mgChl/hr) Control (Comp)	Photo-reactivated (20 min) (-Mn^{2+})	(-Ca^{2+})	(-Asc)	(-Light)	(0 min)	
Normal	346						
Heat-inactivated	30	208	80	54	162	23	36

As shown in Table 1 heat-inactivated chloroplasts can restore the O_2-evolving activity by 20 min photo-reactivation added with the essential factors, Mn^{2+}, Ca^{2+} and ascorbate. When the time of photo-reactivation was extended to 30 min further increase of 10% activity was expectable. The half time of photo-reactivation was 8.1 min. The half light-intensity for the reactivation was 1.4. The half concentration of Mn^{2+} and Ca^{2+} for the photo-reactivation were 1 μM and 0.12 mM, respectively. The actinic light-induced chlorophyll fluorescence rise of chloroplasts was depressed by the heat-inactivation and recovered greatly by the photo-reactivation strongly suggesting the site of inactivation and reactivation to be the rise of chlorophyll fluorescence was depressed by the omission of essential factors and conditions of the photo-reactivation well paralleling with the decrease of O_2-evolving activity in the same conditions in Table 1. The ratio of high-potential form of cytochrome-b_{559} was decreased to 9% by the heat-inactivation and increased to 25% by the photo-reactivation. The rate of recovery is so small at present but by improving the treatment of reactivated chloroplasts and measuring condition the rate will be further increased.

Discussion

The chloroplasts pretreated with reduced DCPIP increased the susceptibility for the heat-inactivation greatly that the O□-evolvimg activity was reversibly removed by heating chloroplasts for only 30 sec time heating will be mild and enabled the inhibited chloroplasts to regain high activity by photo-reactivation and also to hold active thylakoid membrane to accumulate concentrated H^+. Such excellent effect of reduced DCPIP for the chloroplast pretreatment was not substituted by another good reductant, ascorbate although it could reduce DCPIP and/or cytochrome-f and cytochrome-b_{559} in chloroplasts. A special function of reduced DCPIP in the heat-inactivation must be found out. In the chloroplast preparation they were washed with S(0.2)HN twice because this washing was a slightly useful for the non-DCPIP-pretreated chloroplasts in the stimulation of heat-inactivation. Perhaps chloroplasts swelled more in S(0.2)HN (contained 0.2M sucrose) of lower osmotic pressure than in SHN (contained 0.4 M sucrose) . In the reactivation factors omission of light or 20 min incubation brought the largest decrease for the recovery of O_2-evolving activity. However, as the optimum light intensity was low (between 3-6) the injury by stronger light must be avoided. That the weak light is optimum suggests the dark reaction is the limiting step in reactivation.

The large half time of reactivation (8.1 min) will be another evidence of some slow reaction. One of the light function may be photooxidation of Mn^{2+} as reported by Tamura and Cheniae (1987) and Miyao-Tokutomi and Inoue (1992), however, there may be unknown additional function as the active Mn^{2+} incorporation into the thylakoid lumen or the rearrangement of the membrane structure and configuration for rebinding some intrinsic peptides and Mn^{n+} to reconstitute the proper structure of OEC. The contribution of Ca^{2+} was also large but their function was not obvious and must be explained by further investigation. Different from the strongly Mn^{2+}-requiring photo-reactivation of Tris-washed chloroplasts the reversibly heat-inactivated chloroplasts could recover about a half activity without the addition of Mn^{2+} in the photo-reactivation (Table 1). Some Mn of OEC might not be eluted out from the inside of thylakoid lumen as the thylakoid membrane was kept rather intact as mentioned above. Effect of reductant ascobate is always observed as a contributor for the photo-reactivation althought their effect is very smaller. Perhaps the heat-inactivated chloroplasts still contained enough reducing substrate in them.
Because the method of this reversible heat-inactivation is simple and convenient, it may be useful for the study and the demonstration of OEC photo-reactivation.

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