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A Mechanistic Study on the Early Stage-Events Involved in Low Temperature Stress in *Clamydomonas reinhardtii*

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Abstract : The exposure of *Clamydomonas reinhardtii* to low temperatures resulted in an accumulation of cellular pyruvate that dissipated when the chilled cells returned to ambient temperature. The dissipation of pyruvate accumulation was accompanied by an increase in the production level of superoxide radicals (O_2^-) in cells. The formation of O_2^- at an excessive level during the post-chilling period was apparently countered by a substantial activation of superoxide dismutase (SOD). All these results are similar to those observed previously in rice seedlings subjected to the cold-treatment, implicating that a common mechanism is probably underlying for the primary processes of chilling injury both in higher plants and in algae. It was also observed that the activation of Mn-containing SOD contributes the major share in the increase of SOD activity of whole algal cells. Because Mn-SOD is present only in mitochondria, the observation corroborates the concept that the O_2^- scavenging enzyme would be induced to cope with the cold treatment-caused adverse situation in mitochondria where the toxic active oxygen is produced at rates far exceeding the normal rate. (Received October 18, 1994; accepted November 21, 1994)

Introduction

Chilling injury in plants is defined as a physiological disorder that occurs when plant species of tropical and subtropical origin are exposed to low temperatures usually in the range of 0 to 15°C.[1] Although physiological dysfunction and visible morphological symptoms shown by plant species are various, chilling injury has been regarded as a consequence of disruption of normal metabolic processes induced by the phase transition of cellular membranes. As is now generally accepted, cellular membranes of plants undergo a physical phase change from a normal liquid crystal to a solid gel structure at temperatures lower than the phase transition temperature.

It has been inferred that intramitochondrial production of superoxide radicals (O_2^-) at a markedly elevated level is closely associated with the early stage events of low temperature stress in higher plants.[2] It has also been indicated that the end product of glycolysis, pyruvate, accumulates in cells to a significant extent during the cold treatment period of rice seedlings,[3] supporting the concept that the dynamic balance of cellular metabolism for energy production is severely disturbed by low temperatures. However, rice seedlings, placed in a cold (5°C) chamber for a rather long time up to 4 days, do not show any significant symptoms identifiable as chilling injury, even though their growth activity is apparently inhibited, implying that the disruption of metabolic processes is not in itself directly linked to chilling injury in higher plants. Furthermore, previously this laboratory observed that, when rice seedlings subjected to cold treatment were translocated to ambient temperatures (ca 25°C), the pyruvate accumulation started to dissipate, concomitantly accompanying a remarkable

Key words : Chilling injury, phase transition, pyruvate accumulation, superoxide dismutase, superoxide radical
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increase in cellular O_2^- level which was followed by the activation of superoxide dismutase (SOD) in leaf tissues with a considerable lag time.[3,4]

The present investigation was undertaken to address questions whether biochemical mechanism for the primary processes of chilling injury in higher plants, as deduced from the observations in rice seedlings, is equally applicable to the explanation for deleterious effects of chilling treatment in algae and whether the increase in SOD activity of plant cells is due principally to the induction of Mn-SOD which is known to be present only in mitochondria. We herein report that the pyruvate accumulation in *Clamydomonas reinhardtii* by low temperature treatment is the immediate cause of the overproduction of cellular O_2^- during the post-chilling period, as in rice seedlings, and that the activation of Mn-SOD does indeed contribute the major share in a marked increase in SOD activity of plant cells upon being translocated to ambient temperature after the cold treatment.

Materials and Methods

Chemicals and instruments

Biochemicals used were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and obtained from either Fluka Chemie AG (Buches, Switzerland) or Wako Pure Chemical Ind. (Osaka, Japan). These were of the highest purity available and used without further purification. Throughout the work, spectrophotometric measurements were done with a HP 8452 A diode-array spectrophotometer (Hewlett Packard Co., Palo Alto, CA) and oxygen in samples was assayed by using a polarograph constructed in this laboratory. The light fluence rates of visible light (λ>400 nm) were determined by using a IL 1700 Research Radiometer/Photometer (International Light Inc., Newburypot, MA).

Growth conditions of organism

The unicellular green algae *Chlamydomonas reinhardtii* L. (wild type strain 137c, mating type +) were grown in a Tris-acetate phosphate (TAP) medium in a 2-liter Erlenmeyer flask with air bubbling at 25℃ under fluorescent light (20 W/m^2). The TAP medium was prepared by dissolving 2.42 g of Trizma base in 1 liter of the mixture of a salt solution (25 m*l*), 1M potassium phosphate buffer (1 m*l*), a trace element solution (1 m*l*) and distilled water (974 m*l*); the final pH was titrated to 7.0 with glacial acetic acid. The salt solution contained NH_4Cl (1.5 M), $MgSO_4$ (8 mM) and $CaCl_2$ (9 mM) respectively. The trace element solution containing Na_2-EDTA (0.13 M), H_3BO_3 (0.18 M), $ZnSO_4$ (0.14 M), $MnCl_2$ (26 mM), $FeSO_4$ (18 mM), $CoCl_2$ (6.8 mM), $CuSO_4$ (6.3 mM), and $(NH_4)_6Mo_7O_{24}$ (0.9 mM), was prepared according to Surzycki.[5] Synchronous growth was achieved by a 12h light-12h dark cycle. Cultures were used after 48h of total growth. The strain was maintained on 1.5% agar plates.

Measurement of respiratory activity

Respiration of intact algal cells in the TAP medium was measured by monitoring oxygen uptake, as described previously.[6] Chlorophyll (Chl) content of the algal cells was determined by the method of MacKinney.[7]

Determination of superoxide and pyruvate

Cellular level of O_2^- in the algal cells was assayed by the reduction of nitro blue tetrazolium (NBT), as described previously.[3] Cells grown to early stationary phase were harvested by centrifugation and washed with a cold 50 mM potassium phosphate buffer (pH 7.8). Washed cell pastes were suspended in the potassium phosphate buffer containing 0.1 mM EDTA ($1\sim1.5\times10^6$ cells/m*l*), admixed with 5 mM KCN and 20 μM NBT, and then sonicated at 23 kHz for 4×15 s at 4°C, using a Soniprep 150 Ultrasonic disintegrator (MSE-Scientific, Sussex, England). Centrifuging at 20000 g for 10 min at 4°C, the supernatant was used to measure the amounts of the reduction product of NBT.

The procedure of Dawkins *et al*,[8] as modified by Kim *et al*,[3] was employed for the assay of pyruvate. The pastes of algal cells were suspended in

the potassium phosphate buffer ($1\sim1.5\times10^6$ cells/ml) and disrupted. Disintegrated cell suspensions were admixed with chloroform and centrifuged at 20000 g for 10 min to prepare the supernatant free of contaminations by pigments and cell debris. The pyruvate content was measured by the decrease in absorption at 340 nm of NADH in a reaction mixture containing 0.8 ml of the crude extract, 0.1 ml of NADH (1.26 mM) and 0.1 ml of L-lactic dehydrogenase (50 units/ml) in the potassium phosphate buffer. Packed cell volume (PCV) was determined by centrifuging the aliquots of the cell suspensions in hematocrite tubes for 5 min at 1400 g.

Measurement of SOD activity in cells

Cells were harvested by centrifugation, washed, resuspended, and disrupted by sonication as described above. Cell debris and unbroken cells were removed by centrifugation for 10 min at 20000 g. Superoxide dismutase (SOD) contained in the cell extract was assayed by the NBT-xanthine-xanthine oxidase method of Beauchamp and Fridobich[9] as modified by Britton.[10] The cell extract, 40 μM NBT, 32 μM xanthine and 2.5 mu/ml xanthine oxidase were incubated at 25°C in the potassium phosphate buffer. The SOD-inhibitable reduction of NBT was determined by monitoring the increase of absorbance at 530 nm. The rate of NBT reduction in the absence of the cell extract was used as the reference rate. Protein content was measured by the method of Bradford[11] with bovine serum albumin as the standard.

Separation of enzyme and eletrophoresis

Ammonium sulfate fractionation of protein was initiated by the addition of solid $(NH_4)_2SO_4$ to the cell extract to a final concentration of 30% saturation. Removing the precipitated protein by centrifugation, the $(NH_4)_2SO_4$ concentration of the supernatant fraction was increased to 80% saturation. The pellet collected was resuspended in and dialyzed against 10 mM potassium phosphate buffer (pH 7.8). Nondenaturing PAGE was performed on 10% acrylamide gels according to the method of Davis.[12] SOD bands were visualized by soaking the gels in the photochemical NBT stain solution, that contained 2.45 mM NBT, 28 mM tetramethylethylenediamine, 28 μM riboflavin, 0.1 mM EDTA and 36 mM potassium phosphate (pH 7.8), and illuminating them with light from an incandescent lamp, as described by Beauchamp and Fridovitch.[9] To differentiate Mn-SOD from Fe-SOD, the gels were soaked for 1h in the stain solution containing additionally 1 mM KCN and 5 mM H_2O_2.

Results and Discussion

Phase transition of mitochondrial innermembranes

Changes in physical structure of cellular membranes are usually reflected by alterations in biochemical activity of membrane proteins: the membrane-associated enzymic activities can therefore be

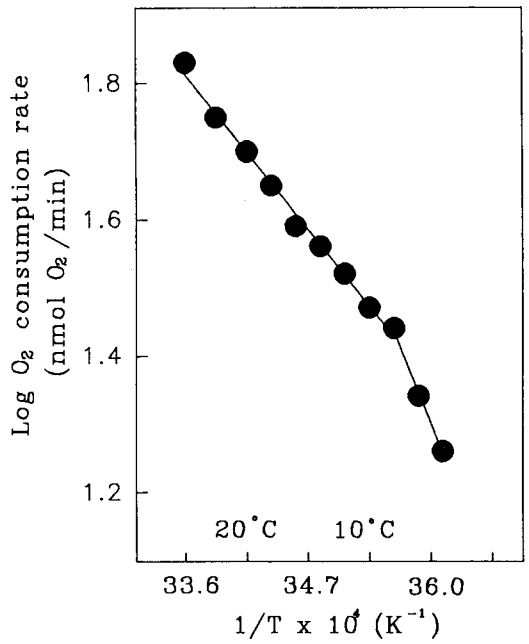

Fig. 1. The Arrhenius plot for the respiratory activity of *C. reinhardtii*. Oxygen uptake by the intact algal cells (30 μg Chl/ml), adapted to the dark for 5 min in the TAP medium at various temperatures, was measured polarographically. Data are averages of duplicate measurements.

biphasic before and after the temperature where the phase transition of membrane takes place. In case of mitochondria the Arrhenius plot of electron transport of respiration versus absolute temperature has often provided convincing evidence as to whether the inner membrane undergoes phase transition.[1,13]

When oxygen uptake by *Clamydomonas reinhardtii* was measured at various temperature in the ranges from 2°C to 25°C and the Arrhenius plot for the respiratory activity was prepared, it turned out that discontinuity clearly appeared at ca 9°C, indicating the occurrence of the fluid-to-solid transition of the algal mitochondrial membranes in temperature ranges where higher plants of tropical or subtropical origin often incur chilling injury. From the data shown in Fig. 1, it was estimated that the activation energy of overall respiratory reaction occurring in the membrane of solid gel phase is about twice as much as that of the electron transport through mitochondrial inner membrane that is in normal liquid crystal phase.

Changes in cellular levels of pyruvate and O_2^-

In *C. reinhardtii* cultured at 25°C, the steady state concentration of pyruvate was measured to be 15 μmol per mL of packed cell volume: this pyruvate remained nearly constant as long as the algal cells were stored at ambient temperature. When the culture was exposed to low temperature in a cold (5°C) chamber, however, the content of cellular pyruvate started to gradually increase, finally attaining the maximum value of 43 μmol/mL PCV in 3 days; thereafter it was leveled off, as shown in Fig. 2.

Low temperature-induced pyruvate accumulation in plant cells was previously observed for the first time in this laboratory.[3] This observation provided

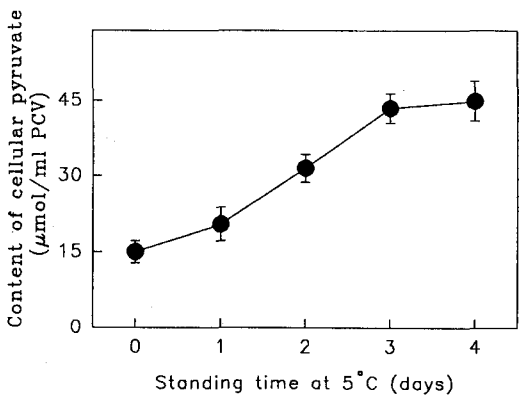

Fig. 2. Accumulation of cellular pyruvate as a function of time of cold-treatment of *C. reinhardtii*. The Algae in the TAP medium at $\times 10^5$ cells/ml were kept in a 5°C chamber and taken in every 24h to measure pyurvate. Data are averages of triplicate measurements with the spread shown as bars.

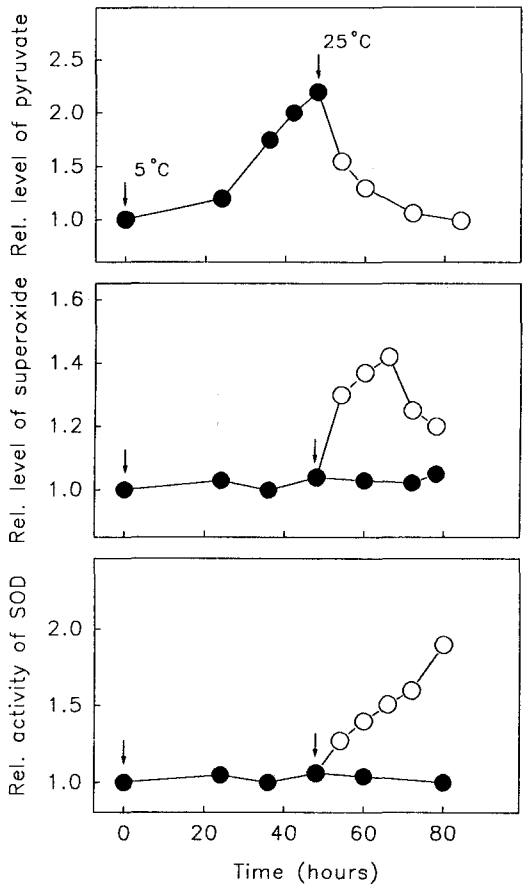

Fig. 3. Changes in the relative levels of pyruvate (A), superoxide radicals (B) and SOD activity (C) in *C. reinhardtii* during the period of exposure to 5°C (●—●), as compared with those observed during the post-chilling period at 25°C subsequent to the cold-treatment for 48h (○—○).

a rationale for our proposition on the mechanism of the early phase events of chilling injury in rice plants: that is, the structural change of mitochondrial membranes results in disturbing the dynamic balance of carbohydrate metabolism sequentially involving glycolysis, the citric acid cycle (CAC) and respiration electron transport, which in turn leads to accumulation of the final product of glycolysis in plant cells; for all enzymatic reactions of glycolysis, occurring in cytosol and thus being independent of cellular membranes, presumably proceed rather normally even at low temperature, whereas the CAC and particularly the respiratory reactions are apparently affected by a change in structural integrity of mitochondrial membranes.

As soon as the cold-treated algal cells returned to ambient temperature, the pyruvate accumulation started to dissipate, as shown in Fig. 3(A). Further, the dissipation was accompanied by an increase in cellular level of O_2^-. As can be seen in Fig. 3(B), the O_2^- level reached to the maximum value in 18 hours and then was rapidly reduced. All these phenomena at molecular level in the algal cells under low temperature conditions are very similar to those observed in rice seedlings, implying that a common mechanism is likely underlying for chilling injury both in higher plants and in algae in so far as the primary processes are concerned.

In relation to the injurious effects of the cold-treatment, whose visual symptoms usually appear during the post-chilling period rather than the chilling period, the overproduction of O_2^- in cells that occurs during the post-chilling period is particularly noteworthy. Because the presence of O_2^- at high concentrations in mitochondrial suspensions has brought about a substantial loss of the respiratory activity[14] that can result in deleterious effects in cell physiology, the formation of O_2^- in mitochondria at an excessive level can be the immediate cause of chilling injury. For the present, it is not known whether O_2^- per se takes part in redox reactions in cells, resulting in chemical modification of important cellular components, or transforms into other activated oxygen species such as peroxides and hydroxyl radicals that could be rather directly involved in the injury-initiating reactions.

Induction of Mn-SOD

Assays of SOD of *C. reinhardtii* revealed that the activity of the O_2^- scavenging enzyme in the algal cells remained almost unchanged from the beginning to the end of the cold treatment, but it started to increase upon translocation of the chilled cells

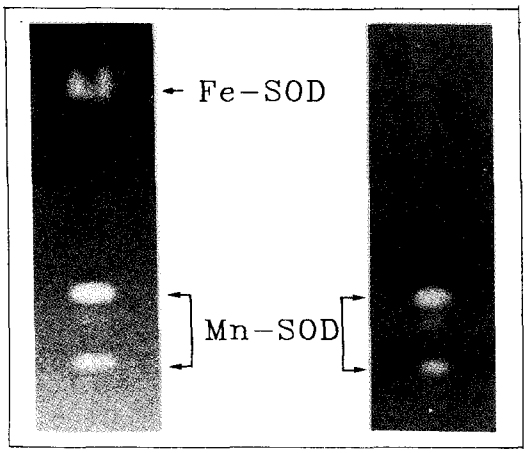

Fig. 4. Nondenaturing PAGE of SOD isozymes from *C. reinhardtii*. Loading 44 μg protein on each slot and developing, the gels were stained by using the photochemical NBT staining method either in the presence (the right) or in the absence (the left) of H_2O_2 and KCN.

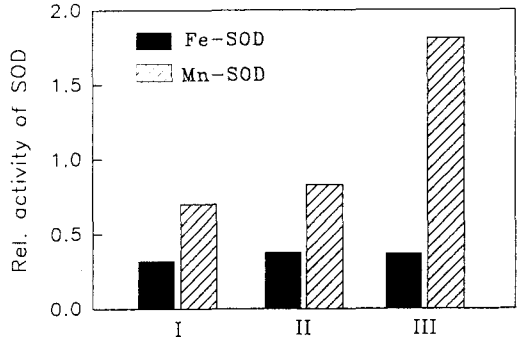

Fig. 5. Activities of SOD isozymes from *C. reinhardtii* harvested at three different stages of the temperature-treatment.
I: at the onset of the cold-treatment at 5°C; II: after 48h at 5°C; III: after 48h at ambient temperature (25 °C) from the point of II

to 25°C. This phenomenon is virtually identical with that observed in rice seedlings, as shown in Fig. 3(C), again corroborating the concept of cellular strategy of plants to counter the overproduction of O_2^- in cells.

It is known that higher plants usually have three types of SOD isozymes, *viz.* Cu/Zn-SOD, Mn-SOD and Fe-SOD. However, it has been reported that algal cells do not contain Cu/Zn-SOD.[15,16] Electrophoretic fractionation done in this work also confirmed that SOD in *C. reinhardtii* consists of Mn-SOD and Fe-SOD only. Fig. 4 shows that the most slowly moving SOD band disappeared completely when the gels were soaked in the photochemical NBT stain solution which contained additionally 5 mM H_2O_2 plus 1 mM KCN: meanwhile, the fast moving bands were affected to a far less extent by H_2O_2 in the presence of KCN, which was added to exclude the possibility of catalase action decomposing H_2O_2. Because H_2O_2 is an effective inhibitor for Fe-SOD and because Mn-SOD is generally insensitive to H_2O_2 as well as to CN^-, the above result not only shows various isozyme types of SOD in *C. reinhardtii* but also enables us to locate the position of protein bands corresponding to each isozyme type on gels.

Nondenaturing PAGE was performed using the $(NH_4)_2SO_4$-fractionated SOD preparations from the algae harvested at different stages of temperature treatment, that is, at the onset of the cold-treatment, right after the cold-treatment for 48 hours, and after 48 hours of exposure of the cold-treated algae to ambient temperature, respectively. Cutting out the sections of gel plates that contain each isozyme type of SOD, enzymes were eluted and then assayed. It turned out that, while the activity of Fe-SOD of *C. reinhardtii* was insensitive to changes in environmental conditions in so far as growth temperature is concerned, Mn-SOD was significantly activated when the cold-treated cells returned to room temperature. Comparing this result (Fig. 5) with the result shown in Fig. 3(C), it is evident that the increase in SOD activity of the algal cells during the post-chilling period is mainly due to the activation of Mn-SOD. Considering that Mn-SOD is present only in mitochondria and that SOD is a substrate-inducible enzyme,[17] this observation seems to be consistent with the suggestion that Mn-SOD is rapidly induced in mitochondria to cope with an adverse situation where the potentially harmful active oxygen O_2^- is formed at rates far exceeding the normal rate.

Conculsions

We have demonstrated in the present study (1) that the physical structure of mitochondrial inner membrane of *C. reinhardtii* undergoes transition from a normal liquid crystalline phase to an abnormal solid gel phase at temperatures lower than 9°C, (2) that pyruvate accumulates in the algal cells to a significant extent when they are kept exposure to low temperatures, (3) that the pyruvate accumulation dissipates when the chilled cells return to ambient temperature, (4) that the dissipation of the pyruvate accumulation is accompanied by an increase in cellular content of O_2^- as well as by an increase in SOD activity of the cells, and (5) that O_2^--dependent induction of Mn-SOD that occurs in mitochondria appears to contribute the major share in the increase in SOD activity of the algal cells during post-chilling period. Taking these results all together, the accumulation of pyruvate in the algal cells that occurs during the chilling period as a consequence of the disruption of dynamic balance maintained in the carbohydrate metabolic pathway linking glycolysis and the citric acid cycle, which are compartmentalized by mitochondrial membranes, is the immediate cause of the overproduction of O_2^- in mitochondria during the post-chilling period, as is the case in rice plant. Because O_2^- not only is potentially harmful to cellular structure and function but also can produce much more toxic active oxygen such as hydroxyl radicals, O_2^- overproduction is supposed to be closely associated with the early phase events of chilling injury in algae as well as in higher plants. Further, it may also be concluded that the induction of Mn-SOD in mitochondria is the primary defensive measure of al-

gal cells, operating to counter against the injury-initiating chemical species.

Acknowledgement

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*Clamydomonas reinhardtii*의 냉해 초기과정에 관한 기작론적 연구

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초록 : 상온에서 배양된 녹색조류 *Clamydomonas reinhardtii*를 5°C의 저온에 세워두었을 때 세포내에는 괄목할 만한 pyruvate의 축적이 일어났다. 그러나 저온처리된 세포를 다시 상온으로 옮기면 세포내 pyruvate 수준은 감소하기 시작하며 이에 수반하여 superoxide radicals ($O_2^{\cdot}$)의 발생수준이 증가하였다. 일정기간 후 최고치에 도달한 $O_2^{\cdot}$ 수준은 다시 비교적 빠르게 떨어지기 시작하였는데, 이때를 전후하여 세포내 superoxide dismutase(SOD)가 현저하게 활성화 되었다. 이러한 결과는 벼의 냉해와 관련하여 얻었던 기존(본 연구실)의 관찰 사실과 매우 유사한 것으로서 고등식물이나 조류가 공히 냉해의 초기과정에 관한한 동일한 기작의 지배를 받고 있음을 시사한다. 아울러 본 연구에서는 저온처리에 의해 야기된 SOD의 활성 증가가 대부분 Mn-SOD의 활성화에 기인하는 것으로 관찰되었다. Mn-SOD는 세포 소기관 중 미토콘드리아에만 존재하므로, 이러한 관찰은 독성이 큰 산소화학종이 과생성되는 부정적 상황 즉 냉해유발 상황에 대처하기위해 항산소성 효소가 냉해의 초기과정이 진행되는 미토콘드리아에서 유도된다는 개념과 부합된다.