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Effect of osmotic shock on the redox system in plasma membrane of *Dunaliella salina*

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ABSTRACT

The unicellular halotolerant alga *Dunaliella salina* had the ability to oxidize NADH and reduce $Fe(CN)_6^{3-}$. The redox reactions were to some extent stimulated by slight hyperosmotic shock (2.0 mol/L → 2.6 mol/L NaC1), but markably inhibited by abrupt hyperosmotic shock (2.0 mol/L → 3.5 mol/L NaC1) and hypoosmotic shock (2.0 mol/L → 1.0 mol/L NaC1; 2.0 mol/L→0.67 mol/L NaC1). With the adaptation of algal cells to osmotic shock by accumulating or degrading intracellular glycerol, the plasmalemma redox activities were also restored. The O_2 uptake stimulated by NADH could be promoted by FA and SHAM. Hypoosmotic shock increases the basal respiration rate of alga cells, but weakened the stimulating effects of NADH, FA and SHAM on O_2 uptake. On the other hand, hyperosmotic shock reduced the basal respiration rate, but relatively enhanced the above effects of NADH, FA and SHAM. H^+ extrusion of alga cells was inhibited by NADH and stimulated by $Fe(CN)_6^{3-}$. Vanadate and DES could inhibit H+ efflux, but had little effect in the presence of NADH and $Fe(CN)_6^{3-}$. Both hyper- and hypoosmotic shock stimulated H^+ extrusion. This effect could be totally inhibited by vanadate and DES, but almost unaffected by 8-hydroxyquinoline. It was suggested that H^+-ATPase probably played a more important role

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in H^+ extrusion and osmoregulation under the conditions of osmotic shock.

Key words: *Osmotic shock, Dunaliella salina, plasmalemma redox system.*

INTRODUCTION

Oxidation-reduction (redox) system found in mitochondria and chloroplasts also universally exists in plasma membrane of plant cells[1, 2]. Like H^+-ATPase, it may participate in the establishment of transplasmalemma electrochemical potential of H^+ which provides energy for solute transport, and plays significant roles in the physiological and biochemical processes in the course of plant growth and development. Evidence is accumulating that active oxygen is generated when trans-plasmalemma electron transfer and O_2 absorption occur. This process is very important in the reaction and adaptation of plants to various environmental stress, such as pathogen, drought, low temperature, hypoosmotic shock and UV radiation. Barr and Crane[3] found that the redox system in the plasmalemma of carrot cells was very sensitive to salt stress, both NADH oxidation, $Fe(CN)_6^{3-}$ reduction and H^+ extrusion were inhibited. Qiu et al[4] also demonstrated that the redox activities in the plasmalemma of wheat root cells were greatly decreased under water stress. So the redox system in plasmalemma may have close relation to plant's reaction to stress. *Dunaliella salina* is a kind of unicellular green algae without rigid cell wall. It is extremely halotolerant and has strong ability of osmoregulation, capable of growth in salinities as different as 0.05 mol/L and 5.5 mol/L NaC1. Furthermore, it reacts sensitively to many extracellular signals, thus becomes a model system for the study of plant resistant mechanisms. In our laboratory, much research work on the osmoregulation of *D. salina* have been conducted[5, 6] and a redox system in its plasmalemma was characterized[7]. A basic question arises regarding the role of plasmalemma redox system in osmoregulation. In the present study, we report the response and possible functions of plasmalemma redox system of *D. salina* cells in the course of osmotic shock.

MATERIAL AND METHODS

Material

The source of the *Dunaliella salina* strain used in this study was grown as described[8] in aseptic medium containing 2.0 mol/L NaCl, under 12 h light (60 μmol/m^2/s PAR) at 26℃. Those used in experiment were at logarithmic growing phase.

Osmotic shock

Osmotic shock was applied by dilution with media containing 5.0 mol/L NaCl or no NaCl to

the desired final NaCl concentrations[6]. 2 min after shock, we began to measure the rates of redox activity, oxygen uptake and proton efflux.

Redox assays

Culture samples containing ($1\text{-}3 \times 10^6$ cells/ml) were centrifuged at 1000 g for 5 min: The cells were rinsed twice with the isoosmotic solution containing 0.3 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$ and 10 mmol/L Tris-MES (pH 8.4) and resuspended in the same solution. A 2.5 ml aliquot was taken for various treatments. NADH or $Fe(CN)_6^{3-}$ were added to start reaction. NADH oxidation was measured in the incubation medium as a loss of absorption at 340 nm in a Shimadzu UV-190 double-beam spectrophotometer using a millimolar extinction coefficient of 6.23. $Fe(CN)_6^{3-}$ reduction was determined by a loss in absorption at 420 nm using a millimolar extinction coefficient of 1.00.

Detection of glycerol content

Glycerol estimation was carried out according to the procedures of Oren-Shamir et al.[9]

Measurement of oxygen uptake

Oxygen consumption was measured by Clark-type oxgen electrode, which was fixed at the bottom of experimental cup made of polyesteramide. Assay mixture consisted of 2.5 ml algal suspension and the cup was tightly sealed. Oxygen absorption was determined at 25℃ in the dark and lasted 15 to 30 min.

Proton extrusion

After resuspension in medium containing 2.0 mol/L NaCl, the algal cells were transferred to a polyethylene cone with flat bottom of 50 ml and stirred slightly. After bubbled with air not containing CO_2 for 10 min, the measurement began with a PD-2 research pH meter and lasted for 30 min. The amount of H^+ extrusion of different samples was determined by back titration with 0.09879 mol/L NaOH at the same time of measurements.

Cell number was determined for each sample in a Coulter counter for more than 3 times until the average deviation less than 10%.

RESULTS

Effect of osmotic shock on plasmalemma redox activities of D. salina

Dunaliella salina cell has the ability to oxidize and reduce extraeellular non-permeable electron donor NADH and electron acceptor $Fe(CN)_6^{3-}$ respectively. In the presence of both NADH and $Fe(CN)_6^{3-}$, the redox activity was greatly increased (Tab 1). Slight hyperosmotic shock (2.0 mol/L → 2.6 mol/L NaCl) could, to some extent, enhance the plasmalemma redox activity, while abrupt hyperosmotic shock (2.0 mol/L → 3.5 mol/L NaCl) and hypoosmotic shock (2.0 mol/L → 1.0 mol/L NaCl; 2.0 mol/L → 0.67 mol/L NaCl) notably inhibited the redox activity, and the inhibition effect had some correlation with the extent of shock (Tab 1).

Glycerol is primarily an only osmoticum in *D. salina* cells[5, 9]. In responding to hyper- or hypoosmotic changes in the medium, the cells first react as osmometers,

Tab 1. Effect of osmotic shock on the activity of plasmalemma redox system in *D. salina*

NADH oxidation rate (nmol / 10^8 cells / min)					
Substrate	Control	Hyperosmotic shock		Hypoosmotic shock	
(mmol/L)	2.0 mol/L NaCl	2.0→2.6 mol/L	2.0→3.5 mol/L	2.0→1.0 mol/L	2.0→0.67 mol/L
NADH(0.3)	28.1±6.2 (100.0)	36.7±7.4 (130.6)	5.1±1.7 (18.1)	23.4±3.3 (83.3)	10.0±0.2 (35.6)
NADH(0.3) +$Fe(CN)_6^{3-}$(0.6)	113.9±6.3 (100.0)	/	25.3±4.6 (22.2)	/	3.9±2.8 (33.2)
$Fe(CN)_6^{3-}$ reduction rate (nmol/10^8 cells/min)					
$Fe(CN)_6^{3-}$(0.6)	116.8±5.8 (100.0)	195.2±23.5 (168.1)	53.0±9.3 (45.7)	81.2±6.8 (70.0)	35.8±2.7 (30.7)
$Fe(CN)_6^{3-}$(0.6) +NADH(0.3)	330.3±6.9 (100.0)	357.1±21.8 (108.2)	152.5±20.1 (46.2)	270.6±15.2 (81.8)	55.2±9.5 (16.7)

shrinking or swelling, soon followed by rapid synthesis or elimination of glycerol (Fig 1). Full osmotic adaptation is marked by reassumption of the original cell volume.

During the adaptation process, the plasmalemma redox activity was also gradually recovered (Fig 2).

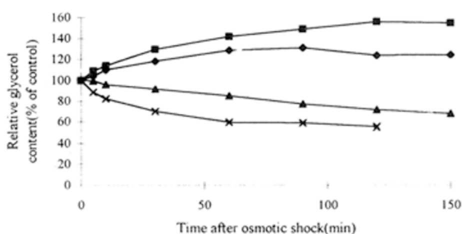

Fig 1. Effect of osmotic shock on glycerol metabolism in *D. salina*
-◆- 2 to 2.6 mol/L NaCl,
-■- 2 to 3.5 mol/L NaCl,
-▲- 2 to 1 mol/L NaCl,
-×- 2 to 0.67 mol/L NaCl

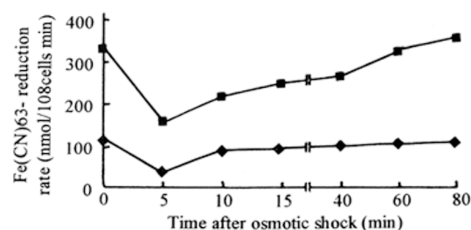

Fig 2. Recovery of the plasmalemma redox activity of *D. salina* cells during osmotic shock.
-◆- 2 to 0.67 mol/L NaCl,
-■- 2 to 3.5 mol/L NaCl,
+ NADH (0.3 mol/L)

Effect of osmotic shock on O_2 absorption in the presence of NADH

NADH stimulated O_2 absorption of algal cells, an effect which could greatly enhanced by adding SHAM (1.0 mmol/L) and ferulic acid (FA, 1.0 mmol/L) (Fig 3, 4). Hypoosmotic shock increased the basal respiration rate, but weakened the stimulating effects of NADH, SHAM and FA on O_2 uptake (Fig 3).

On the contrary, hyperosmotic shock reduced the basal respiration rate of the cells, but relatively enhanced the stimulating effects of NADH, SHAM and FA on O_2 uptake (Fig 4).

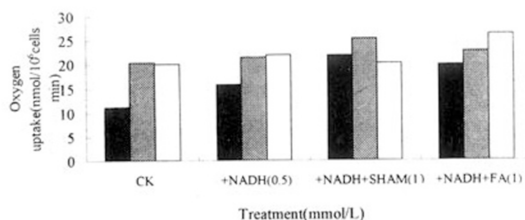

Fig 3. Effect of hypoosmitic shock on oxygen uptake by *D. salina* cells in the presence of NADH
■ control
▨ 2.0 to 1.0 mol/L NaCl
□ 2.0 to 0.67 mol/L NaCl

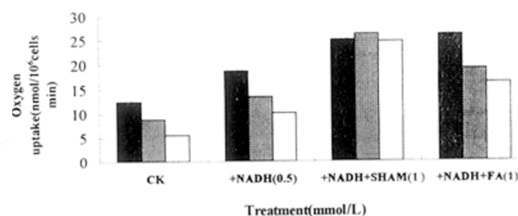

Fig 4. Effect of hyperosmotic shock on oxygen uptake by *D. salina* cell in the presence of NADH
■ control
▨ 2.0 to 2.6 mol/L NaCl
□ 2.0 to 3.5 mol/L NaCl

Plasmalemma redox system and H^+ transport

Addition of exogenous NADH inhibited H^+ efflux of *D. salina* cells, while $Fe(CN)_6^{3-}$ stimulated H^+ efflux. In the presence of both NADH and $Fe(CN)_6^{3-}$, the rate of H^+ efflux increased dramatically to about 409.7% of control (Tab 2).

Tab 2. Effect of NADH and $Fe(CN)_6^{3-}$ on H^+ efflux of *D. salina*

Addition (mmol/L)	H^+ efflux (nmol/10^8 cells/h)
None (CK)	3.42 ± 0.20 (100.0)
NADH (1.0)	-4.03 ± 0.84 (-117.84)
$Fe(CN)_6^{3-}$ (2.0)	5.16 ± 0.50 (150.88)
NADH (1.0)+$Fe(CN)_6^{3-}$ (2.0)	14.01 ± 3.47 (409.65)

Plasmalemma H^+ -ATPase inhibitors such as vanadate and DES could almost completely inhibit acidification of the medium. The addition of NADH and $Fe(CN)_6^{3-}$ was then accompanied by a renewed acidification of medium. The above effect of NADH and $Fe(CN)_6^{3-}$ on H^+ efflux could be inhibited by 8-hydroxyquinoline, an inhibitor of plasmalemma redox system (Tab 4). The stimulatory effect of $Fe(CN)_6^{3-}$ on H^+ efflux was enhanced by ethanol treatment, and the enhancement was inhibited by arsenite treatment (Tab 3).

Tab 3. Influence of inhibitors on H^+ efflux stimulated by NADH and $Fe(CN)_6^{3-}$

Inhibitors (mmol/L)	H^+ efflux (nmol/lO^8 cells/h)
None (CK)	3.46
Vanadate (0.08) + DES (0.1)	0.54
Vanadate (0.08) + DES (0.1) + NADH (1.0) + $Fe(CN)_6^{3-}$ (2.0)	13.67
8-hydroxyquinoline (1.0) + NADH (1.0) + $Fe(CN)_6^{3-}$ (2.0)	2.79
Ethanol (200)[a] + $Fe(CN)_6^{3-}$ (2.0)	6.69
Ethanol (200) + Arsenite (10.0)[b] + $Fe(CN)_6^{3-}$ (2.0)	0.86

a. the cells were treated with ethanol (200 mmol/L) for 1 h.
b. the cells were treated with arsenite (10.0 mmol/L) for 30 min.

Osmotic shock and plant plasma membrane redox system

Both hypo- and hyperosmotic shock markable increased the H^+ efflux rate of agal cells. This effect was totally inhibited by vanadate and DES, but almost unaffacted by 8-hydroxyquinoline (Tab 4)

Tab 4. Effect of osmotic shock on H^+ effiux of *D. salina*

Treatment (mmol/L)	H^+ efflux (nmol/10^8 cells/h)
Control	3.66±0.63
Hypoosmotic shock (2.0→0.67 mol/L NaCl)	5.57±0.65
Hypoosmotic shock (2.0→0.67 mol/L NaCl)+Vanadate (0.08)+DES (0.1)	1.36±0.10
Hypoosmotic shock (2.0→0.67 mol/L NaCl)+8-hydroxyquinoline (1.0)	5.29±0.83
Hypoosmotic shock (2.0→3.5 mol/L NaCl)	7.24±1.25
Hypoosmotic shock (2.0→3.5 mol/L NaCl)+Vanadate (0.08)+DES (0.1)	0.83±0.06
Hypoosmotic shock (2.0→3.5 mol/L NaCl)+8-hydroxyquinoline (1.0)	6.82±0.46

DISCUSSION

Dunaliella salina has attracted great attention of scientists as a model system to study the response of plants to environmental stress[5, 10]. On receiving stress signals, the plasma membrane composition, structure and function are altered. This is very important for the cells to transduce extracellular signals, to lead to physiological reactions and to adapt to stress conditions. We found that plasmalemma redox system of *D. salina* is very sensitive to osmotic shock. Except of slight hyperosmtic shock, abrupt hyperosmotic shock and hypoosmotic shock greatly decreased the rates of NADH oxidation and $Fe(CN)_6^{3-}$ reduction (Tab 1), which is in accordance with the phenomenon observed by Crane and Barr under salt stress[3] and Qiu et al under water stress[4].

The electron transport chains of plasmalemma redox system are multiple [11, 12]: innerside or outerside cis-plasmalemma electron transfer; inward or outward trans-plasmalemma electron transfer etc. The enzymes responsible for electron transfer are NAD(P)H: $Fe(CN)_6^{3-}$ standard system oxidoreductase, NAD(P)H: Fe^{3+} chelator oxidoreductase, NAD(P)H: O_2 oxidoreductase, peroxidase, nitrate reductase, and so on[2, 12]. Generally speaking, exogenous NADH increased O_2 uptake through plasmalemma electron transport. This process was enhanced by two peroxidase activitors FA and SHAM (Fig 3, 4), suggesting that the oxidoreductase in the plasmalemma of *D. salina* might have peroxidase properties. The stimulation of basal respiration rate of algae by hypoosmotic shock was favorable to provide energy for the conversion of glycerol to starch. That hypoosmotic shock weakened the O_2 uptake stimulated by NADH, SHAM and FA was in agreement with the decrease of plasmalemma redox activity at the time, and also suggested that mitochondria electron transfer was primarily responsible for O_2 uptake during hypoosmotic shock (Fig 3). The conversion of starch to glycerol in the course of hyperosmotic shock was an energy producing process, thus required little energy from the respiration chain, accordingly the basal respiration rate of algal cells was reduced (Fig 4). Abrupt hyperosmotic shock notably inhibited NADH oxidation, but relatively enhanced the

stimulatory effects of NADH, SHAM and FA on O_2 uptake. The inconsistence indirectly showed that O_2 might not be the final electron acceptor of exogenous NADH oxidation enhanced by SHAM and FA; or NADH might cause the "wound" response of agal cells during hyperosmotic shock[13], which stimulated other O_2 consumption pathways related to SHAM and FA. It was also possible that hyperosmotic shock initiated another redox pathway in plasma membrane different from standard system. This new redox pathway used O_2 as terminal electron acceptor and probably reduced glutathione and ascorbate, not NADH, as terminal electron donors. From the different effects of hypo- and hyperosmotic shock on plasmalemma redox activities and O_2 uptake, we could see that hypo- and hyperosmotic shock probably influenced the operation of plasmalemma redox system through different mechanisms, and the responses of various oxidoreductases in plasmalemma to osmotic shock might also be different.

Up to now, it is thought that only plasmalemma H^+-ATPase was responsible for H^+ extrusion in *D. salina*[8, 14]. Our evidence showed that the plasmalemma redox system could also be proton pumping and therefore involved in energization of the plasma membrane (Tab 2, 3). Ethanol, which could increase the intracellular NADH level through the action of cytoplasmic alcohol dehydrogenase[15], led to an increase of H^+ efflux. In contrast, arsenite, which could decrease the intracellular NADH level, inhibited H^+ efflux (Tab 3). The cytoplasmic NADH generated might stimulate trans-plasmalemma electron transport, which in some way coupled to H^+ efflux. The H^+ efflux in the presence of both NADH and $Fe(CN)_6^{3-}$ was not inhibited by plasmalemma H^+-ATPase inhibitors, but inhibited by plasmalemma redox system inhibitors (Tab 3). This supported the idea that plasmalemma redox system was directly coupled to H^+ efflux, not dependent on the activation of H^+-ATPase. Moreover, the redox-driven H^+ pump possibly had regulatory connections with H^+-ATPase. Accompanying trans-plasmalemma electron transport, H^+ may extrude through a protonated electron carrier (ubiquinone), or H^+ channels, or the conformational change of redox proteins[2, 11]. Under osmotic shock conditions, H^+ efflux was inhibited by vanadate and DES, but almost unaffected by 8-hydroxyquinoline (Tab 4). This indicated that the contribution of plasmalemma redox system to H^+ efflux was small. This was consistent with the reduction of redox activities at the time. So the stimulatory effect of hypo- or hyperosmotic shock on H^+ efflux is mainly through the activation of H^+-ATPase. It was reported that water stress increased plasmalemma H^+-ATPase activity[4]. Chen et al.[5] also found that osmotic shock stimulated H^+-ATPase, decreased the intracellular ratio of ATP to Pi, and thus activated glycerol metabolism. The increase of H^+-ATPase activity under environmental stress is thought to play a central role in the adjustment of algal cells against stress [5, 9], but the effect of plasma membrane redox system on the adaptation to stress can not be easily excluded and remains to be investigated.

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