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Contribution of nitrate to H^+ permeability of *Synechococcus* PCC 6311 plasmalemma vesicles

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Abstract

The fluorescent pH-sensitive probe 9-aminoacridine was used to show the contribution of nitrate to proton permeability of purified plasmalemma vesicles from the cyanobacterium *Synechococcus* PCC 6311. Kinetics of the fluorescence quenching of 9-aminoacridine were performed with a proton gradient of 1.5 ΔpH across the plasma membrane, acidic inside, in the presence of different salts ($NaNO_3$, KNO_3, NaCl, KCl) inside or outside the plasmalemma vesicles. It was observed that the proton gradient dissipation was accelerated when the NO_3^- anion was in the same compartment as H^+, whatever the cation. When the salt was external, $NaNO_3$ had a more efficient effect than KNO_3 on the H^+ gradient dissipation, which could be explained by the H^+/Na^+ antiport system present in this membrane. Analysis of the data suggested the existence of an H^+/NO_3^- symport.

Keywords: Plasmalemma; Nitrate transport; Proton permeability; Fluorescence quenching; (Cyanobacterium)

1. Introduction

Since nitrate is the principal nitrogen source for all photosynthetic organisms and particularly for cyanobacteria, the mechanism of nitrate transport at the level of the plasmalemma deserves particular attention [1–6]. Purified membrane vesicles provide a suitable experimental system for studying the molecular mechanism of transport across the membrane in the absence of interactions with other components of the cell metabolism [3,7,8]. When the nitrate permeability of *Synechococcus* plasmalemma vesicles was examined, by EPR [9], significant differences were observed between potassium and sodium nitrate, depending on ΔpH (unpublished data). Since this method is not suitable for low salt concentrations and kinetic studies in the minutes time-scale, pH-dependent fluorescence quenching of 9-aminoacridine [7,10–13] was used to examine the contribution of nitrate to protons movements. Comparative kinetic analysis of the fluorescence quenching of this probe incorporated into the plasmalemma vesicles with a ΔpH of 1.5, in the presence of potassium and sodium nitrate or chloride with or without addition of nigericin, suggested the possibility that an H^+/NO_3^- symport could play a role in nitrate transport into plasmalemma vesicles.

2. Material and methods

The experiments described in this report were performed on the cyanobacterium strain *Synechococcus* PCC 6311.

2.1. Culture conditions

The cyanobacteria were grown at 30° C in sterilized culture medium according to Kratz and Myers [14], under gentle stirring and bubbling with 2% CO_2 enriched air. The culture was illuminated by 50 $\mu E/m^2$ per s white light.

2.2. Membrane preparation and purification

The preparation and purification of the plasmalemma vesicles as well as the protein determination were per-

Abbreviations: PCC, Pasteur Culture Collection; 9-AA, 9-aminoacridine; ΔpH, pH gradient between inner and outer space of the vesicles; CM, cytoplasmic membrane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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formed as previously described [15]. These vesicles were 50–65% right-side-out oriented [15] and 0.25 μm in diameter [16].

2.3. Treatment of CM vesicle samples for fluorescence assays

Before fluorescence intensity measurements, the CM vesicles were equilibrated in various conditions as described below. CM were incubated for 2 h, at 4° C in a large volume of 5 mM Hepes-KOH buffer (0.4 mM K^+) pH 6.35, containing 300 mM sorbitol (the incubation medium), centrifuged for 1 h at 100 000 × g, at 4° C then resuspended in the same medium and divided into 40-μl aliquots, each containing 50 μg of CM protein. CM aliquots were kept in an ice-bath until use in fluorescence assays. Four different salts were used: KNO_3, $NaNO_3$, KCl and NaCl. To load the CM vesicles with the salt in question, 40 μl aliquots were incubated for 2 h in the ice-bath with 10 μl of the incubation medium containing 2.5 mM salt, final concentration.

2.4. Fluorescence measurements

9-AA was used as ΔpH probe across cyanobacteria plasmalemma vesicles [7,10–13]. This dye, a weak base, is distributed across the membrane according to ΔpH [7], which is not the case for all acridine dyes. However, the use of 9-AA necessitated the choice of particular experimental conditions. The 9-AA concentration chosen was 0.1 μM, to avoid self quenching of the dye in the absence of CM vesicles which could occur at higher concentrations. After stabilization of the fluorescence intensity, the experiment was initiated by injection of 50 μl of CM vesicle suspension (50 μg of CM protein) into the reaction medium. The hyperbolic variation of the initial quenching (see the definitions section below) with the CM vesicle volume was checked. The variation of fluorescence intensity induced by the ΔpH of 1.5 was constant in the protein concentration range 25 μg–50 μg. Usually, 50 μg (0.03 mg/ml) of CM protein were used to measure the variation of fluorescence quenching during pH equilibration between the internal CM vesicle volume (pH 6.35) and reaction medium (pH 7.85). The reaction medium was composed of 1.5 ml of 5 mM Hepes-KOH buffer (4 mM K^+), pH 7.85, containing 300 mM sorbitol and 0.1 μM 9-AA. The reaction medium (pH 7.85) for fluorescence measurements differed from the incubation medium (pH 6.35) by a 10-fold higher K^+ concentration. For investigation of the effect of nigericin on the permeability of CM vesicles with the various salts inside or outside, a final concentration of 0.01 nM nigericin was added to the reaction medium before injection of CM vesicles [17]. The nigericin stock solution was prepared in ethanol. We observed that the addition of ethanol alone had no effect on the fluorescence intensity of 9-AA. The remaining ΔpH after 10 min was dissipated by addition of gramicidin-D (0.02 mg/mg of total CM protein) to each fluorescence assay [17]. The gramicidin stock solution was prepared in ethanol. During all measurements the reaction mixture was maintained at 25° C under gentle stirring. Fluorescence emission measurements were performed with a JY3D Jobin Yvon spectrofluorometer and the fluorescence intensity variations were recorded with a Sefram recorder. Excitation and emission wavelengths were 400 nm and 460 nm respectively.

2.5. Some definitions used for fluorescence kinetic analysis

Initial quenching = F_{100}: the maximal quenching of the fluorescence emission after CM vesicle injection. This quenching is due to the compartmentalization of 9-AA within the inner space of CM vesicles.

F_0: the fluorescence level measured after gramicidin addition, i.e., after total dissipation of the ΔpH. This fluorescence level was the same whatever the time of gramicidin addition. It was equal to the fluorescence level observed after complete equilibration, for at least 1 h.

time-dependent ΔpH quenching = $F_t - F_0$: the amplitude at time t of the fluorescence emission, which depends on ΔpH dissipation rate.

maximum ΔpH-dependent quenching: the amplitude of fluorescence corresponding to the difference between F_{100} and F_0.

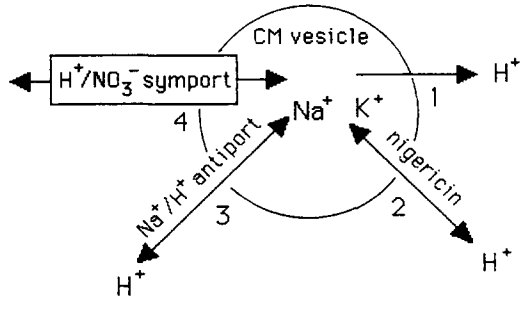
dissipated ΔpH: the difference $F_t - F_{100}$.

percentage of fluorescence quenching = $Q\%$ at time (t): this value was calculated from the following formula.

$$Q\%_{(t)} = \frac{F_t - F_0}{F_{100} - F_0} \times 100$$

percentage of dissipated ΔpH = $Q\%$ (t) calculated at 10 min.

The terms used above are presented in the following diagram:



The kinetic data were fitted as a sum of two exponentials according to the following relationship in which k_1 and k_2 are the rate constants of fast and slow kinetic phases of ΔpH-dependent quenching, respectively. F_1, F_2 are the corresponding amplitudes.

$$Q\%_{(t)} = F_1 \exp^{-k_1 t} + F_2 \exp^{-k_2 t}$$

2.6. Statistics

The data were the result of seven experiments, except those illustrating the effect of nigericin which were based on five experiments. Each kinetic profile was repeated three times in each experiment. The kinetic data were fitted as described above and standard deviations were calculated.

3. Results

An example of a kinetic profile is presented in Fig. 1. The calculated rate constants and the percentages of dissipated ΔpH for different experimental conditions are summarized in Fig. 2.

The reaction medium (pH 7.85) for fluorescence measurements differed from the incubation medium (pH 6.35) by a 10-fold ratio of K^+ concentration (4 mM and 0.4 mM, respectively). However, when K^+ salts were included in the vesicles this ratio decreased to 1.5 and when they were added to the external medium it increased to 15. When sodium salts were studied the Na^+ concentration difference was always 2.5 mM. The conditions for cations were therefore not equivalent. To eliminate this difference, one solution would have been to use another organic buffer as a substitute for K^+, but in this case complex quenching of 9-AA would have occurred, as pointed out by Deamer et al. [10]. Another solution would be to block the Na^+/H^+ antiporter naturally present in the *Synechococcus* plasmalemma [18–20], but our pH conditions were incompatible with an effective inhibitory effect of amiloride [21]. To obtain a parallelism with the Na^+/H^+ antiporter, nigericin was used as an artificial K^+/H^+ antiporter [17]. According to the kinetic profile presented in Fig. 1, the nigericin concentration was chosen in order to increase the ΔpH decay rate and to maintain this rate at a measurable level. Once these experimental conditions were established, the following kinetic data were obtained.

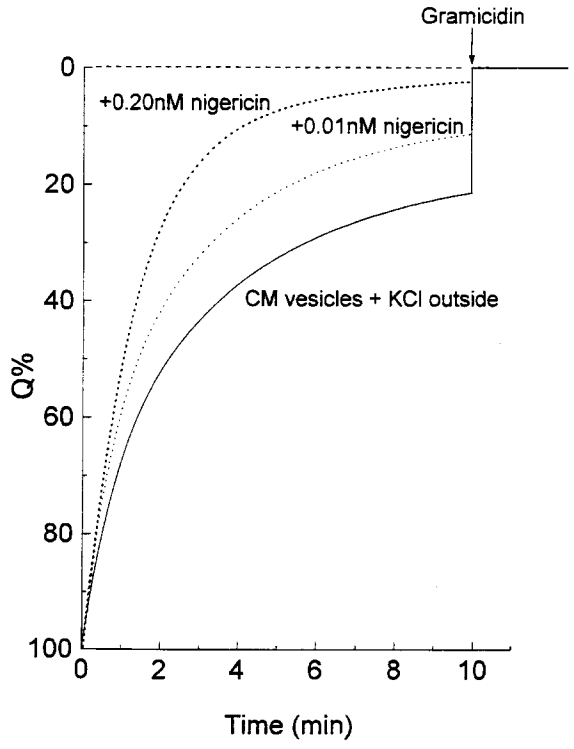


Fig. 1. Kinetics of ΔpH-dependent quenching with KCl outside the CM vesicles in the presence (dashed lines) or absence (solid line) of nigericin. The reaction medium at pH 7.85 contained 2.5 mM KCl. The kinetics were initiated by the injection of 50 μg CM vesicles preincubated at pH 6.35.

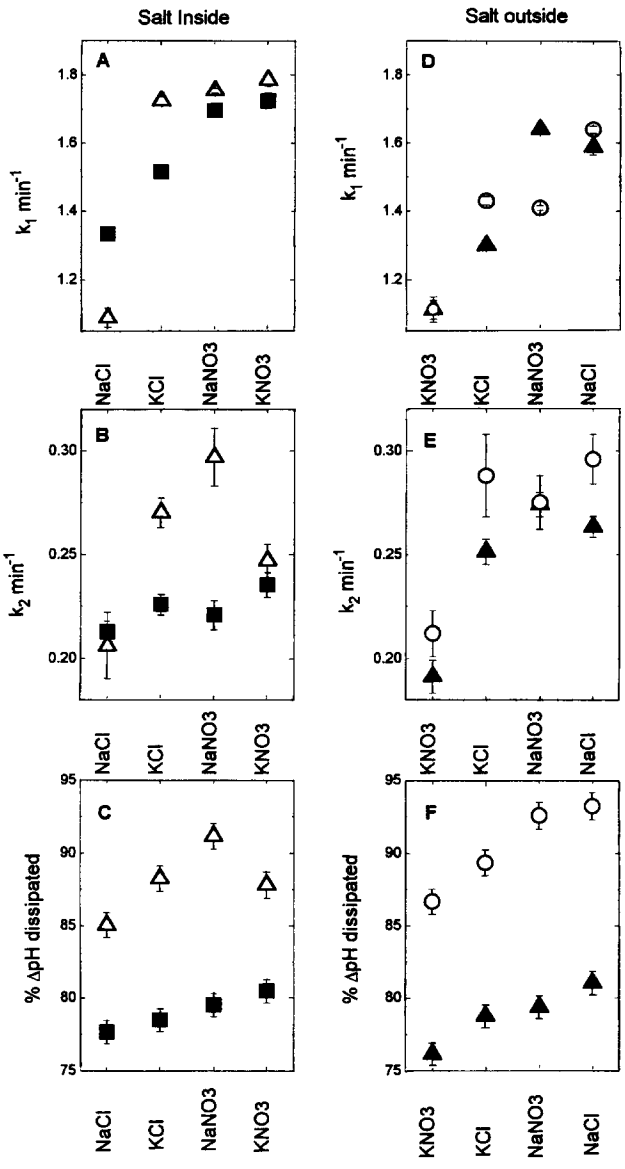


Fig. 2. Effect of salts on values of percentages of dissipated ΔpH (t = 10 min) and rate constants for fast (k_1) and slow (k_2) phases of kinetics. Closed symbols correspond to values obtained in the absence of nigericin and open ones to those obtained in the presence of nigericin (means ± standard deviations).

3.1. Effect of salt inside

Fig. 2A shows that the rate constants calculated for the fast phase k_1 were in the following ascending order: NaCl, KCl and $NaNO_3 \approx KNO_3$. The same rank order was obtained for values of the rate constant of the slow phase, k_2 (Fig. 2B) and the percentage of dissipated ΔpH (Fig. 2C).

3.2. Effect of salt outside

The values of k_1 could be ranked in the following increasing order: KNO_3, KCl and $NaNO_3 \approx$ NaCl (Fig. 2D). The same order was noted for the values of k_2 (Fig. 2E) and the percentage of dissipated ΔpH (Fig. 2F)

Comparison of the data presented in Fig. 2 (closed symbols) shows that the dissipated ΔpH percentages and the H^+ export rate constants were dependent on the location of KNO_3 and NaCl. The values were minimal for NaCl and maximal for KNO_3 when these salts were inside. An opposite result was observed when NaCl and KNO_3 were added to the external medium. Indeed, despite the high external K^+ concentration, KNO_3 yielded the lowest rate constants and percentage of dissipated ΔpH. The $NaNO_3$ rate constants were close to those of KNO_3 when these salts were inside, i.e., in the same compartment as H^+, and to those of NaCl when these salts were outside. The rate constants measured in the presence of KCl had intermediate values whatever its location.

3.3. Effect of salt inside in the presence of nigericin

As expected, the percentage of dissipated ΔpH was increased by the presence of nigericin, to a different extent depending on the salt (Fig. 2C). A comparison of the kinetic parameters after addition of nigericin revealed that the minimal and the maximal values of rate constants were observed in the presence of the same salts : NaCl and $NaNO_3$, respectively (Fig. 2A, B, C).

3.4. Effect of salt outside in the presence of nigericin

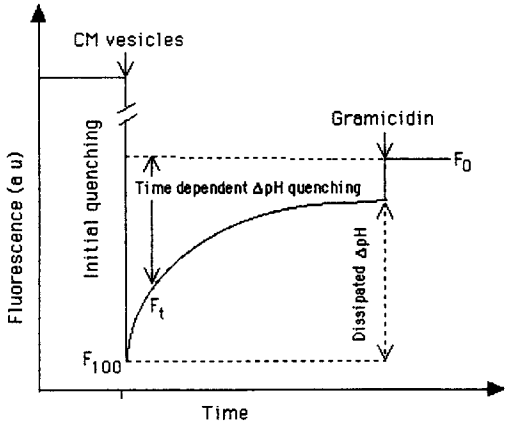
An enhancing effect of nigericin on the dissipated ΔpH was also observed (Fig. 2F). These kinetic profiles revealed that the minimal and the maximal values for percentage of dissipated ΔpH as well as both rate constants were obtained in the presence of KNO_3 and NaCl, respectively (Fig. 2D, E, F).

Again, it was observed that the percentage of dissipated ΔpH and export rate constants were extremely dependent on the location of KNO_3 and NaCl. As expected, nigericin increased the percentage of dissipated ΔpH for all the salts whatever their location. In the presence of KCl, nigericin also increased the both rate constants. However, an unexpected effect on the H^+ flux was observed in the presence of NaCl inside or $NaNO_3$ and KNO_3 outside. In the presence of the sodium salts, the rate constant k_1 decreased and k_2 did not vary. In the presence of KNO_3 outside, the lowest k_1 value was observed, despite the amplitude of the potassium gradient.

4. Discussion

The fluorescent pH-sensitive probe 9-AA was used to show the contribution of potassium, sodium nitrate and chloride to proton permeability in purified plasmalemma vesicles of the cyanobacterium *Synechococcus* PCC 6311. Generally, 9-AA is used in steady-state conditions in order to measure the ΔpH between different compartments [7,10–13]. In this report, we used the comparative kinetic analysis of fluorescence relaxation quenching of 9-AA incorporated into CM vesicles to determine the relationship between H^+ export and salt passage across this membrane.

All the kinetic profiles representing the H^+ export were biphasic (Fig. 1) and could be fitted using a two exponential model. The corresponding kinetic parameters could be informative about the influence of the different symport and antiport mechanisms involved in this process. From the calculated rate constants of the ΔpH decay and the percentage of dissipated ΔpH, the variations of the outward H^+ flux can be discussed. The following scheme is proposed in order to clarify the discussion of the results:



The H^+ flux across the membrane is controlled by four components:

(1) The spontaneous outward flux of protons.

(2) The artificial K^+/H^+ antiport created by nigericin [17] when it was added to the medium.

(3) The Na^+/H^+ antiport described for cyanobacteria [18–20].

(4) A putative H^+/NO_3^- symport.

The Na^+/NO_3^- symport [22,23] is not shown on the scheme, since it is not directly related to the H^+ flux in this isolated system.

The overall H^+ flux is the algebraic sum of the fluxes

of these four components of the system which is described by a simple mathematical model. Components (1) and (2) always contributed to an outward flow of H^+, in our experimental conditions, whereas components (3) and (4) contributed to inward or outward H^+ flow according to the location of the studied salt.

First, we discuss the results concerning the effect of NaCl on the outward H^+ flux (Fig. 2). The strong dependence of both rate constants and the percentages of dissipated ΔpH on NaCl location demonstrated the functioning of the Na^+/H^+ antiport. The lowest rate constants were observed for the internal location and the greatest for the external one. Indeed, the contribution of process (1) was increased by the additional contribution of process (3) to ΔpH dissipation when NaCl was present outside the vesicles. In the presence of nigericin, involving process (2), when NaCl was inside, the first rate constant was paradoxically the lowest, indicating that the Na^+/H^+ antiporter was able to buffer the H^+ efflux. This example illustrated the sensitivity of the comparative kinetic studies for demonstrating the role of different factors affecting H^+ movements.

The outward flux of H^+ was not so sensitive to the location of KCl. The kinetic parameters had intermediate values under all experimental conditions and nigericin had the expected effect of increasing the both rate constants and the percentage of dissipated ΔpH (Fig. 2).

Nitrate salts modified the H^+ efflux in different ways (Fig. 2). Since the presence of KNO_3 or $NaNO_3$ inside the vesicles resulted in the same effect on the H^+ efflux and since the Na^+/H^+ antiporter could not work efficiently under these conditions, it was necessary to postulate another mechanism contributing to the higher H^+ efflux induced by nitrate salts inside the vesicles. When these salts were outside, $NaNO_3$ was more efficient than KNO_3 in increasing the outward H^+ flux. Indeed, under these conditions, NaCl had a similar effect to $NaNO_3$, which could be ascribed to the Na^+/H^+ antiporter. The simultaneous presence of nigericin and $NaNO_3$ outside the vesicles resulted in a decrease of the rate of outward H^+ movement which indicated some phenomenon moderating the H^+ efflux. The kinetics observed with KNO_3 outside could be explained by the same mechanism. It slowed down the rate of H^+ output, despite the high outer concentration of K^+, too, in the presence of nigericin, where k_1 had the same value as in its absence and k_2 was only slightly affected. All these observations could be explained by a putative H^+/NO_3^- symport, which would account for the effects of salts location on the H^+ outward flux. Obviously, the H^+ flux was increased either by the presence of nitrate when it was in the same compartment as H^+ or when the Na^+/H^+ antiporter was able to function.

On the other hand, if the unique functional presence of an Na^+/NO_3^- symport was assumed, these observations could not be explained, particularly those concerning KNO_3. However, this kind of analysis on isolated membranes in the absence of transport coupled with a natural driving force was not sufficient to display the large variety of mechanisms of NO_3^- transport observed in intact cells. Theoretically, symport mechanisms allow the naturally generated electrochemical potential to be converted from one ionic species to other. The strong endergonic process of active NO_3^- transport [24] could depend on a chemiosmotic circuit primary based on $\Delta\mu H^+$ [19,25,26] and H^+/NO_3^- symport as described for root cells of higher plants by Lu and Briskin [3], Ruiz-Cristin and Briskin [8] and McClure et al. [27]. Ritchie draws attention to a $\Delta\mu Cl^-$ which could play a role in NO_3^- transport [28,29]. According to Lara et al. [22–24], the existence of an Na^+/NO_3^- symport system driven by the energy of $\Delta\mu Na^+$ [28–30] created by light or ATP in cyanobacteria [30,31] may explain the NO_3^- uptake. On the basis of genetic data and kinetics of nitrate reduction, Omata et al. have assumed the existence of two independent mechanisms of NO_3^- uptake [32].

The data reported here illustrate this complexity and suggest that these bacteria might use different ways of nitrate transport: an H^+-dependent system and an Na^+-dependent one activated by light, according to the environment. Further correlations between our observations on isolated membranes and those obtained from intact cells could be reinforced by biochemical data on the protein composition of the plasmalemma isolated from cells grown in the absence of NO_3^- and also by NO_3^- transport kinetics with an ATPase-generated gradient [15].

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References

[1] Marschner, H. (1987) in Mineral nutrition of higher plants, Academic Press, London.
[2] Guerrero, M.G., Vega, J.M. and Losada, M. (1981) Annu. Rev. Plant Physiol. 32, 169–204.
[3] Lu, Q. and Briskin, D.P. (1993) Phytochemistry 33, 1–8.
[4] Lara, C., Romero, J.M. and Guerrero, M.G. (1987) J. Bacteriol. 169, 4376–4378.
[5] Wray, J.L. (1988) Plant Cell Environ. 11, 369–382.
[6] Guerrero, M.G. and Lara, C. (1987) in The Cyanobacteria (Fay, P. and Van Baalen, C., eds.), pp163–186, Elsevier, Amsterdam.
[7] Cheung Lee, H. and Forte, J.G. (1978) Biochim. Biophys. Acta 508, 339–356.
[8] Ruiz-Cristin, J. and Briskin, D.P. (1991) Arch. Biochem. Biophys. 285, 74–82.
[9] Melhorn, R.J., Candau, P. and Packer, L. (1982) Methods Enzymol. 88, 751–762.
[10] Deamer, D.W., Prince, R.C. and Crofts, A.R. (1972) Biochim. Biophys. Acta 274, 323–335.
[11] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.

[12] Fiolet, J.W.T., Bakker, E.P. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445.
[13] Haraux, F. and De Kouchkovsky, Y. (1980) Biochim. Biophys. Acta 592, 153–168.
[14] Kratz, W.A. and Myers, J. (1955) Am. J. Botany 42, 282–287.
[15] Fresneau, C., Rivière, M.-E. and Arrio, B. (1993) Arch. Biochem. Biophys. 306, 254–260.
[16] Rivière, M.E., Johannin, G., Gamet, D., Molitor, V., Peschek, G.A. and Arrio, B. (1988) Methods Enzymol. 167, 691–700.
[17] Skulachev, V.P. (1988) in Membrane Bioenergetics, pp 157–290, Springer, Berlin.
[18] Paschinger, H. (1977) Arch. Microbiol. 113, 285–291.
[19] Blumwald, E., Wolosin, J.M. and Packer, L. (1984) Biochem. Biophys. Res. Commun. 122, 452–459.
[20] Pescheck, G.A., Czerny, T., Schmetterer, G. and Nitschmann, W.H. (1985) Plant. Physiol. 79, 278–284.
[21] Kleyman, T.R. and Cragoe, E.J., Jr. (1988) J. Membr. Biol. 105, 1–21.
[22] Lara, C., Rodriguez, R. and Guerrero, M.G. (1993) Physiol. Plant. 89, 582–587.
[23] Lara, C., Rodriguez, R. and Guerrero, M.G. (1993) J. Phycol. 29, 389–395.
[24] Rodriguez, R., Lara, C. and Guerrero, M.G. (1992) Biochem. J. 282, 639–643.
[25] Scherer, S., Stürzl, E. and Böger, P. (1984) J. Bacteriol. 58, 609–614.
[26] Molitor, V. and Peschek, G.A. (1986) FEBS Lett.195, 145–150.
[27] McClure, P.R., Kochian, L.V., Spanswick, R.M. and Shaff, J.E. (1990) Plant Physiol. 93, 290–294.
[28] Ritchie, R.J. (1992) Plant Cell Environm. 15, 163–177.
[29] Ritchie, R.J. (1992) Plant Cell Environm. 15, 179–184.
[30] Brown, I.I., Fadeyev, S.I., Kirik, I.I., Severina, I.I. and Skulachev, V.P. (1990) FEBS Lett. 270, 203–206.
[31] Ritchie, R.J. (1992) J. Plant. Physiol. 139, 320–330.
[32] Omata, T., Andriesse, K. and Hirano, A. (1993) Mol. Gen. Genet. 236, 193–202.