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Retardation of electron donation to Photosystem I in aged cyanobacteria and its reversal by metal cations

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The dark reduction of photooxidized P-700 in the cyanobacterium *Anacystis nidulans* becomes slower as a consequence of aging. In cells, of which the envelope has been enzymatically permeabilized to electrolytes, the reduction of P-700 by endogenous electron donors is accelerated by KCl plus valinomycin, but not by KCl alone. The K^+-valinomycin system is ineffective, however, in the case of aged but unpermeabilized cells. These results suggest that a consequence of aging in *Anacystis* is an increase in the negative electric potential at the inner thylakoid surface, which makes electron donation to P-700 by acidic donors (cytochrome *c*-553 and plastocyanin) less probable. This situation is reversed by the permeant K^+-valinomycin system, which suppresses the inner surface potential and collapses the inside-outside potential difference, but not by K^+ alone, since the thylakoid membrane is impermeable to it.

Introduction

Above pH 4.1, the thylakoid membrane is negatively charged on both surfaces [1–3], and this predicts that electrostatic forces should govern, to a certain extent, electron donation to the membrane-embedded P-700 by acidic donors. The prediction has been borne out by numerous reconstitution experiments, designed to study the interaction of P-700 either with artificial [4–7], or with natural donors (i.e., plastocyanin or cytochrome *C*-553; Refs. 7–11). Since P-700 lies on the inner side of thylakoids, and therefore it is inaccessible to medium ions, these experiments were possible only with subthylakoid fragments, at various levels of disorganization, such as osmotically shocked thylakoids [11], sonicated thylakoids [5,6], Photosystem I-enriched particles [4,7,9,11] and highly resolved Photosystem I particles [8]. The conclusion derived from these studies is that a positive shift of the particle surface potential, either by charge screening or by charge neutralization, accelerates electron donation to P-700 by electronegative donors, and decelerates electron donation by electropositive donors.

Subthylakoid particles, however, can hardly be conceived to represent the intact membrane, particularly with regard to the surface density of ionizable groups that contribute the surface electric charge. In addition, all the reconstitution experiments mentioned above were performed with membrane fragments obtained from higher plant thylakoids. In the present work, we examine whether electron donation to P-700 is electrostatically controlled employing intact thylakoids, as they exist in enzymatically permeabilized cells of the cyanobacterium *Anacystis nidulans*. With this system, we obtained evidence indicating that elec-

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Abbreviations: BQ, *p*-benzoquinone; Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; Cyt, cytochrome.

tron donation to P-700 by the natural donor(s) is governed by the surface electric potential of the inner thylakoid face.

Materials and Methods

Anacystis nidulans was cultured photoautotrophically as described in Ref. 12, in the medium C of Kratz and Myers [13]. 4-Day old cultures were harvested by centrifugation at 6000 × *g* for 6 min, and the cells were transferred with one washing to 50 mM Hepes-NaOH (pH 7.5). The final suspension contained 0.15 mg Chl *a*/ml.

To prepare ion-permeable cells, 2 mg lysozyme and 1.25 μmol EDTA were added to each ml of cell suspension, and the mixture was incubated at 36°C for 30 min with slow shaking. Permeabilization to electrolytes was monitored in terms of the ability of *Anacystis* to photoevolve O_2 in the presence of the ferricyanide anion. Insignificant amounts of phycobiliproteins were released during the incubation, and the cells preserved their typical elongated shape. They correspond, therefore, to permeaplasts, rather than to sphaeroplasts [14].

Intact cells and permeaplasts were aged by prolonged storage at 23°C and darkness. For the aging treatment, the cells were resuspended at 0.15 mg Chl *a*/ml in a low-salt medium consisting of 0.4 M sorbitol/1 mM Hepes-NaOH, (pH 7.5). During 7 days of aging, the medium pH decreased to 7.2.

For the spectrophotometric assays, the cells were washed and transferred to fresh sorbitol/Hepes-NaOH medium, at the same cell density. Dark reduction of P-700, following photooxidation, was measured with a Hitachi Model 557 spectrophotometer, operated in the dual-wavelength mode. The measuring and reference beams were set at 700 nm and 720 nm, respectively, at half-band widths of 1 nm. P-700 was photooxidized by an actinic light beam of 93.5 $W \cdot m^{-2}$ at incidence, which was directed to the sample at a right angle to the reference and measuring beam paths by means of a flexible light guide. On the way to the sample, the actinic light passed in sequence through a heat-reflecting filter (Oriel 5740), a heat-absorbing filter (Ealing E26-3681) and a color glass filter (Corning C.S. 4-72). Two color glass filters, Corning C.S. 2-64 and C.S. 7-54 guarded the entrance of the photomultiplier tube. No leaks of actinic light to the photomultiplier could be detected, and no heating effects of actinic light which might have influenced the optical properties of the samples. No detectable photooxidation of P-700 was caused by the weak measuring light beam. On the other hand, the actinic light used to photooxidize P-700 was saturating. The reaction mixtures contained *Anacystis* cells equivalent to 8 μg Chl *a* per ml, in 0.4 M sorbitol/1 mM Hepes (pH 7.5)/10 μM DCMU/50 μM methyl viologen. Further details are given in the legends to the figures.

Photoinduced O_2 evolution was measured with a Clark-type electrode [12]. Chl *a* was determined in methanolic extracts according to McKinney [15]. All preparations and assays were performed at room temperature.

Results

The photooxidation of P-700, the reaction center chromophore of Photosystem I, is reported by an absorption loss centered near 700 nm (see Ref. 16), as shown in Fig. 1. After turning the actinic light off, the absorption loss is reversed, reflecting the reduction of P-700 by endogenous donors. In cyanobacteria [17,18] and in higher plant chloroplasts [19,20], this process occurs at rates which exceed the time resolution capability of double-

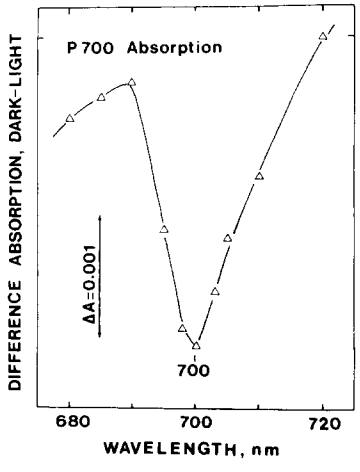

Fig. 1. Dark-light difference absorption spectrum of P-700 in *Anacystis nidulans*. Dual-wavelength mode measurement with $\lambda_{ref} = 720$ nm and $\Delta\lambda = 1$ nm. For further details, see also Materials and Methods.

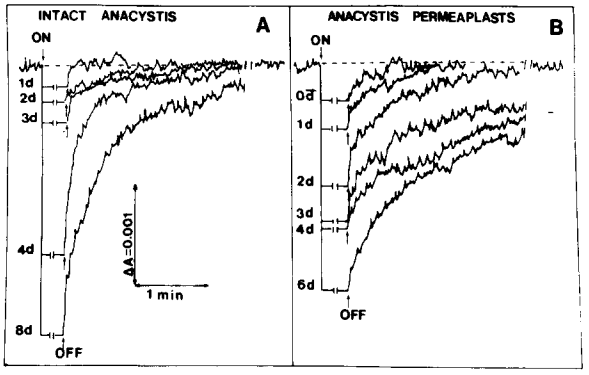

Fig. 2. Effect of cell aging on the dark reduction kinetics of photooxidized P-700 in intact *Anacystis* (A) and *Anacystis* permeaplasts (B). Cell age in days (d) is shown on the figure. Traces are recorded at 24 h intervals. In intact *Anacystis* the 0-day trace coincides with the 1-day trace.

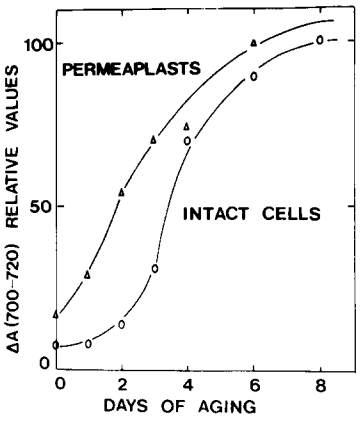

Fig. 3. The recorded amplitude of P-700 dark recovery ($\Delta A_{700-720}$), following a light period, as a function of age of intact *Anacystis* cells and of permeaplasts.

beam spectrophotometers. These instruments are slow because of the time delay imposed by the sharing of the photomultiplier by the two beams.

Accordingly, as shown in Fig. 2, the time courses of dark P-700 reduction, recorded either with freshly harvested *Anacystis* cells (A), or with freshly prepared permeaplasts (B) are characterized by small amplitudes. This implies that the dark reduction of P-700 was nearly over before the double-beam spectrophotometer could register it. As the cells grew older, however, it became possible to record increasingly larger portions of the dark reduction time courses. This indicates a progressive slow down of electron donation of P-700, as a result of the physicochemical changes caused to the thylakoid membrane by cell aging.

Fig. 3 shows that the recorded P-700 reduction amplitudes do not increase indefinitely with cell age, but reach a maximal level after 6–8 days, both in the case of intact cells, and in the case of permeaplasts. Using the millimolar difference absorptivity for P-700, reported by Lien and San Pietro (Ref. 8; $E_{697-720} = 58\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$), it can be calculated that the maximal amplitudes recorded correspond to Chl *a*/P-700 molar ratios of 181/220 : 1, which lie within the range of Chl *a*/P-700 ratios reported for cyanobacteria [18,21]. Evidently, the recorded kinetics account for most, or all the P-700 present in the aged samples.

In view of the negative surface electricity of cyanobacterial thylakoids [22], and the acidic nature of cyt *c*-553, and most likely of plastocyanin in *Anacystis* [23], the possibility exists that the slower reduction of P-700 in the aged cells reflects a state of insufficient charge compensation, and therefore of increased coulombic repulsion between the donor and the acceptor species. To test this possibility, we measured the dark reduction kinetics of P-700 in 7-day old *Anacystis* cells either in the absence, or in the presence of KCl and the cationophore valinomycin. The KCl-valinomycin system is capable of inducing a positive shift of the electric potential of sealed membrane vesicles. Fig. 4A displays an experiment in which the effect of 50 mM KCl and 3 μM valinomycin, added either separately or together, to a suspension of intact cells was examined. Added alone, they had no appreciable effect on the reduction kinetics (traces are omitted from Fig. 4A). When added together, the reduction of P-700 was somewhat speeded up, but still an appreciable reversal of the retardation caused by aging was not observed.

This may mean either that the slower P-700 reduction is not the consequence of a more negative surface potential in the aged thylakoids, or that the cationophore is incapable of transporting K^+ ions across the Gram-negative envelope of the cyanobacterium cell. The second alternative has been documented for the photosynthetic bacterium *Rhodopseudomonas sphaeroides* by Matsuura and

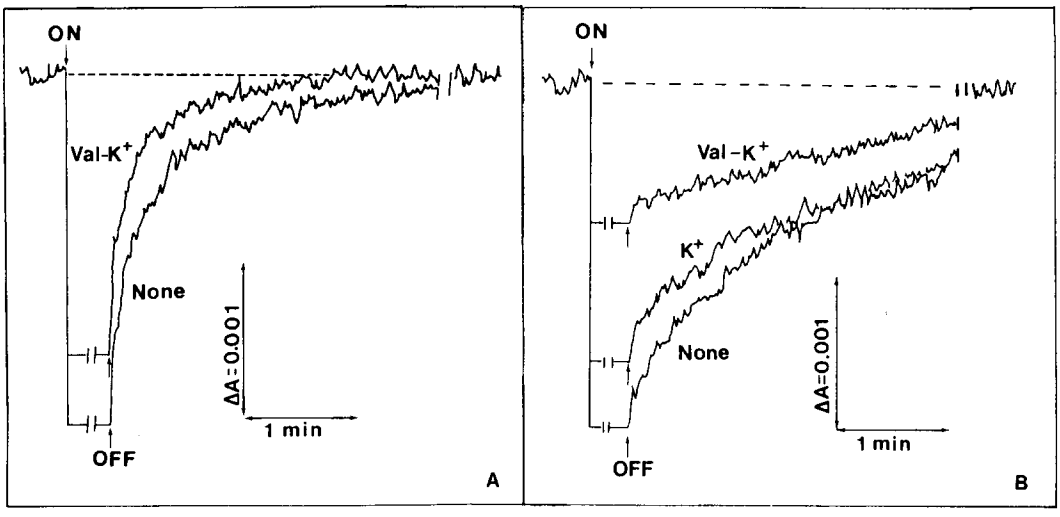

Fig. 4. Effect of valinomycin (3 μM) and KCl (50 mM) on the dark reduction kinetics of photooxidized P-700. (A) In 7-day old intact *Anacystis* cells, (B) in 6-day old *Anacystis* permeaplasts.

Nishimura [24]. To decide between the two possibilities, we tested the effect of KCl-valinomycin on the rate of P-700 reduction with 6-day old *Anacystis* permeaplasts (Fig. 4B). Added alone, KCl caused some acceleration. A remarkable acceleration, exceeding 60% of the maximal amplitude, was observed, however, when the two reagents were added together.

Employing the Gouy-Chapman theory (reviewed in Refs. 25, 26), Itoh [5] derived the following expression

$$\log k = a - bC^{-1/2} \quad (1)$$

which links the apparent rate constant of interaction between membrane sites and solute molecules (k), both negatively charged, to the concentration of monovalent cations in the aqueous bulk phase (C); a and b are physical constants of the system. In our assay system, the greater the recorded P-700 reduction amplitude, the slower the interaction between the electron-donating solute and the P-700-containing membrane site. Setting k proportional to the recorded amplitude ($k \propto \Delta A^{-1}$) we may modify the above-mentioned equation as follows:

$$-\log \Delta A = a' - b'C^{-1/2} \quad (2)$$

In Fig. 5, we plot the recorded P-700 reduction amplitudes against the KCl concentration in the suspension medium. Valinomycin, 1.5 μM was present in all samples. The amplitude decreases with increasing KCl concentration down to about 40% of its original value, remaining constant above 2 mM KCl. When these results were plotted according to the modified Itoh equation (Eqn. 2; Fig. 5, inset) a straight line was obtained.

The effect of aging on the overall electron

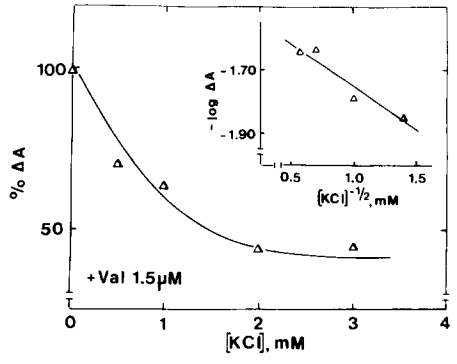

Fig. 5. Dependence of the amplitude of the dark recovery of the photoinduced P-700 absorption change in 6-day old *Anacystis* permeaplasts on the concentration of KCl in the suspension medium. Valinomycin, 1.5 μM, was present in all samples. The absorption amplitudes are normalized by setting $\Delta A_{700-720} = 100$ for the minus-KCl sample.

transport across Photosystem II ($H_2O \rightarrow BQ$) and across Photosystem I ($DCIPH_2 \rightarrow$ methyl viologen) in intact cells and permeaplast was examined in the experiment represented by Fig. 6. In permeaplasts, both photoreactions are inactivated with $t_{1/2} = 18–19$ h. A slower Photosystem II inactivation ($t_{1/2} = 53$ h) was observed with intact *Anacystis*, using the permeant oxidant *p*-benzoquinone to monitor photosynthetic O_2 evolution. Intact cells are impermeable, however, to the cationic oxidant methylviologen. Accordingly, low electron-transport rates are measured with them. Furthermore, on the basis of the $DCIPH_2 \rightarrow$ methyl viologen rate, it appears that aging has virtually no effect on the permeability of the cell envelope to methyl viologen.

It is interesting to compare the effect of aging on electron donation to P-700 (Fig. 3) and on overall electron-transport across Photosystems I and II (Fig. 6). In the case of permeaplasts, electron donation to P-700 is inactivated at a slower pace ($t_{1/2} > 48$ h) than electron transport from $DCIPH_2$ to methyl viologen, across Photosystem I. This, then, may imply a site more sensitive to aging than the donor-P-700 pair. Lastly, both with regard to electron donation to P-700, and with regard to the overall electron transport across Photosystem II, intact cells resist aging more successfully than the permeabilized cells.

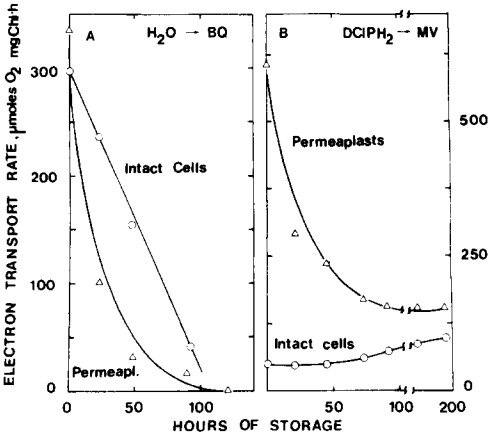

Fig. 6. The rate of electron transport across Photosystem II ($H_2O \rightarrow BQ$) and across Photosystem I ($DCIPH_2 \rightarrow$ methyl viologen (MV); in the presence of 10 μM DCMU) as a function of cell age in intact *Anacystis* cells (A) and in permeaplasts (B).

Discussion

The soluble Cu protein plastocyanin is an obligatory intersystem electron carrier in higher plants, but not in algae and cyanobacteria, where electron donation to Photosystem I is performed interchangeably by plastocyanin and Cyt *c*-553 [27–29]. It is possible that electron donation to P-700 by these proteins is mediated by subunit III of the Photosystem I reaction center complex [30,31]. The donor proteins dissociate easily from the membrane and may exist in more than one kinetic pools. Two such pools have been identified in the cyanobacterium *Plectonema boryanum* by Hiyama and Ke [17], and three pools in spinach chloroplasts by Haehnel et al. [11] on the basis of P-700 reduction kinetics, following a microsecond oxidizing light flash.

The relative proportion of Cyt *c*-553 and plastocyanin depend on the availability of Cu in the nutrient media, with Cu deficiencies leading to higher levels of the heme protein [29]. Acidic Cyt *c*-553 (p*I* = 3.84–3.87), most likely the major donor, has been isolated from *Anacystis nidulans* by Ho and Krogmann [23]. Presence of plastocyanin in the same cyanobacterium has been documented only indirectly, on the basis of the $g = 2.05$ EPR signal [32]. In view of the Cu content (20 ng/ml) of the culture medium we used, presence of plastocyanin in our samples cannot be ruled out. In analogy to Cyt *c*-553, this plastocyanin should also be acidic, since from the cases examined higherto, it appears that within the same cyanobacterium genus the two proteins appear to have approximately equal isoelectric points [23].

A consequence of aging of *Anacystis* is a slower rate of electron donation to P-700 by the endogenous donors. These donors must communicate with pools of reductants produced by respiration or photosynthesis, during the light period that precedes the dark reduction of P-700. Aging, therefore, may affect either the transfer of electrons from the reductant pools to the donors, or from the donors to P-700, or both. Our results suggest that the major influence is on the second process.

Addition of KCl to a suspension of *Anacystis* cells accelerates electron donation to P-700 by the natural donors only when the following three conditions are simultaneously satisfied: (i) The K^+-

specific cationophore valinomycin is present. (ii) The cell envelope is enzymatically permeabilized. (iii) The permeabilized cell is aged. The first two conditions prove that the intact cell envelope is impermeable to the K^+-valinomycin system, and that K^+ cannot cross the thylakoid membrane unaided by valinomycin. In addition, it has been shown that *Anacystis* permeaplasts display photosynthetic control ratios exceeding 2.0 [33]. This indicates coupling of photoinduced electron transport to energy conservation, which in turn requires that permeaplast thylakoids are not passively permeable to H^+.

The third condition hints to a fundamental difference between fresh and aged permeaplasts with regard to the electrostatic properties of the inner thylakoid surface. The most logical explanation for the acceleration of electron donation to P-700 by KCl is a positive shift of the electrostatic potential at the inner surface, brought about by the diffusion of K^+ ions from the medium to the thylakoid interior. Since no appreciable acceleration is observed in fresh permeaplasts, we must infer that, in this case, the inner surface potential is already positive. Accordingly, the surface concentration of the negatively charged reactants is not affected much by the important K^+ ions. In aged permeaplasts, on the other hand, we observe both lower rates of electron donation to P-700 and appreciable acceleration upon addition of KCl to the suspension. Both these effects suggest a negatively charged inner thylakoid surface.

Imported K^+ ions may modify the electrostatic properties of membrane-solution and donor protein-solution interfaces both by accumulating at the negatively charged interface (charge screening) and by attaching to acidic ligands (charge neutralization). While both effects must be contributing to the suppression of coulombic repulsion between the inner thylakoid surface and the negatively charged electron-donating protein, the applicability of the Itoh equation to KCl concentrations of less than 2 mM (Fig. 6) suggests that screening of membrane charges by the imported cations must be the dominant cause for the accelerated electron donation to P-700. On the other hand, charge neutralization, brought about by the binding of cations to negative membrane sites, may be more expressed at higher concentrations of added salts. This, then, may be the cause of the inhibition of electron donation to P-700 by exogenous plastocyanin [19] and ferrocyanide [5], observed at such concentrations.

The physicochemical, structural and functional consequences of aging on thylakoid membranes have been investigated, almost exclusively, with isolated higher plant chloroplasts. Aging causes oxidative and hydrolytic damage of membrane lipids [34], which may be propagated by a free radical chain mechanism. With regard to the surface electric properties, Barber et al. [35] established that aging suppresses the total charge density (outside plus inside) of osmotically shaked pea chloroplasts, and increases the rigidity of the membrane. Our results, which refer to the inner surface charge of intact cyanobacterial thylakoids, point to the opposite, namely an increase in the surface charge as a result of aging.

In summary, our results suggest an internal location of the electron donor(s) to P-700 in *Anacystis nidulans* and an electrostatic control of the electron-transfer process. Aging of the cells suppresses electron donation to P-700 by increasing the coulombic repulsion between donor molecules and the inner thylakoid surface, without affecting the impermeability of the thylakoid membrane to ions.

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