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Photosynthetic properties of rapidly permeabilized cells of the cyanobacterium *Anacystis nidulans*

George C. Papageorgiou and Thoula Lagoyanni

Nuclear Research Center Demokritos, Department of Biology, Aghia Paraskevi, Athens, 153 10 (Greece)

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A study has been made (i) of the effect of the suspension medium composition on the kinetics of permeabilization of *Anacystis nidulans* to ions by lysozyme, and (ii) of the consequences of permeabilization on the photosynthetic apparatus of the cyanobacterium. Ion-permeable, osmotically resistant cells (permeaplasts) were prepared by a 15 min lysozyme treatment in 0.05 M sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate. Permeabilization causes detachment of phycobilisomes from the thylakoid surface and partial dissociation of phycobiliprotein multimers. The permeaplasts contain, however, virtually intact thylakoids, as testified by their fully functional photosynthetic electron-transport chain and their capacity for photosynthetic control. Supplementation of the lysozyme incubation medium with osmotica delays cell permeabilization, while prolongation of the lysozyme treatment inactivates the photosynthetic electron transport at a site preceding electron donation by 1,5-diphenylcarbazide.

Introduction

The Gram-negative envelope of cyanobacteria, consisting of the cell wall (outer membrane, periplasm and peptidoglycan layer) and the cell membrane, is not freely permeable to ions [1,2]. Therefore, in order to expose the thylakoids of the cytoplasm to the electrolytes of the suspension medium, either the cells must be fractured or their envelope must be permeabilized. Cell-free cyanobacterial thylakoid fragments are very fragile, both structurally and functionally [3–8]. Cell permeabilization to ions, on the other hand, offers the advantage of better preserved thylakoids, and can be achieved by mild procedures such as the enzymatic hydrolysis of peptidoglycan by lysozyme [4,5,9–14].

Some cyanobacteria are unusually resistant to enzymatic permeabilization, presumably because of a more effective shielding of peptidoglycan by the outer membrane. One such species is the unicellular cyanobacterium *Anacystis nidulans*. Anacystis spheroplasts can be formed only after several hours' incubation with lysozyme and they do not photoevolve O_2 [15,16]. Shorter incubations (1–2 h) yield osmoresistant, ion-permeable cells (permeaplasts) with full photoelectron transport activity [11,12].

In the present report, we describe a rapid (15–20 min) enzymatic permeabilization procedure for *Anacystis*, capable of producing highly active and stable permeaplasts. We further examine the consequences of the rapid permeabilization on the

Abbreviations: Chl, chlorophyll; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; PC, plastocyanin; PD, PD_{ox}, reduced and oxidized *p*-phenylenediamine; PQ, plastoquinone.

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integrity of the photosynthetic apparatus in terms of several indices, such as absorption and fluorescence spectra of pigments in situ, photoinduced electron transport, photosynthetic control and slow induction of Chl *a* fluorescence.

Materials and Methods

Anacystis nidulans (reclassified as *Synechococcus AN*; Ref. 1) was cultured photoautotrophically in medium C of Kratz and Myers [17]. The cultures were aerated with a mixture of 5% CO_2 in air, and illuminated with 3500 lux of white fluorescent light. Temperature was maintained at 30°C. The bacteria were harvested in the late logarithmic phase, 4–5 days after inoculation.

All media used in this work were buffered at pH 7.5 with 0.05 M Hepes-NaOH. Egg-white lysozyme (3 × recrystallized; grade I) was purchased from Sigma. Three different suspension media were tested for the permeabilization reaction: (i) 0.05 M Hepes-NaOH; (ii) 0.05 M Hepes-NaOH/0.5 M sorbitol; and (iii) 0.05 M Hepes-NaOH/0.5 M sorbitol/0.03 M sodium potassium phosphate. Cells were transferred to these media from the culture with one intervening washing. After temperature equilibration at 37°C in a shaker water bath, 1 μmol/ml EDTA and 10 mg/ml lysozyme were introduced. The cell density corresponded to 0.15 mg Chl *a*/ml. Progress of the permeabilization reaction was monitored in terms of the rate of ferricyanide-dependent O_2 evolution in aliquots withdrawn from the reaction mixture. The reaction was stopped by centrifuging the cells out and resuspending them in 0.05 M Hepes-NaOH/0.5 M sorbitol at 0.15 mg Chl *a*.

Photoinduced electron transport was measured with a Clark-type O_2 electrode, as in Ref. 13. The reaction mixture (3 ml) was made in 0.25 M sorbitol/0.25 M KCl, buffered with 0.05 M Hepes-NaOH (see above). It contained *Anacystis* equivalent to 15–20 mg Chl *a* per ml, and depending on the particular assay, the following additions: 1 mM potassium ferricyanide; 34 μM 2,6-dichlorophenolindophenol; 0.6 mM *p*-phenylenediamine; 0.1 mM methyl viologen; 0.5 mM 1,5-diphenylcarbazide; 2 mM ADP; 5 mM KH_2PO_4; and 10 μM DCMU. Saturating actinic light was provided by a 1000 W photographic lamp, operated through a Variac. The samples were thermostated at 25° by circulating water in a surrounding mantle and by filtering the actinic light with a 5 cm layer of 5% (w/v) $CuSO_4$ solution.

Light absorption was measured with a microprossessor-controlled Hitachi Model 557 dual-wavelength spectrophotometer, with $\Delta\lambda = 1$ nm. Turbidity artifacts were minimized by positioning the samples close to the photomutiplier photocathode. Second derivative absorption spectra were recorded using $\Delta\lambda = 2$ nm. Fluorescence emission spectra were measured with a Perkin-Elmer Model MPF-3L spectrofluorimeter, which provides for a 90° angle between the directions of excitation and emission. Further details are given in the legend of Fig. 4.

Chl *a* was determined in methanolic extracts according to McKinney [18]. Extracted phycobiliproteins were determined according to Kursar and Alberte [19].

Results

Permeabilization of the *Anacystis* cell envelope to ions by lysozyme becomes manifest by the acceleration of the photoinduced, ferricyanide-dependent O_2 evolution, which presumably reflects the increasing cytoplasmic concentration of the ferricyanide anion. Cell permeabilization facilitates, however, not only the importation of medium solutes, but also the exportation of cytoplasmic solutes, and this eventually inhibits O_2 evolution. The interplay of the two effects results in kinetic profiles such as those shown in Fig. 1. Following the terminology of Ward and Myers [11], we shall hereafter designate the ascending branch of these curves as phase 1 and the descending branch as phase 2.

Fig. 1. illustrates how critical the composition of the suspension medium can be for the permeabilization of *Anacystis* by lysozyme. Of the three media tested, the 0.05 M Hepes-NaOH medium is undoubtedly superior, yielding the most avtive permeaplasts in the shortest time. In fact, this happens to be the fastest permeabillization of *Anacystis nidulans* ever reported. When this medium is supplemented with sorbitol, or with sorbitol and phosphate, phases 1 and 2 become

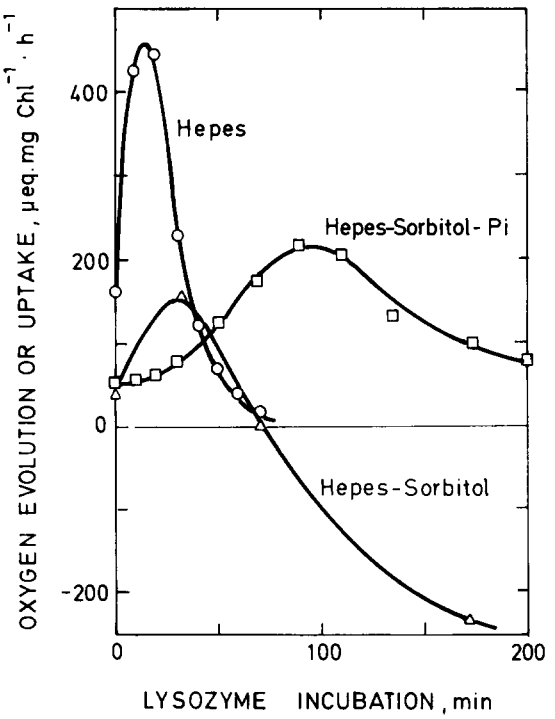
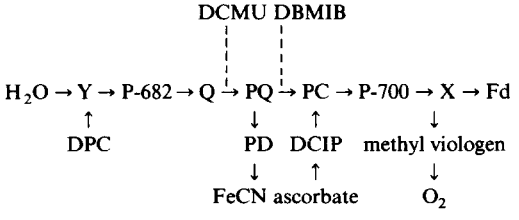


Fig. 1. The rate of light-induced O_2 evolution, or uptake, by *Anacystis nidulans* as a function of the length of lysozyme treatment in three incubation media: (i) 0.05 M Hepes-NaOH; (ii) 0.05 M Hepes-NaOH/0.5 M sorbitol; and (iii) 0.05 M Hepes-NaOH/0.5 M sorbitol/0.03 M sodium potassium phosphate. All media were buffered at pH 7.5. Saturating actinic light was used in this and in subsequent experiments.

slower, and the resulting permeaplasts are less active. In the case of the Hepes-NaOH/sorbitol medium, but not in the other media, phase 2 crosses into the region of negative rates, indicating net O_2 uptake.

In order to locate the site of phase 2 inactivation, we examined the effect of lysozyme treatment on several partial electron-transport processes, which are shown in Scheme I. The results are displayed in Fig. 2,

Scheme I. FeCN, $K_3Fe(CN)_6$; FD, ferredoxin.



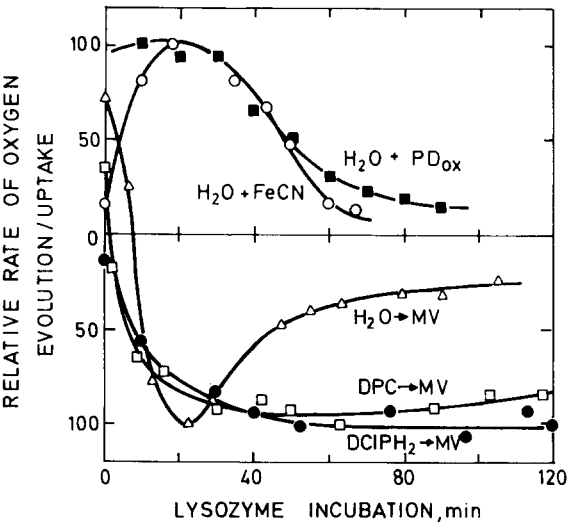


Fig. 2. The rate of light-induced O_2 evolution, or uptake, by *Anacystis nidulans* in the presence of various electron donors and acceptors, as a function of the duration of lysozyme treatment. Incubation medium, 0.05 M Hepes-NaOH (pH 7.5). Actual maximal rates, in μequiv·mg Chl^{-1}·h^{-1}, are as follows: $H_2O \rightarrow FeCN$ (426); $H_2O \rightarrow MV$ (200); $H_2O \rightarrow PD_{ox}$ (610); $DPC \rightarrow MV$ (240); and $DCIPH_2 \rightarrow MV$ (910). MV, methyl viologen; FeCN, $K_3Fe(CN)_6$.

where positive rates denote net O_2 evolution and negative rates net O_2 uptake. For easier comparisons, the curves have been normalized to equal heights.

Phase 2 is present in all electron-transport sequences beginning with H_2O ($H_2O \rightarrow$ ferricyanide; $H_2O \rightarrow$ oxidized *p*-phenylenediamine; $H_2O \rightarrow$ methyl viologen), but absent when H_2O is superseded by another electron donor (1,5-diphenylcarbazide → methyl viologen; reduced 2,6-dichlorophenolindophenol → methyl viologen). Of the latter two electron donors, 1,5-diphenylcarbazide is known to donate electrons to an intermediate on the oxidizing side of Photosystem II [20], and ascorbate-reduced 2,6-dichlorophenolindophenol to post-plastoquinone intermediates [21]. These results, then, indicate a site preceding the site of electron donation by 1,5-diphenylcarbazide as responsible for phase 2 inactivation. The absence of a post-plastoquinone inhibition site is supported also by the fact that phase 1 and 2 kinetics were not affected when the ferricyanide Hill reaction was measured in the presence of dibromothy-

quinone, an inhibitor of plastoquinol oxidation by Photosystem I.

Phase 1 is present in all electron-transport sequences except that which employs the lipophilic mediator oxidized *p*-phenylenediamine (PD_{ox}) in order to shuttle electrons between thylakoids and ferricyanide. Similar results were obtained also with *p*-benzoquinone, another permeant electron acceptor. Phase 1, therefore, represents a gradual permeabilization of *Anacystis* to anions and cations, such as $(Fe(CN_6)^{3-}$ and $(methyl\ viologen)^{2+}$. O_2 evolution by intact *Anacystis*, observed initially instead of O_2 uptake in the presence of methyl viologen, should be attributed to electron donation to endogenous HCO^{3-}. The evolution ceases after the cells are sufficiently permeabilized in order to import methyl viologen. It appears, then, that unlike other cyanobacteria [22], *Anacystis* is impermeable to methyl viologen.

The rationale for incorporating osmotica (usually sorbitol, mannitol or sucrose) in the lysozyme incubation media is to forestall cell lysis after the breakdown of peptidoglycan, which serves as an exoskeleton to the cell. In the hypoosmotic 0.05 M Hepes-NaOH medium, cell lysis is indeed a possibility. However, as Fig. 3A shows, less than 1% of total C-phycocyanin is released after 15 min incubation of *Anacystis* with lysozyme, although this time suffices for the preparation of permeaplasts. Even after 4 h incubation, less than 6% of C-phycocyanin escapes from the cells, indicating virtual absence of cell lysis. In consequence, the absorption spectra of intact cells and permeaplasts (Fig. 3B) are nearly identical. It is noteworthy that more C-phycocyanin is released when sorbitol is present in the lysozyme incubation medium. On subsequent storage of permeaplasts in sorbitol-Hepes, more C-phycocyanin is released (approx. 40% of total in 2 h). Retention of the phycobiliprotein pigments by the permeaplasts, during or shortly after lysozyme treatment, does not necessarily imply an intact system of light-harvesting phycobilisomes. In fact, in view of their tendency to break down in low ionic strength media [23], some impairment is due as a result of the permeabilization.

Phycobiliprotein-sensitized Chl *a* fluorescence is a criterion for the attachment of phycobilisomes to the thylakoid surface. When excited within the

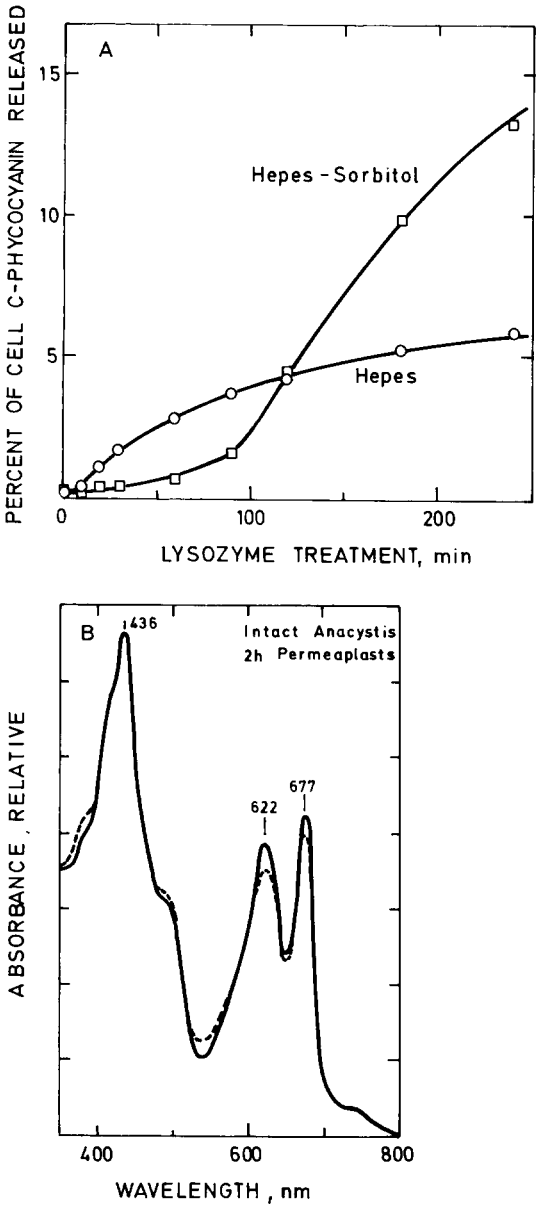


Fig. 3. (A) Percent of total C-phycocyanin released during lysozyme treatment of *Anacystis* in two incubation media. (B) Absorption spectra of intact cells (solid line) and of permeaplasts (dashed line), obtained with *Anacystis* treated for 2 h with lysozyme in 0.05 M Hepes-NaOH (pH 7.5).

C-phycocyanin absorption band (580 nm), intact cells emit both allophycocyanin (662 nm) and Chl *a* fluorescence (680 nm; Fig. 4). Permeabilized cells, on the ohter hand, emit only allophycocyanin fluorescence, possibly with some admix-

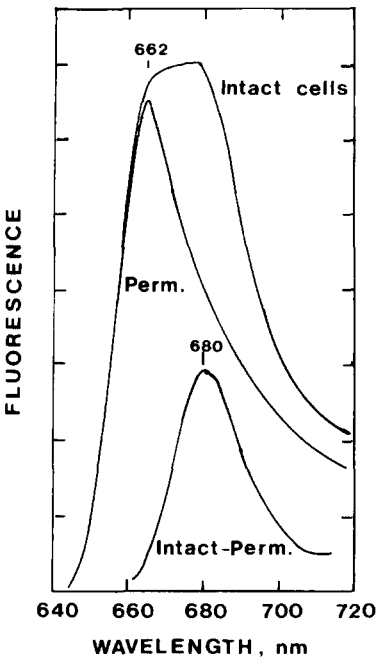


Fig. 4. Fluorescence emission spectra of intact *Anacystis* cells and of permeaplasts, obtained by a 15 min lysozyme treatment of *Anacystis* in 0.05 M Hepes-NaOH (pH 7.5). Excitation: $\Delta\lambda = 580$ nm, $\Delta\lambda = 2$ nm. Scanning: $\Delta\lambda = 2$ nm.

ture of C-phycocyanin fluorescence, originating from detached pigment molecules. After cell permeabilization, therefore, C-phycocyanin is no longer a sensitizer of Chl *a* fluorescence, indicating dissociation of phycobilisomes from the thylakoid surface.

Further information on the consequences of permeabilization on phycobilisomes is provided by the second-derivative absorption spectra of intact cells and of permeaplasts (Fig. 5). Inverted second derivatives of Gaussian and Lorentzian curves have major bands of narrower bandwidth, located in the same position and pointing to the same direction as the parent curves. They are therefore very useful in identifying individual contributions in the case of overlapping spectra. The second-derivative absorption spectrum of intact *Anacystis* has four major bands in the 600–660 nm region, which are contributed by the phycocyanobilin chromophore of C-phycocyanin and allophycocyanin. After permeabilization, these bands become less distinct, as if they have fused partially into a single band. Complete fusion signifies the

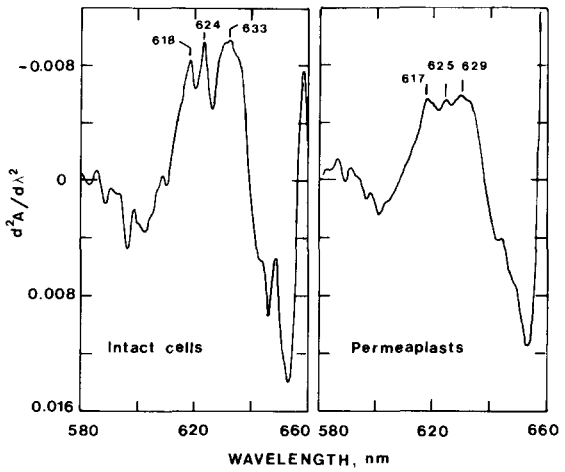


Fig. 5. Inverted second derivative absorption spectra of intact *Anacystis* cells and of permeaplasts, obtained by a 15 min lysozyme treatment of *Anacystis* in 0.05 M Hepes-NaOH (pH 7.5).

dissociation of multimeric C-phycocyanin and allophycocyanin to monomers [24]. We may therefore interpret the results of Fig. 5 as indicating a partial dissociation of phycobiliproteins to monomers in the permeabilized *Anacysts*.

A sensitive criterion of thylakoid integrity is photosynthetic control, defined as the acceleration of the electron transport by cofactors, or by uncouplers of the photophosphorylation. Its physical basis is a properly sealed vesicle, capable of main-

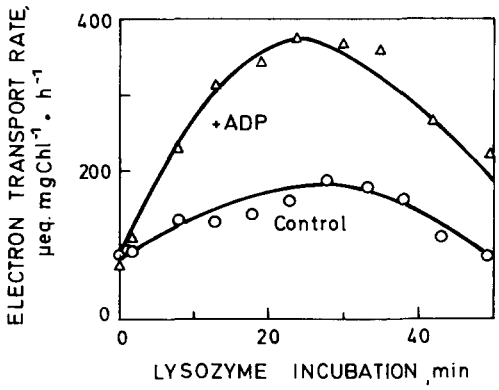


Fig. 6. The rate of ferricyanide-supported O_2-evolution, in the absence (control) and in the presence of photophosphorylation cofactors (ADP, KH_2PO_4, $MgCl_2$), as a function of the duration of lysozyme treatment. Incubation medium, 0.05 M Hepes-NaOH (pH 7.5).

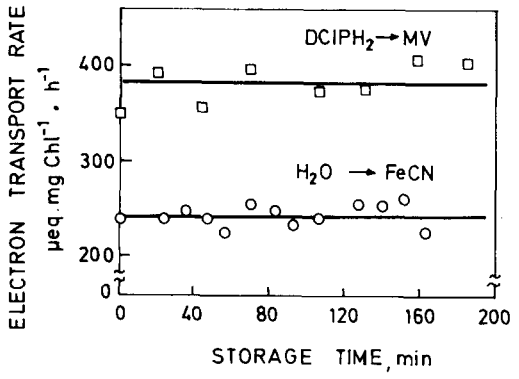


Fig. 7. Electron-transport activities of *Anacystis* permeaplasts, obtained by a 15 min lysozyme treatment in 0.05 M Hepes-NaOH (pH 7.5), as a function of storage time at room temperature and in 0.05 M Hepes-NaOH/0.5 M sorbitol (pH 7.5).

taining a steady-state ΔpH difference (acid inside) across the membrane, which prevents the reoxidation of plastoquinol by Photosystem I [25,26]. Fig. 6 shows that photophosphorylation cofactors stimulate electron transport is permeabilized *Anacystis*. Control ratios are in the range 2–2.3. Electron transport across Photosystem I (reduced 2,6-dichlorophenolindophenol → methyl viologen) or across both photosystems (H_2O → ferricyanide) was accelerated also upon collapsing the electrochemical potential difference across the thylakoid membrane with FCCP and potassium valinomycin (Table I). These results prove the presence of properly oriented, sealed and functionally intact thylakoid membranes in the permeaplasts.

Finally, we examined whether the sequestration of permeaplasts from the lysozyme reaction mixture, near the peak of their activity, suffices to block the inactivation represented by phase 2. In the experiment of Fig. 7, 15 min after the initiation of the lysozyme reaction, the bacteria were transferred to 0.05 M Hepes-NaOH/0.05 M sorbitol, and were left to stand at room temperature. Electron-transport rates, either across Photosystem I (reduced 2,6-dichlorophenolindophenol → methyl viologen), or across both photosystems (H_2O → ferricyanide) remained stable for at least 4 h.

TABLE I

EFFECT OF ADP, PHOSPHATE AND UNCOUPLERS ON THE PHOTOELECTRON-TRANSPORT RATES OF *ANACYSTIS* PERMEAPLASTS

Concentrations of added compounds: KH_2PO_4, 5 mM; $MgCl_2$, 5 mM; ADP, 5 mM; FCCP, 2 μM; valinomycin, 2 μM. Other additions as in Materials and Methods.

Additions	Electron μequiv. per mg Chl per h	Relative activity
H_2O → FeCN		
No addition	155	100
KH_2PO_4; ADP; $MgCl_2$	330	213
KH_2PO_4; $MgCl_2$; FCCP; Valinomycin	490	316
$DCIPH_2$ → methyl viologen		
No addition	378	100
FCCP; valinomycin	589	156

Discussion

Osmotic or mechanical rupture of spheroplasts is a universally employed method for the preparation of cell-free thylakoid vesicles from cyanobacteria. However, as a result of the long lysozyme treatment required for spheroplast formation and the cell rupture and membrane fractionation procedures, the end preparations are often inactive. In addition, since such preparations consist of mixed populations of properly and improperly sealed vesicles, as well as of imperfectly sealed vesicles, the definition of membrane orientation is impossible.

For reasons that are not clearly understood, the cyanobacteria become permeable to ions after partial hydrolysis of the cell-wall peptidoglycan with lysozyme, which has no effect on the cell membrane. The resulting osmoresistant cells (permeaplasts) offer certain advantages to the experimenter that are unattainable with cell-free thylakoid fragments such as ion-accessible intact thylakoids with superior structural and functional stability.

Anacystis nidulans is a cyanobacterium which does not easily lend itself to permeabilization. In the present work, we sought to establish first the conditions for a rapid preparation of *Anacystis* permeaplasts, and subsequently to characterize the integrity of their photosynthetic apparatus in terms of several structural and functional indeces. The fastest permeabilization, which resulted also in the

most active permeaplasts, was achieved by incubating *Anacystis* with lysozyme in 0.05 M Hepes-NaOH (pH 7.5). In analogy with earlier observations, we assume that this hypoosmotic medium facilitates the penetration of the enzyme molecules through the outer membrane of the cell wall [13,27].

The lysozyme modified *Anacystis* envelope is passively permeable to ions and most likely to small hydrophilic molecules, as suggested by the increased permeaplast fragility in the presence of sorbitol (cf. Fig. 3A). On the other hand, larger molecules, such as phycobiliprotein monomers (molecular weight, approx. 35 000) are not allowed to pass. A state of unimpeded ion diffusion across the cell envelope will tend to equalize electrolyte concentrations in the medium and the cytoplasm (disregarding Donnan effects), with, as observed, severe consequence on the phycobilisome light harvesting system. However, unless the lysozyme reaction is allowed to proceed to phase 2, permeabilization has no effect on the operation of the membrane-embedded photosynthetic electron-transport chain.

Ward and Myers [11] proposed that lysozyme treatment causes two kinds of electron-transport inhibition in *Anacystis*: a phase 1 inhibition occurring at a site located between the photoacts, which decreases the rate of electorn donation to methyl viologen; and a phase 2 inhibition occuring to the Photosystem II apparatus. In osmotically lysed *Phormidium luridum* spheroplasts, Binder et al. [4] obtained evidence for damage near the Photosystem II reaction center, and in Photosystem I. The latter was attributed to the loss of a soluble protein factor, in corroboration of earlier observations by Biggins [9], also in osmotically lysed *Phormidium* spheroplasts.

Our results preclude a phase 1 inactivation, since otherwise we should have observed a rate decline in the presence of lipophilic oxidants (e.g., oxidized *p*-phenylenediamine; cf. Fig. 2) immediately after the initiation of the lysozyme reaction. Furthermore, there is no evidence of a Photosystem II (1,5-diphenylcarbazide → methyl viologen) or Photosystem I (reduce 2,6-dichlorophenol indophenol → methyl viologen) inhibition upon protracted lysozyme treatment of the cyanobacterium. We must therefore exclude the loss of soluble protein factors, such as those described by Biggins [9] and by Binder et al. [4]. It is clear from our results that the site of phase 2 inhibition lies closer to the H_2O-splitting function of Photosystem II, ahead of the 1,5-diphenylcarbazide coupling site, than to the Photosystem II reaction center. Various monovalent and divalent metal cations, including Ca^{2+}, failed to reverse the phase 2 inhibition. Its occurrence should be attributed, therefore, to the loss of other unidentified diffusible cytoplasmic substances.

A trivial cause for the inactivation of photoelectron transport in the permeabilized cells, particularly under light-limiting conditions, could be an insufficient supply of electronic excitation to the photosynthetic reaction centers, as a result of the destruction of phycobilisomes. This, however, must be ruled out as an explanation of our experimental results for the following two reasons. (i) Electron transport rates were measured under light-saturating conditons (cf. Materials and Methods). (ii) Insufficient excitation supply would have affected all the photoinduced electron-transport processes examined. However, as Fig. 2 shows, phase 2 inactivation was expressed only when the terminal electron donor was H_2O.

The cyanobacteria combine certain characteristic properties, which make them higly suitable for the biotechnological production of low-potential reducing power by light-energy transduction [13]. Two conditions should be satisfied for such applications: unrestricted importation and exportation of oxido-reducible compounds on the one hand and functional stability on the other. The rapidly permeabilized *Anacystis* cell, described in this report, is a promising prospect in this direction.

Acknowledgements

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