Biochimica et Biophysica Acta, 461 (1977) 379–391
© Elsevier/North-Holland Biomedical Press

BBA 47352

PHOTOSYNTHETIC ACTIVITY OF DIIMIDOESTER-MODIFIED CELLS, PERMEAPLASTS, AND CELL-FREE MEMBRANE FRAGMENTS OF THE BLUE-GREEN ALGA *ANACYSTIS NIDULANS*

GEORGE C. PAPAGEORGIOU

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

(Received January 25th, 1977)

SUMMARY

On treating the blue-green alga *Anacystis nidulans* with dimethylsuberimidate up to 70 % of the free NH_2 of the photosynthetic membrane is amidinated, and presumably inter- and intramolecular cross-links are established in the membrane proteins. Amidination destroys the ability of *A. nidulans* to photoreduce HCO_3^- but leaves the photochemical activities of Photosystems II and I nearly intact. With added electron acceptors, photosynthetic O_2 evolution can be demonstrated both with permeable cells (permeaplasts) prepared by digestion of the cell wall of dimethylsuberimidate-reacted *A. nidulans* with lysozyme, as well as with heavy membrane particles ($36\,000 \times g$) prepared from dimethylsuberimidate-reacted cells.

Permeaplasts prepared from dimethylsuberimidate-reacted cells resist damage in hypoosmotic medium, whereas those prepared from unreacted cells are induced to release C-phycocyanin. On the other hand, the former are inactivated more easily by heat stress than the latter. On this basis, it is concluded that cross-linking with dimethylsuberimidate confers functional instability to photosynthetic membranes.

INTRODUCTION

Cross-linking of membrane proteins with inter- and intramolecular covalent linkages is useful in correlating mobility parameters to function [1], and in stabilizing membrane-bound enzymes for technological purposes [2–4]. In the particular case of the photosynthetic membranes, the most widely employed cross-linking agent is glutaraldehyde. When it is reacted with isolated higher plant chloroplasts, glutaraldehyde produces fixed structures [5] that preserve partially the capacity for photoinduced electron transport [6–8]. In addition, glutaraldehyde affords some protection to chloroplasts against trypsin [9], chaotropic compounds [9], detergents [10], and

Abbreviations: BSA, bovine serum albumin; Chl, chlorophyll; DCIP, $DCIPH_2$, oxidized and reduced 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, diphenylcarbazide; EDTA-Na_4, tetrasodium salt of ethylene diamine tetraacetate; MV, methyl viologen; SDS, sodium dodecyl sulfate; TNBS, 2,4,6-trinitro-1-benzenesulfonic acid.

aging during cold storage [6, 11]. Algae treated with formaldehyde and glutaraldehyde are still capable of some photoinduced electron transport [12, 13]. and they become permeable to artificial electron acceptors, such as MV and DCIP [12].

In spite of its popularity as a protein cross-linker, glutaraldehyde has the disadvantage to be a multifunctional reagent, especially at alkaline pH where it condenses to unsaturated derivatives [14, 15]. Recently, the applications of aliphatic diimidoesters as cross-linkers have been extended to mitochondrial [16] and chloroplast [17] membranes. Between pH 7 and 10, the imidoester function reacts exclusively with α-NH_3^+ and ε-NH_3^+ to yield amidines with the advantage of charge conservation, since for each $-NH_3^+$ amidinated an $=NH_2^+$ group appears in the vicinity [18]. It is expected, therefore, that imidoesters will preserve the native state of a biological membrane more faithfully than glutaraldehyde.

In this study we have examined the effect of the imidoester dimethylsuberimidate on the molecular and functional properties of the photosynthetic membranes of the blue-green alga *Anacystis nidulans*. We have chosen to study a procaryote, after we had established that higher plant chloroplasts lose the H_2O-splitting ability upon fixation with dimethylsuberimidate. (Packer, L. and Papageorgiou, G., unpublished experiments). We shall show here that *A. nidulans* cells, permeaplasts, and cell-free membrane preparations preserve the ability to photolyse H_2O, even after heavy amidination with dimethylsuberimidate. Contrary, to the expectation however, dimethylsuberimidate confers no increased functional stability, since cells reacted with it are less tolerant to heat stress than the unreacted controls.

MATERIALS AND METHODS

Sterile *A. nidulans* was grown in medium C of Kratz and Myers [19], with a stream of 5 % CO_2 in air passing through the cell suspension. Some cultures were grown on air only. These, as reported also by Goedheer and Kleinen Hammans [20], were found to be less active and contained less phycobilin than the "CO_2-grown" cells. Illumination was provided by a combination of fluorescent and tungsten filament lamps, which also assisted in keeping the temperature of the culture area at 30 °C. After 2–4 days (or longer in the case of air-grown cultures), the cells were precipitated at $6000 \times g$ for 5 min, were washed once with sorbitol/phosphate buffer (500 mM sorbitol, 30 mM KH_2PO_4, pH 7.5) and were resuspended in the same buffer at 400 μg chlorophyll *a*/ml.

The dimethylsuberimidate reagent (0.1 mol/l in sorbitol/phosphate buffer adjusted to pH 8–10) was prepared fresh. A calculated volume was added to an aliquot of alga suspension that had been preadjusted to pH 8.5 with NaOH. The mixture was incubated at room temperature and darkness for 1 h, after which the cells were spun down at $6000 \times g$ for 5 min, and then resuspended in sorbitol/phosphate buffer.

Cell-free membrane preparations were obtained by intermittent sonication of ice-cold *A. nidulans* for a total of 5 min in an MSE sonicator. Unbroken cells were sedimented at $6000 \times g$ for 10 min, and two membrane fractions were collected by centrifuging the supernatant first at $36\,000 \times g$ for 30 min, and then at $144\,000 \times g$ for 90 min in refrigerated ultracentrifuge (Beckmann Model L2-65B). The membrane precipitates were transferred to cold sorbitol/phosphate with the aid of a glass

homogenizer, and were stored at 0–4 °C and darkness.

Cells permeable to ferricyanide, DCIP, and MV (permeaplasts) were prepared both from unfixed and from dimethylsuberimidate-fixed *A. nidulans* by lysozyme digestion of their cell walls according to Ward and Myers [21]. The reaction was carried out in sorbitol/phosphate at 37 °C in a mixture that contained in a total volume of 14 ml: *A. nidulans* equivalent to 2 mg Chl *a*; EDTA-Na_4, 12 μmol; and lysozyme (Sigma, grade 1) 175 mg. Progress was followed by monitoring ferricyanide permeation into the cells in terms of ferricyanide-dependent O_2 evoluation in aliquots removed from the reaction mixture at various time intervals. When this activity was maximized (after 40–60 min), the cells were spun down at 12 000 × *g* for 10 min, and were resuspended in cold sorbitol/phosphate at 400 μg Chl/ml.

Free NH_2 was determined in acetone-extracted membranes by an adaptation of the method of Satake et al. [22], as it has been modified by Habeeb [23]. The cell sonicate was first cleared of unbroken cells by centrifugation at 6000 × *g* for 10 min, and then the membranes were precipitated at 144 000 × *g* for 90 min. The resulting residue was extracted twice with 80 % (v/v) aqueous acetone, each time followed by pelleting at 144 000 × *g* for 90 min. The final grey-white precipitate was resuspended with the aid of a glass homogenizer in 100 mM $Na_2B_4O_7$, 200 mM NaCl, pH 5.0, to a concentration of 2–3 mg protein/ml. 0–1 ml of this stock was brought to 1 ml with the borax/NaCl buffer, and 1 ml of 0.1 % (w/v)TNBS was added. Incubation was carried out at 40 °C for 2 h in tubes wrapped in aluminium foil. At the end, 1 ml of 10 % (w/v) SDS was added to solubilize the membranes, followed by 0.5 ml of 1 M HCl. Absorbance was read at 335 nm relative to a minus membranes blank, after dilution 1 : 10 with borax/NaCl buffer. Serially diluted BSA samples were assayed concomitantly to ascertain the linearity of the measured absorbance with the concentration of NH_2.

O_2 evolution was measured with a concentration electrode (Rank Brothers, Cambridge, England). The reaction mixtures are given in the figure legends. The samples were illuminated with saturating light, which passed through a $CuSO_4$ filter (1 % (w/v), 5 cm thick). Temperature control was possible by circulating thermostated H_2O around the sample. In heat stress experiments, the temperature of the sample was allowed to rise to a constant rate of 1 °C/3 min. The sensitivity of the electrode at various temperatures was established independently by consuming the O_2 of air-saturated H_2O aliquots with $Na_2S_4O_6$. O_2 content of air-saturated H_2O was obtained from the literature [24].

Chl *a* was determined in methanol extracts spectroscopically [25]. Protein was determined according to Lowry et al. [26] using BSA as standard. Scatter-free absorption spectra were recorded with a Hitachi 356 dual wavelength spectrophotometer by placing the sample and the reference in the primary compartment, and operating the instrument in the split beam mode.

RESULTS

Fig. 1 shows the absorbance at 335 nm to be proportional to the membrane protein content (therefore to the NH_2 content) of the TNBS assay mixture. Cell-free membrane fractions prepared from *A. nidulans* that had been reacted with 200 μmol dimethylsuberimidate/mg Chl *a* have fewer detectable NH_2 groups. From the slopes of

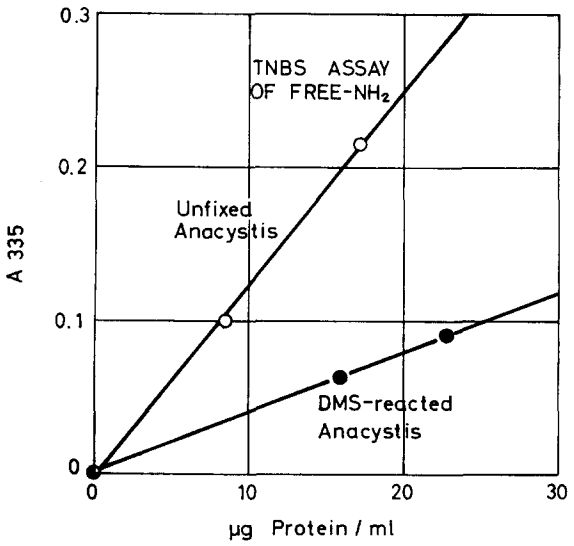

Fig. 1. Concentration of triphenylated NH_2 of photosynthetic membranes prepared from unreacted and from dimethylsuberimidate-reacted (200 µmol/mg Chl *a*) *A. nidulans*. The absorbance at 335 nm is proportional to the concentration of NH_2 that had been triphenylated with TNBS. The abscissa gives the protein content of the final reaction mixture. Further details, as in Materials and Methods.

the linear plots in Fig. 1, it can be calculated that at 200 µmol dimethylsuberimidate/mg Chl *a* nearly 70 % of the membrane protein NH_2 has been amidinated. This is within the saturation range since a further increase in the proportion of the diimidoester did not increase the fraction of amidinated NH_2.

A. nidulans cannot photoreduce HCO_3^- with electrons derived from H_2O after exhaustive amidination with dimethylsuberimidate (Table I). In order to investigate whether dimethylsuberimidate inhibits the photochemical part of photosynthesis, we needed to have cells that were permeable to the artificial oxidoreduction compounds that are used in such studies. *A. nidulans* cells become permeable (permeaplasts; ref. 21) upon digestion of their cell wall with lysozyme. These modified cells are capable of photoinduced electron transport from H_2O to *p*-benzoquinone (Photosystem II

TABLE I

ELECTRON TRANSPORT ACTIVITY (H_2O TO HCO_3^-) OF DIMETHYLSUBERIMIDATE-TREATED *ANACYSTIS NIDULANS*

Air-grown cells. The reaction mixture contained *Anacystis* cells (90–150 µg Chl *a*) in 3 ml of 200 mM $NaHCO_3$, pH 8.5. Temperature of the assay, 24 °C.

µmol dimethylsuberimidate/mg chlorophyll	µequiv./mg chlorophyll per h
0	172
50	126
100	76
200	28

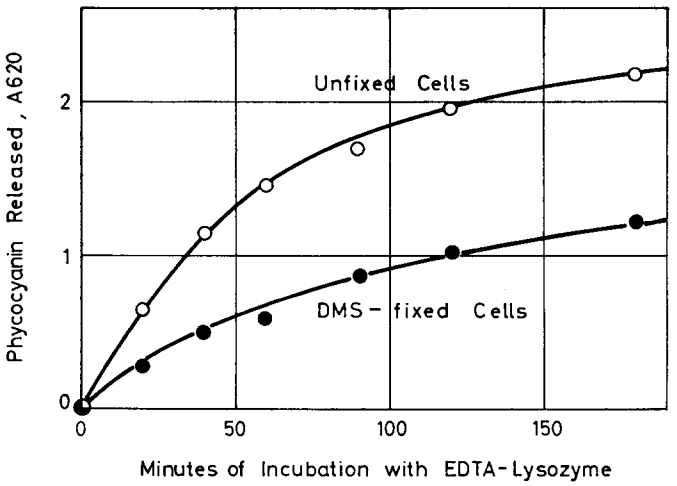

Fig. 2. Release of C-phycocyanin to the medium during digestion of the *A. nidulans* cell wall with lysozyme. C-phycocyanin is determined in terms of the 620 nm absorbance in the supernatant of a 6000 × *g* for 10 min centrifugation. Dimethylsuberimidate-fixed cells have been reacted with 200 μmol dimethylsuberimidate/mg Chl *a*.

activity) and to ferricyanide (Photosystem II and Photosystem II+I activities; ref. 27), as well as from $DCIPH_2$ to MV (Photosystem I activity).

Digestion of the *A. nidulans* cell wall with lysozyme is attended by a progressive release of C-phycocyanin to the medium, which can be monitored in terms of its characteristic absorbance at 620 nm (Fig. 2). Cells that had been reacted at 200 μmol dimethylsuberimidate/mg Chl *a* lose C-phycocyanin less easily than unreacted cells. Although digestion of the *A. nidulans* cell wall requires a higher concentration of lysozyme compared to other blue-green algae (e.g. *Phormidium luridum*; ref. 28), C-

TABLE II

EFFECT OF OSMOTIC SHOCK ON THE PHYCOCYANIN CONTENT OF PERMEAPLASTS PREPARED FROM UNFIXED AND FROM DIMETHYLSUBERIMIDATE-FIXED *ANACYSTIS NIDULANS* CELLS

The permeaplasts were shocked osmotically by a 15 min incubation in 1/10 strength sorbitol/phosphate. The shocked cells were pelleted at 12 000 × *g* for 10 min and then resuspended in buffer. The relative phycocyanin content of the cell suspension was estimated from the scatter-free absorbance at 620 nm. Dimethylsuberimidate was administered at a level of 200 μmol/mg Chl.

Type of cells	Relative phycocyanin content
Unfixed cells	100
Dimethylsuberimidate-fixed cells	100
Unfixed permeaplasts	78
Unfixed permeaplasts, osmotically shocked	42
Dimethylsuberimidate-fixed permeaplasts	90
Dimethylsuberimidate-fixed permeaplasts, osmotically shocked	84

phycocyanin is released to the medium not because of cell disintegration, as no Chl *a*-containing material could be detected in the supernatant of low speed centrifugation ($6000 \times g$ for 10 min).

Permeaplasts prepared from normal cells are more susceptible to osmotic damage of the cell membrane than those prepared from dimethylsuberimidate-reacted cells. On the basis of phycobilin released to the medium, osmotic shock appears to have little effect on the second type of permeaplasts (Table II). It has been reported that after cross-linking with bifunctional imidoesters red blood cells resist hemolysis [29] and that *Escherichia coli* ghosts resist lysis in boiling 1% SDS [30]. Similarly, whole chloroplasts prepared from spinach resist osmotic rupture of their envelopes after fixation with dimethylsuberimidate (Krause, G. H., and Pagageorgiou, G., unpublished experiments). Dimethylsuberimidate, however, does not appear to establish covalent links between phycobilins and membrane proteins, since the absorption spectra of cell-free membrane fractions of diimidoester-treated *A. nidulans* do not include the typical C-phycocyanin absorption band at 620 nm (Fig. 3).

The effect of progressive digestion of the cell wall by lysozyme on the electron transport rates of *A. nidulans* is illustrated in Fig. 4. HCO_3^--dependent photosynthetic O_2 evolution falls off with the elapsed reaction time, changing at the end to light-induced uptake of O_2. *Anacystis* that had not been treated with lysozyme (i.e. the zero time control) was also capable of O_2 evolution in the presence of ferricyanide, especially after preincubation of the cells with this oxidant under illumination. It is possible, that the zero time O_2 signal is due to ferricyanide that permeated slowly into the cell, and also to CO_2 dissolved in the reaction mixture.

As reported by Ward and Myers [21], the rate of ferricyanide reduction increases first with the elapsed time of lysozyme digestion of the cell wall (phase 1), and then decreases (phase 2). The rate of electron transport across Photosystem I, assayed in terms of MV-mediated O_2 uptake with ascorbate/$DCIPH_2$ as the electron donor, also increases after an initial lag, and stays high during phase 2 of the ferricyanide reduction process.

An analogous experiment, in which electron transport activities of dimethylsuberimidate-reacted *A. nidulans* were monitored during digestion of the cell wall with lysozyme is illustrated in Fig. 5. Cells that have been reacted with 200 μmol dimethylsuberimidate/mg Chl *a* are nearly incapable of photoreducing HCO_3^- (cf. Table I also). The rates of electron transport from H_2O to ferricyanide, and ascorbate/$DCIPH_2$ to MV, however, follow the same kinetic pattern as in the case of unfixed cells (cf. Fig. 4). The important fact demonstrated here is that even after heavy fixation with dimethylsuberimidate, *A. nidulans* is still capable of producing low potential reducing equivalents by photolysing H_2O, at rates comparable to those of the unreacted cells.

Cell-free fragments from *A. nidulans* that are capable of transporting electrons across Photosystem II at rates ranging between 100 and 200 μequiv./mg Chl *a* per h have been prepared by several research groups [31–33]. In this series of experiments, it was of interest to us to compare electron transport activities of membrane fragments prepared from normal and from dimethylsuberimidate-reacted cells by sonication and centrifugal fractionation. Such fragments are free, or nearly free, of C-phycocyanin (cf. Fig. 3). The results are displayed in Table III, which shows that heavy particles (precipitated at $36\,000 \times g$) are capable of photoinduced electron transport across

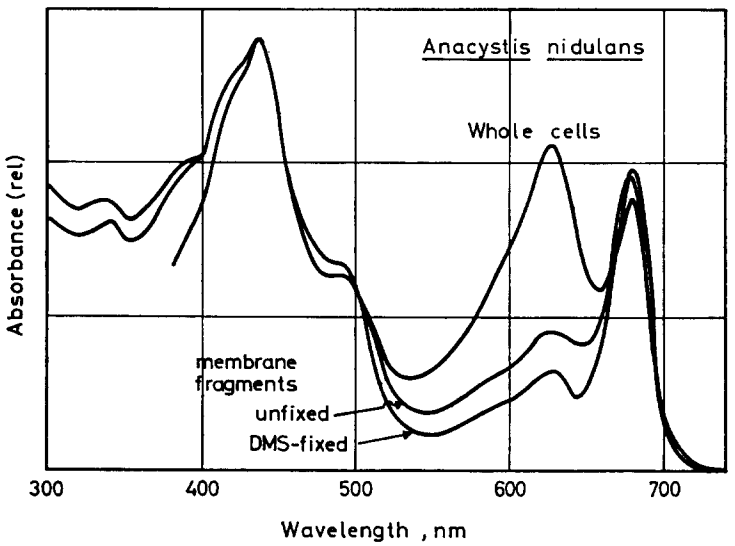

Fig. 3. Absorption spectra of suspensions of whole *A. nidulans* cells, and of cell-free membrane fragments, prepared from unreacted and from dimethylsuberimidate-reacted (200 μmol dimethylsuberimidate/mg Chl *a*) cells.

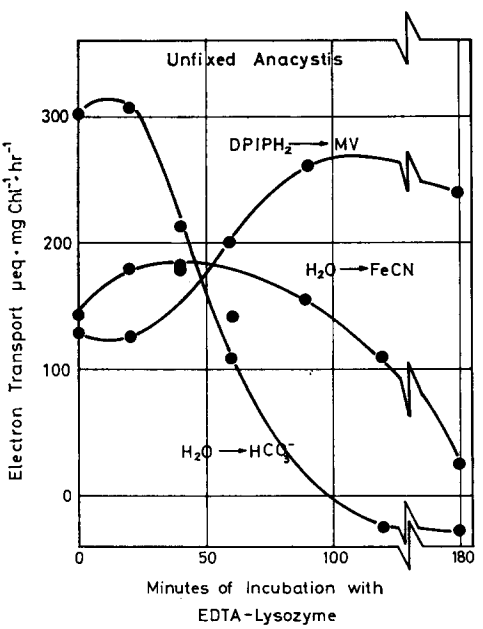

Fig. 4. Electron transport activities of *A. nidulans* (grown in air enriched with 5 % CO_2) during incubation with lysozyme. The reaction mixtures contained 80–120 μg Chl *a* in 3 ml sorbitol/phosphate buffer, plus the following. H_2O to HCO_3^-: 0.3 mmol $NaHCO_3$, pH 8.5, H_2O to ferricyanide: 3 μmol $K_3Fe(CN)_6$. $DCIPH_2$ to MV: 6 μmol sodium ascorbate, 0.1 μmol DCIP, 60 nmol DCMU, and 0.3 μmol MV. The assay was carried out at 24 °C.

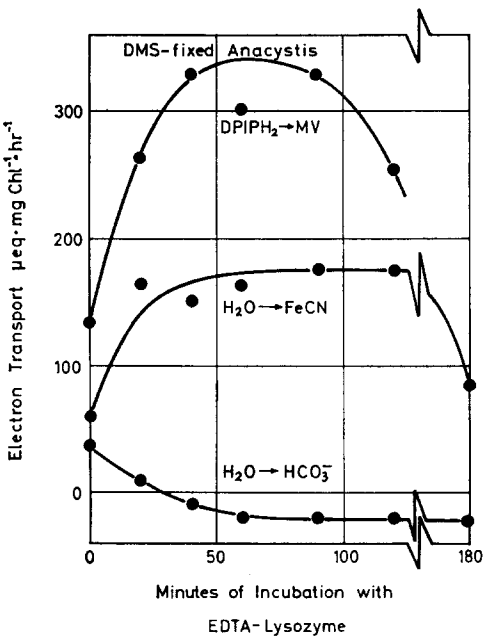

Fig. 5. Electron transport activities of dimethylsuberimidate-reacted (200 μmol dimethylsuberimidate/mg Chl *a*) *A. nidulans* during incubation with lysozyme. Cultures grown on 5 % CO_2 in air. Other conditions, as in the legend to Fig. 4.

TABLE III

ELECTRON TRANSPORT ACTIVITIES OF CELL-FREE MEMBRANE PREPARATIONS FROM UNFIXED AND DIMETHYLSUBERIMIDATE-FIXED *ANACYSTIS NIDULANS* CELLS

Air-grown cells. Cell-free fragments were prepared by sonication and centrifugal fractionation as described in Materials and Methods. The reaction mixtures contained in 3 ml: H_2O to MV. Membrane fragments containing 90–150 μg Chl *a*; 0.3 μmol methyl viologen, 1.5 mmol sorbitol, 90 mol KH_2PO_4, pH 7.5. DPC to MV, same composition as the H_2O to MV reaction mixture, plus 1.5 μmol diphenylcarbazide. $DCIPH_2$ to MV. Membrane fragments containing 60–90 μg Chl *a*; 60 nmol DCMU, 6 μmol sodium ascorbate, 72 nmol DCIP, and approx. 0.3 nmol ethanol added with DCMU. Temperature of the assay, 22–24 °C. Results are expressed in μequiv./mg chlorophyll per h.

Membrane precipitate	μmol dimethyl-suberimidate/mg Chl	$H_2O \rightarrow MV$	$DPC \rightarrow MV$	$DCIPH_2 \rightarrow MV$
36 000 × *g*	0	71	80	410
	100	63	78	378
	200	57	74	377
144 000 × *g*	0	0	0	210
	100	0	0	236

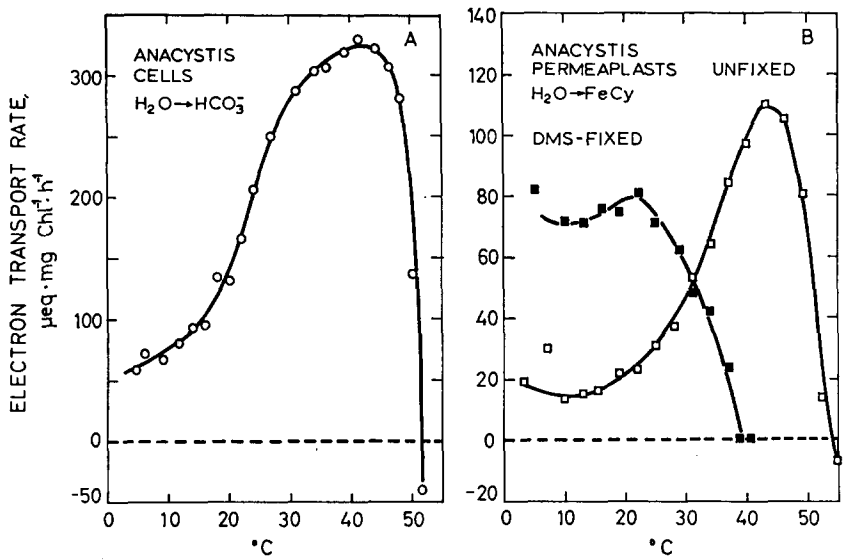

Fig. 6. Temperature profiles of electron transport activities of whole *A. nidulans* cells (A), and of permeaplasts prepared from unreacted and from dimethylsuberimidate-reacted cells. Air grown cultures. Treatment with dimethylsuberimidate at 100 μmol/mg Chl *a*. The temperature of the reaction mixture was allowed to rise at a constant rate of 1 °C/3 min. Reaction mixtures, as in the legend to Fig. 4. Other details, as in Materials and Methods.

Photosystem I ($DCIPH_2$ to MV) as well as across both photosystems (H_2O to MV, and DPC to MV). Light particles, ($144\,000 \times g$) on the other hand, are capable only of Photoreaction I. In all cases, inhibition by 200 μmol dimethylsuberimidate/mg Chl *a* does not exceed 20 %.

Several reports exist describing increased functional stability of enzymes after cross-linking with bifunctional reagents [2]. To test whether dimethylsuberimidate confers increased stability to *A. nidulans*, we examined the effect of heat stress on the photochemical activities of intact cells and permeaplasts. Fig. 6B depicts the temperature profile of Photosystem II activity (H_2O to ferricyanide) of permeaplasts prepared from unfixed cells and from cells that had been reacted with 100 μmol dimethylsuberimidate/mg Chl *a*. Fig. 6A depicts the temperature profile of HCO_3^--supported O_2 evolution of intact unfixed cells, for comparison.

Both the intact cells and the unfixed permeaplasts exhibit optimal H_2O-splitting activity at 43 °C. Beyond this temperature, O_2 evolution activity deteriorates rapidly, possibly assisted by a progressively increasing light-induced O_2 uptake. Above 50 °C, only light-induced O_2 uptake is observed. In contrast, permeaplasts prepared from dimethylsuberimidate reacted cells show a strikingly different temperature profile. Below 22 °C, H_2O-splitting activity is fairly constant with temperature, but beyond that point it begins to drop gradually. At 40 °C, i.e. ahead of the temperature optimum of the unfixed permeaplasts and of the unreacted intact cells, the dimethylsuberimidate-fixed permeaplasts are completely inactivated. No light-induced O_2 uptake is observed beyond the point of inactivation. In all cases studied, thermal inactivation was found to be irreversible.

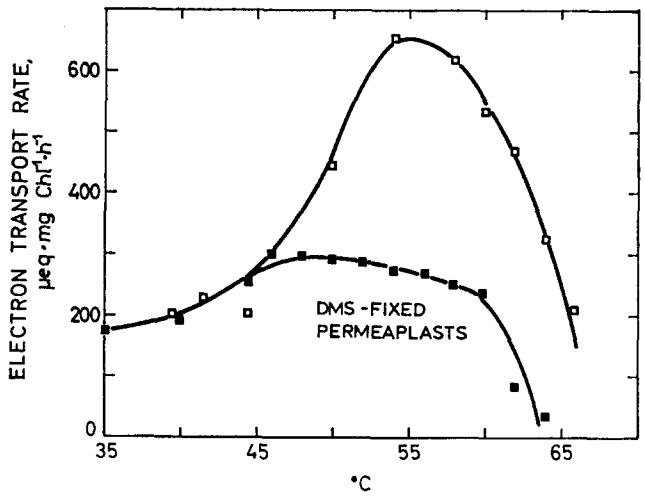

Fig. 7. Temperature profile of Photosystem I activity of permeaplasts prepared from unreacted and from dimethylsuberimidate-reacted (100 mol dimethylsuberimidate/mg Chl *a*) *A. nidulans*. $DCIPH_2$ to MV reaction mixture, as in the legend to Fig. 4. Other conditions, as in the legend to Fig. 6.

Temperature profiles of Photosystem I activity of permeaplasts prepared from unreacted and from dimethylsuberimidate-reacted *A. nidulans* are illustrated in Fig. 7. With unfixed permeaplasts, the assay is complicated by the cofactor-independent light-induced O_2 uptake that sets in at about 45 °C, which makes them appear more active than their dimethylsuberimidate-fixed counterparts. Nevertheless, Fig. 7 shows that treatment with dimethylsuberimidate entails no particular increase in the thermostability of Photosystem I, since the fixed preparation is inactivated ahead of the unfixed. On the other hand, since dimethylsuberimidate-fixed permeaplasts carry out $DCIPH_2$-dependent uptake of O_2 only, this result is indicative of a remarkable tolerance of Photosystem I to heat stress, which should be contrasted with the earlier inactivation of Photosystem II both in the unfixed and the dimethylsuberimidate-fixed permeaplasts (Fig. 6B).

DISCUSSION

Dimethylsuberimidate may react with the free NH_2 of proteins either monofunctionally, or bifunctionally. In the first case, the unreacted imidoester group is eventually hydrolysed to the carbonic acid ester, whereas in the second an 8-carbon atom bridge is formed, linking either two polypeptide chains, or two loci on the same polypeptide chain.

For the following reasons we assume that dimethylsuberimidate establishes cross-links in and among the protein subunits of the photosynthetic membranes of *A. nidulans*: (1) At alkaline pH, the rate of reaction of the imidoester group with NH_2 exceeds the rate of hydrolysis by a factor 10^2–10^3 [34]. (2) At alkaline pH, dimethylsuberimidate has been shown to cross-link soluble [35] and membrane-bound proteins [16], and to be a suitable fixative for electron microscopy preparations [36]. (3) Dimethylsuberimidate cross-links chloroplast proteins to aggregates with molecular

weights in excess of 300 000 daltons (Isaakidou, J. and Papageorgiou, G., unpublished experiments). (4) As shown in Fig. 1, about 70 % of the free NH_2 of *A. nidulans* lamellae disappears upon exhaustive amidination with dimethylsuberimidate. Presumably, the remaining 30% of membrane NH_2 is inaccessible to the diimidoester, but accessible to the highly reactive TNBS after extraction of the membrane lipids (cf. Materials and Methods).

In contrast to the effects of formaldehyde and glutaraldehyde [12], the envelope of intact cells of *A. nidulans* does not become permeable to MV upon treatment with dimethylsuberimidate. Following digestion of the cell wall with lysozyme, dimethylsuberimidate-reacted cells become permeable both to small ions and to fairly large proteins such as C-phycocyanin (molecular weight of the monomer, 30 000 daltons; ref. 37). At the same time, photoreduction of HCO_3^- is inhibited although the permeaplast is capable of producing low potential reductants by photolysing H_2O (Fig. 4). This inhibition should most likely be attributed to the loss of ferredoxin, and of other soluble proteins, to the medium. In this attribute, *A. nidulans* resembles the cyanophyte *Fremyella diplosiphon*, which cannot fix CO_2 after treatment with lysozyme [38]. On the other hand, spheroplasts prepared by lysozyme digestion of *P. luridum* can reduce CO_2 at rates of 95–155 μmol CO_2/mg Chl per h [28].

Dimethylsuberimidate-treated *Anacystis* loses C-phycocyanin to the medium less readily than untreated controls during digestion of the cell was with lysozyme (Fig. 2). This should be attributed to the altered properties of the cell membrane, rather than to a covalent attachment of the soluble phycobilins to the lamella, since cell-free membrane preparations from normal and dimethylsuberimidate-treated *A. nidulans* are both devoid of the typical 620 nm absorption of C-phycocyanin (Fig. 3). The decreased permeability of cell membrane, however, manifests only relative to the passage of large proteins, since permeaplasts prepared from dimethylsuberimidate-reacted cells are capable of importing MV and ferricyanide (Fig. 5).

A second effect of dimethylsuberimidate is the mechanical strengthening of the cell membrane which is evidenced by the fact that permeaplasts of dimethylsuberimidate treated cells resist osmotic lysis, whereas those of normal cells do not (Table II). This may be due either to the covalent cross linking of cell membrane proteins, or to the incomplete digestion of the cell wall because of such cross links, or to both.

Amidination of about 40 % of the free NH_2 of rat liver mitochondria with dimethylsuberimidate causes 70–90 % inhibition of electron transport activities [16]. Similarly, isolated spinach chloroplasts cannot photoevolve O_2 following treatment with dimethylsuberimidate at room temperature, although partially active preparations are obtained by carrying out the cross-linking reaction at 0–4 °C (Packer, L. and Papageorgiou, G., unpublished results). It appears, therefore, that among the studied systems, which possess membrane-integrated electron transport chains, only that of *A. nidulans* can function in a state of heavy amidination with dimethylsuberimidate. This is true both with intact permeable cells (Fig. 5), and with phycocyaninless cell-free membrane preparations (Table II). Dimethylsuberimidate fixed cells and permeaplasts, however, cannot photoreduce HCO_3^-, suggesting a diimidoester effected inactivation of the non-photochemical reactions of photosynthesis (cf. Table I and Fig. 5).

The randomization of protein structure, caused by diverse physical and chemical process, is recognized as the main cause of enzyme inactivation [2]. Accordingly,

conditions which minimize the chance for structural change are expected to prolong the function of enzymes. Storage at low temperature and cross-linking with glutaraldehyde, applied simultaneously, have been shown to assist in preserving the activity of resting chloroplasts in vitro [6, 11]. Such preparations, however, are readily inactivated when subjected to continuous working stress. This, then, raises the question whether a chemically immobilized membrane is more stable than its native counterpart, in which, because of smaller molecular size, the lateral motion of proteins is easier. In the case of *A. nidulans* (Fig. 6), the answer is it is not. It is very characteristic that the threshold of temperature inactivation of the H_2O-splitting activity of dimethylsuberimidate treated permeaplasts is in the room temperature range (20–25 °C), and that complete inactivation occurs ahead of the temperature optimum (43 °C) of untreated permeaplasts and cells.

It is recognized now that the physical state of the lipid phase influences the integral processes of biological membranes, in the sense that lipid fluidity is essential for functional expression [39]. Because of the high proportion of *cis*-unsaturated fatty acids, most biological membranes, including those of higher plant chloroplasts and algae, are fluid above 0 °C. *A. nidulans*, however, is devoid of linolenic acid, the major unsaturated fatty acid of photosynthetic organisms [40]. Because of that it undergoes reversible lipid phase transition at temperatures as high as 24 °C [41]. At the temperaoptimum, therefore, of the photosynthetic activity of normal *A. nidulans* (43 °C) the membrane lipids are in the liquid disordered state. We may account then, for the observed threshold of temperature inactivation of dimethylsuberimidate reacted permeaplasts at 20–25 °C by assuming that the cross-linked protein subunits can no longer move laterally into fluid lipid domains. In such preparations, phase transitions appear to be irreversible, since the H_2O splitting function is not reactivated on lowering the temperature to approx. 20 °C.

In conclusion, we may say that the artificial cross-linking of proteins contributes to the instability of integral membrane functions by making state transitions in the membrane irreversible. This differentiates the membranes from the functional oligomeric and monomeric proteins, where covalent cross-linking may stabilize activity by preventing denaturation.

ACKNOWLEDGEMENT

I wish to thank Mrs. Thoula Lagoyanni for expert technical assistance throughout this work.

REFERENCES

1 Tinberg, H. M. and Packer, L. (1976) in Mitochondria: Bioenergetics, Biogenesis, and Membrane Structure, pp. 349–366, Academic Press, New York
2 Zaborsky, O. R. (1973) Immobilized Enzyme, CRC Press, Inc., Cleveland
3 Kestner, A. I. (1974) Usp. Khim. 43, 1480–1511
4 Bowers, L. D. and Carr, P. W. (1976) Anal. Chem. 48, 545A–559A
5 Packer, L., Allen, J. M. and Starks, M. (1968) Arch. Biochem. Biophys. 128, 142–152
6 Park, R. B., Kelly, J., Drury, S. and Sauer, K. (1966) Proc. Natl. Acad. Sci. U.S. 55, 1056–1062
7 West, J. and Packer, L. (1970) Bioenergetics 1, 405–412
8 Oku, T., Sugahara, K. and Tomita, G. (1973) Plant Cell Physiol. 14, 385–396

9 Zilinskas, B. A. and Govindjee (1976) Z. Pflanzenphysiol. 77, 302–314
10 Sane, P. V. and Park, R. B. (1970) Plant Physiol. 46, 852–854
11 Hardt, H. and Kok, B. (1976) Biochim. Biophys. Acta 449, 125–135
12 Hallier, U. W. and Park, R. B. (1969) Plant Physiol. 44, 535–539
13 Mohanty, P. K., Papageorgiou, G. and Govindjee (1971) Photochem. Photobiol. 14, 667–682
14 Richards, F. M. and Knowles, J. R. (1968) J. Mol. Biol. 37, 231–233
15 Habeeb, A. F. S. A. and Hiramoto, R. (1968) Arch. Biochem. Biophys. 128, 142–152
16 Tinberg, H. M., Nayudu, P. R. V. and Packer, L. (1976) Arch. Biochem. Biophys. 172, 734–740
17 Packer, L., Torres-Pereira, J., Chang, P. and Hansen, S. (1974) Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), Vol. 2, pp. 867–872, Elsevier Sci. Publ. Co., Amsterdam
18 Hunter, M. J. and Ludwig, M. L. (1962) J. Am. Chem. Soc. 84, 3491–3504
19 Kratz, W. A. and Myers, J. (1955) Am. J. Bot. 42, 282–287
20 Goedheer, J. C. and Kleinen Hammans, J. W. (1975) Nature 256, 333–335
21 Ward, B. and Myers, J. (1972) Plant Physiol. 50, 547–550
22 Satake, K., Okayama, T., Ohashi, M. and Shinoda, T. (1960) J. Biochem. (Japan) 47, 654–660
23 Habeeb, A. F. S. A. (1966) Anal. Biochem. 14, 328–336
24 International Critical Tables (1928) Vol. 3, p. 270, McGraw-Hill Book Co., New York
25 Ozerol, N. H. and Titus, J. S. (1965) Trans. Ill. Acad. Sci. 58, No. 2
26 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
27 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) J. Biol. Chem. 246, 3204–3209
28 Biggins, J. (1967) Plant Physiol. 42, 1442–1446
29 Dutton, A., Adams, M. and Singer, S. J. (1966) Biochem. Biophys. Res. Commun. 23, 730–739
30 Haller, I. and Henning, U. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2018–2021
31 Fredericks, W. W. and Jagendorf, A. J. (1964) Arch. Biochem. Biophys. 104, 39–49
32 Suzuki, R. and Fujita, Y. (1972) Plant Cell Physiol. 13, 427–436
33 de Kouchkovsky, Y. and Sigalat, C. (1975) Physiol. Veg. 13, 831–851
34 Hunter, M. J. and Ludwig, M. L. (1962) J. Am. Chem. Soc. 84, 3491–3504
35 Davies, G. E. and Stark, G. R. (1970) Proc. Natl. Acad. Sci. U.S. 66, 651–656
36 Hassel, J. and Hand, A. R. (1974) J. Histochem. Cytochem. 22, 223–239
37 Neufeld, G. and Riggs, A. (1969) Biochim. Biophys. Acta 181, 234–243
38 Crespi, H. L., Mandeville, S. E. and Katz, J. J. (1962) Biochem. Biophys. Res. Commun. 9, 569–573
39 Overath, P., Thilo, L. and Träuble, H. (1976) Trends Biochem. Sci. 1, 186–189
40 Hirayama, O. (1967) J. Biochem. 61, 179–185
41 Murata, N., Troughton, J. H. and Fork, D. C. (1975) Plant Physiol. 56, 508–517