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Sulfide-dependent electron transport in thylakoids from the cyanobacterium *Oscillatoria limnetica*

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(1) *Oscillatoria limnetica* was grown either aerobically (with water as electron donor), or anaerobically (with sulfide as electron donor). Thylakoids from both types of cells were compared with respect to (a) the content of P-700 and cytochromes, (b) the sulfide-dependence of the re-reduction of P-700 and cytochrome *c*-553 after light-oxidation, and (c) the spectral characteristics of the light-induced absorbance changes in the α-band region of the cytochromes. (2) The molar ratios of P-700 / chlorophyll and P-700 / cytochrome *b*-559 in thylakoids from anaerobically grown cells are more than 2 times higher than in thylakoids from aerobically grown cells. (3) Only thylakoids from anaerobically grown cells possess a membrane-bound sulfide oxidase which feeds electrons into the photosynthetic electron-transport chain at the level of the plastoquinone pool or the cytochrome b_6/f complex. This pathway is characterized by a high sulfide affinity and high rates of re-reduction of P-700 and cytochrome *c*-553. In addition, thylakoids from both types of cell possess 'nonspecific' pathways for electron transfer from sulfide to Photosystem I. These pathways bypass the cytochrome b_6/f complex and exhibit a much lower sulfide affinity, and lower rates of re-reduction of P-700 and cytochrome *c*-553.

Introduction

Oscillatoria limnetica shares with a number of other Cyanobacteria the peculiar property that it can use either water or sulfide as the ultimate electron donor for photosynthesis [1–3]. It has been suggested that organisms with this capacity may have provided the evolutionary link between photosynthetic bacteria sensu stricto, and higher plants [4]. Sulfide-dependent photosynthesis has been studied in some detail in whole cells of *O. limnetica*, in which either CO_2 assimilation or hydrogen evolution was measured. Addition of sulfide to an aerobically grown culture leads to an immediate inhibition of Photosystem-II activity [5], as a result of which photosynthesis stops; however, after an 'induction period' of a few hours photosynthesis is resumed, but the process is now DCMU-insensitive and involves Photosystem I only [6–8]. Inhibitor studies with whole cells indicated that electrons from sulfide enter the photosynthetic electron-transport chain at the level of plastoquinone or the cytochrome b_6f complex [9]. The induction of sulfide-dependent photosynthesis is inhibited by chloramphenicol, indicating that during the induction period one or more proteins are synthesized which are essential for this process [7]. The hydrogenase activity of the cells increases dramatically during growth on sulfide [9–11]; much of this activity is recovered in the soluble enzyme fraction of cell-free extract [12].

In order to study the electron transport from sulfide to Photosystem I in more detail, we worked with thylakoid preparations from *O. limnetica*. We have shown recently that in these thylakoids two pathways for sulfide-linked NADP-reduction exist: a 'non-specific pathway' and a 'specific pathway'. The 'nonspecific pathway' occurs in thylakoids from both aerobically and anaerobically grown cells; it bypasses inhibitor-sensitive sites located on the cytochrome b_6/f complex and is characterized by a low sulfide affinity (K_m(sulfide) in the order of mmolar). The 'specific pathway' occurs only in thylakoids from anaerobically grown cells; it is inhibited by inhibitors of the cytochrome b_6/f complex and is characterized by a much higher sulfide affinity (K_m(sulfide) $\approx$ 24 μM) [13]. These experiments were

Abbreviations: PMSF, phenylmethylsulphonyl fluoride; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; NQNO, 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide; DBMIB, 2,5-dibromothymoquinone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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performed with thylakoids in the presence of a soluble enzyme fraction (see Methods) which contained the ferredoxin required for NADP-reduction. It was, therefore, not clear whether the sulfide oxidase involved in the 'specific pathway' of NADP-reduction was solubilized or membrane-bound. For this reason we measured the kinetics of light-induced cytochrome- and P-700 absorbance changes in thylakoids without soluble enzyme fraction. The results are presented in this paper.

Methods

O. limnetica was kindly provided by Dr. E. Padan from The Hebrew University, Jerusalem, Israel. The growth medium described in Ref. 6 was modified and contained, per l: 7.3 g Na_2SO_4, 1.5 g Na_2CO_3, 0.32 g KH_2PO_4, 44.6 g NaCl, 20.6 g $MgCl_2 \cdot 6H_2O$, 2.63 g $CaCl_2$, 1.53 g KCl, 0.33 g NH_4Cl, 2.1 mg citric acid, 1.8 mg ferric citrate, 1 ml A5 trace metals [14], and HCl to pH 6.8. The medium was autoclaved in two portions, one (60 ml) containing the first three components, and the other (940 ml) containing the remainder.

The cells were grown in 1-l culture bottles of 9 cm diameter, equipped with a polypropylene lid with holes through which a pH-electrode and glass tubes for gassing, inoculation, additions and sample-drawing were inserted. The bottles were positioned in a thermostatted (36 °C) water bath at 40 cm from two 20-W fluorescent tubes (Sylvania Gro-Lux). The cultures were stirred periodically (1 min on, 5 min off) with a magnetic stirrer. The bottles were inoculated with the settled suspension of a 200-ml culture. During aerobic growth the cultures were gassed with 5% CO_2 in air. The doubling time was approx. 36 h. After 4 days of growth the cells were either harvested (aerobically grown cells), or grown on sulfide. In the latter case 10 μM DCMU was added and the culture was purged with nitrogen; then 4 mM sulfide was added, the pH was adjusted to 7.7 and the culture was kept under nitrogen pressure. Sulfide consumption started at approx. 2 h after this transition; however, the chlorophyll concentration remained constant for approx. 20 h and then increased with a doubling time of 35 h. Twice daily the sulfide content of the culture was determined and sulfide, carbonate and HCl were added in a 2 : 1 : 4 ratio so as to restore the sulfide and carbonate content as well as the pH to the original level. After 48 h of growth on sulfide the cells were harvested (anaerobically grown cells).

The cells were washed (by centrifugation and resuspension) 2–3 times in fresh growth medium, until they were free from greyish precipitates (probably calcium salts); then they were washed once in a medium containing 1.24 M NaCl, 5 mM KCl, 10 mM Hepes, 10 mM KH_2PO_4, 5 mM EDTA (pH 7.0) and once in the same medium but without EDTA. Finally the cells were resuspended in 8 ml of 'basal medium' supplemented with 0.1 mM PMSF. The basal medium contained 10 mM Hepes, 10 mM K_2HPO_4 and 5 mM $MgCl_2$ at pH 7.0. The cells were sonicated for 1 min in an ice-cooled waterbath in 4-ml portions with a probe-type sonifier (MSE, 150 W) operated at 3 μ amplitude (1/8 of full output). The suspension was centrifuged at 3° C for 10 min at $3000 \times g$ to remove cell debris, and then for 30 min at $75\,000 \times g$. The sediment (thylakoids) was resuspended in approx. 3 ml of basal medium, at a chlorophyll concentration of 0.5–1 mg/ml, and stored on ice for up to 4 days. The supernatant ('soluble enzyme fraction') was stored on ice as such.

All experiments were carried out in stirred and stoppered cylindrical cuvettes of 1 cm optical path length and 2.3 ml capacity. The lid contained a narrow hole, suitable for additions by means of syringes. The cuvettes were filled to capacity with basal medium supplemented with the necessary additives. If sulfide (sodium salt) was added, the pH was adjusted with HCl. Continuous, actinic light, provided by a 200-W tungsten-halogen lamp, was filtered through appropriate colour filters and through 10 cm of water.

Absorption spectra, light-induced absorbance changes in the red region and chemically induced difference spectra were measured on a Cary 2300 spectrophotometer equipped with side illumination. Light-induced absorbance changes in the blue region and around 550 nm were measured on a laboratory-built double-beam spectrophotometer used in the single-beam mode. The signal from the photomultiplier was fed into a Beckman pH-meter, which provided an electrical compensation for the signal measured without actinic illumination. The output of the pH-meter was fed into a dual-channel strip chart recorder. The second channel was used to monitor switching actinic light on and off.

Oxygen uptake was measured with a Clark-type electrode. Sulfide was determined according to Ref. 15. Chlorophyll was determined according to Ref. 16 after extraction of the thylakoids with 80% acetone in water. The P-700-content was determined assuming a differential extinction coefficient of 64 $mM^{-1} \cdot cm^{-1}$ for P-700-oxidation [17]. The cytochrome content was determined from chemical reduced minus oxidized difference spectra as outlined in Ref. 18, except that for cytochrome *f* we used isobestic points of 546 and 566 nm. Kinetic data, given as rate vs. substrate concentration, were analyzed on a personal computer using a non-linear least-square regression program.

DBMIB and NQNO were generously donated by Dr. A. Trebst, Ruhr Universität, Bochum, and by Dr. J. Whitmarsh, University of Illinois, Urbana.

Results

In order to obtain optically clear suspensions of thylakoids it proved to be necessary to wash the cells

once with a medium containing EDTA (see Methods). This had no influence on, for instance, the oxygen-evolving capacity of intact cells. However, sonication of the cells resulted in a complete loss of oxygen evolution (not shown).

In agreement with Oren et al. [8] we found little difference in the phycobilin/chlorophyll ratio between cell-free extracts from aerobically and anaerobically grown cells (not shown). However, we have shown [13] that growth on sulfide caused a red-shift in the chlorophyll absorption maxima, amounting to about 5 nm in the red region and to about 2.5 nm in the blue region. The wavelength dependence as well as the shape of light-induced difference spectra in the P-700-region around 700 nm were the same in thylakoids from aerobically and anaerobically gown cells (not shown). Chemically induced and light-induced difference spectra were similar in shape as well as in magnitude in this region (Fig. 1). The chlorophyll/P-700 molar ratios, determined from chemically induced difference spectra, were 254 ± 107 and 104 ± 36, for thylakoids from aerobically and anaerobically grown cells, respectively (mean $\pm$ standard deviation; $n = 5$). Thus on a chlorophyll basis the P-700 content was in thylakoids from anaerobically grown cells on the average about 2.5-times larger than in thylakoids from aerobically grown cells. The fluctuations which we observed in the chlorophyll/P-700 ratios may have been due to slight variations in the culture conditions.

Duroquinol-supported methyl viologen reduction was measured as oxygen uptake. To this end the 'basal

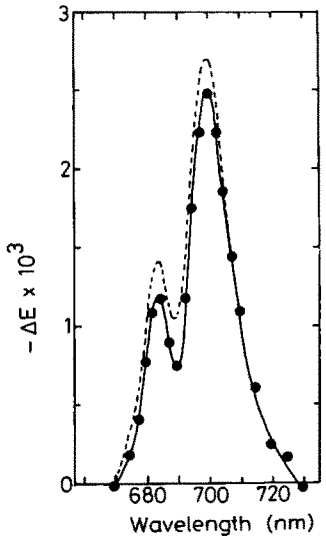

Fig. 1. Comparison of light-induced and chemically induced absorbance changes in thylakoids from aerobically grown cells. Conditions: basal medium supplemented with 20 mM glucose, 10 u/ml glucose oxidase and thylakoids corresponding with 11.1 μg/ml chlorophyll. ------, Chemically induced difference spectrum; 500 μM sulfide + 50 μM ferricyanide minus 50 μM ferricyanide; ●———●, light-induced difference spectrum, measured with 500 μM sulfide plus 0.1 mM methylviologen.

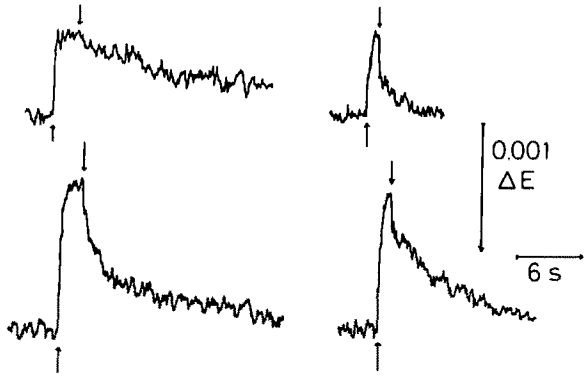

Fig. 2. Kinetics of light-induced absorbance changes at 435 nm in thylakoids from aerobically (left) and anaerobically grown cells (right), in the presence of 20 μM (top) or 200 μM sulfide (bottom). Other additions: 0.1 mM methyl viologen, 20 mM glucose, 20 u/ml glucose oxidase and thylakoids corresponding with 5.1 μg/ml chlorophyll. Actinic light was switched on and off at the times indicated by the upward and downward pointing arrows.

medium' (see Methods) was supplemented with 0.5 mM duroquinol, 0.5 mM KCN, 0.1 mM sodium azide, thylakoids corresponding with 13 μg per ml chlorophyll and an equivalent amount of soluble enzyme fraction. The samples were illuminated with red light ($\lambda > 665$ nm). The rates were 101.5 and 109 μmol O_2 per h per mg Chl with thylakoids from aerobically and anaerobically grown cells, respectively. DBMIB (5 μM) caused 89% inhibition of the rate of methyl viologen reduction in thylakoids from aerobically grown cells, and 72.5% inhibition in thylakoids from anaerobically grown cells. It has been shown that duroquinol donates electrons to the cytochrome b_6/f complex, and that DBMIB competes with duroquinol for a binding site on this complex [19]. Hence these data indicate that the cytochrome b_6/f complex is functionally active in thylakoids from both aerobically and anaerobically grown cells.

As mentioned in Introduction, only thylakoids from anaerobically grown cells possess a 'specific pathway' for electron transport from sulfide to NADP. This pathway involves the cytochrome b_6/f complex and exhibits a high sulfide affinity [13]. In order to make sure that this 'specific' pathway requires only membrane-bound enzymes, we measured the kinetics of light-induced cytochrome and P-700 absorbance changes in thylakoids without soluble enzyme fraction.

Fig. 2 shows kinetics of the photo-oxidation and dark re-reduction of P-700, measured at 435 nm [20]. Methyl viologen was present in these experiments in order to prevent re-reduction of P-700 by a back reaction. With 20 μM sulfide (top) the dark re-reduction of P-700 was slow and apparently monophasic in thylakoids from aerobically grown cells; however, in thylakoids from anaerobically grown cells a very rapid phase in the re-reduction was manifest, in addition to a slow phase. Raising the sulfide concentration to 200 μM (bottom) caused the appearance of a relatively rapid phase in the

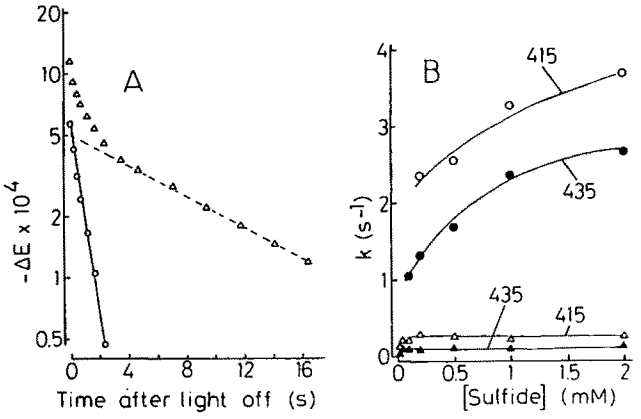

Fig. 3. (A) Analysis of the dark decay of the light-induced absorbance change at 435 nm (triangles) into a slow component (dashed line) and a fast component (circles). Results obtained with thylakoids from aerobically grown cells at 200 μM sulfide. Conditions were as in Fig. 2. (B) Sulfide-dependence of the rate constants for the fast (circles) and slow components (triangles) of the dark decay of the light-induced absorbance changes measured at 415 and 435 nm in thylakoids from aerobically grown cells. Conditions were as in Fig. 2.

reduction kinetics in thylakoids from aerobically grown cells; however, in thylakoids from anaerobically grown cells only the slow phase increased in extent. Similar measurements were carried out at 415 nm (not shown), where the absorbance changes were mainly due to cytochrome *c*-553 (Refs. 20 and 21; see also below). The dark decay of the absorbance changes was analyzed into two components (fast and slow); an example, obtained at 435 nm, is shown in Fig. 3A. In thylakoids from aerobically grown cells, the fast-decaying components exhibited rate constants varying from 1.4 to 2.7 s^{-1} (at 435 nm), or from 2.3 to 3.7 s^{-1} (at 415 nm), when the sulfide concentration was raised from 0.2 to 2 mM (Fig. 3B). By contrast, in thylakoids from anaerobically grown cells we found at both wavelengths instrument-limited values of 6–7 s^{-1} for the rate constants of the fast-decaying components, even at the lowest sulfide concentrations (cf. Fig. 2). For the slowly decaying components we found rate constants of 0.1–0.3 s^{-1} in thylakoids from aerobically grown cells (Fig. 3B), and similar values were obtained with thylakoids from anaerobically grown cells (cf. Fig. 2). Fig. 4A shows the amplitudes of the fast-decaying components of the absorbance changes measured at 415 nm (open symbols) and 435 nm (solid symbols). These amplitudes were saturated at about 20 μM sulfide in thylakoids from anaerobically grown cells (solid lines), and at 200–500 μM sulfide in thylakoids from aerobically grown cells (dashed lines). The amplitude of the slowly decaying components of these absorbance changes were saturated at about 20–50 μM sulfide in both types of preparations (Fig. 4B), with exception of the absorbance change at 435 nm in thylakoids from anaerobically grown cells (solid circles, solid line). Here the slow phase was saturated at about 500 μM sulfide; presumably this reflects the presence in these thylakoids of a population of P-700 which was not properly connected to cytochrome *c*-553, and was reduced directly by sulfide instead, especially at high concentrations of this compound (see also Discussion).

In conclusion, the rapid phases in the kinetics of the dark re-reduction of P-700 and cytochrome *c*-553, in thylakoids from aerobically and anaerobically grown cells (Fig. 4A), exhibited the same characteristic difference in sulfide affinity which we observed earlier in connection with NADP-reduction between thylakoids from aerobically and anaerobically grown cells [13]. In addition, the fast phases in the re-reduction of cytochrome *c*-553 and P-700 at sulfide concentrations of 0.2 mM and below, were much faster in thylakoids from anaerobically grown cells than in thylakoids from aerobically grown cells. This indicates that the sulfide oxidase activity associated with the 'specific' pathway of NADP-reduction (and of P-700 and cytochrome *c*-553 reduction), which is induced by growth of the cells on sulfide, is indeed membrane-bound.

This conclusion was supported by measurements of light-induced absorbance changes in the α-band region of the cytochromes. Fig. 5 shows some typical absorbance changes obtained with thylakoids from anaerobically grown cells, in the presence of 75 μM sulfide, and with or without DBMIB. These experiments were done without methyl viologen, because absorbance changes due to its reduction often interfered with the measurements in the α-band region. Fig. 6A shows the difference spectra obtained in these experiments, in the absence of DBMIB. During the first 0.15 s of illumina-

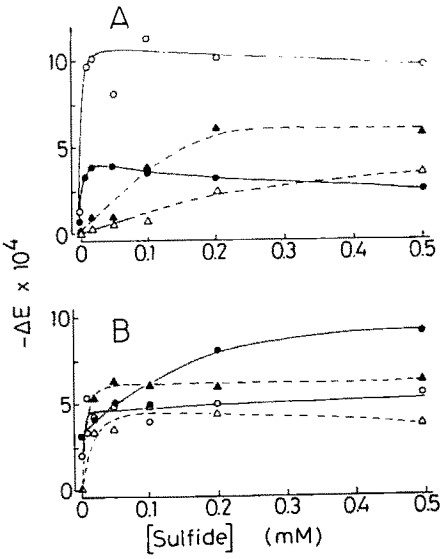

Fig. 4. Sulfide-dependence of the amplitude of the fast-decaying (A) and slowly decaying components (B) of the light-induced absorbance changes measured at 415 nm (open symbols) and 435 nm (solid symbols) in thylakoids from aerobically grown cells (△------△, ▲------▲) and from anaerobically grown cells (○——○, ●——●). Conditions were as in Fig. 2.

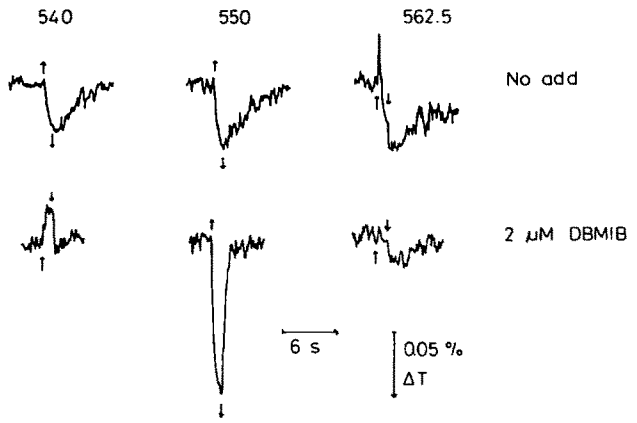

Fig. 5. Kinetics of light-induced absorbance changes in thylakoids from anaerobically grown cells, at the indicated wavelengths (nm). Upward and downward pointing arrows indicate the switching on and off of the actinic light. Additions: 75 μM sulfide, 20 mM glucose, 10 u/ml glucose oxidase and thylakoids corresponding with 18.3 μg/ml chlorophyll.

tion (curve 1) we observed oxidation of cytochrome *c*-553 (with a minimum at about 553 nm), and reduction of cytochrome b_6 (with a maximum at about 564 nm). After 1 s of illumination cytochrome b_6 had been re-oxidized, and oxidation of cytochrome *f* (with a minimum at 555 nm) became apparent (curve 2). At that time the actinic light was switched off, and 0.6 s later cytochrome *f* had been largely re-reduced, whereas some further oxidation of a *b*-type cytochrome (possibly *b*-559) had taken place (curve 3). These absorbance changes were superimposed on an apparently featureless and poorly reproducible absorbance change of unknown origin (possibly due to a scattering change).

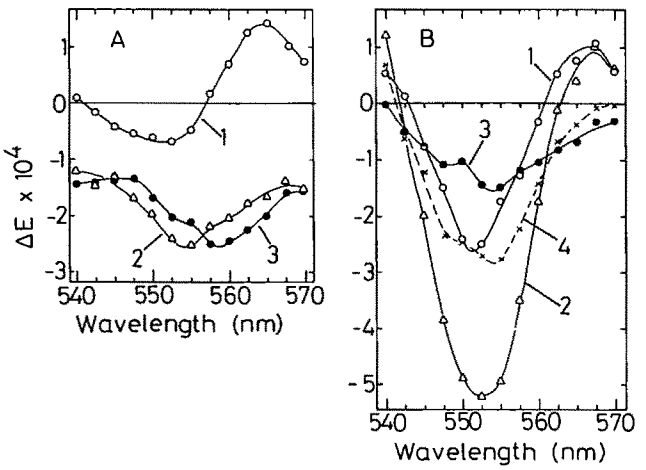

Fig. 6. Spectra of light-induced absorbance changes obtained with thylakoids from anaerobically grown cells in the absence (A) or presence of 2 μM DBMIB (B). Other additions as in Fig. 5. Actinic illumination time, 1 s. Dark time between illuminations, 1 min. Each point is the average of three measurements. The results shown in panel A were obtained with a single sample. A second sample was used for the results shown in panel B. Curve 1: 0.15 s after light on. Curve 2: 1 s after light on. Curve 3: 0.6 s after light off. Curve 4 (panel B): curve 2 minus curve 1.

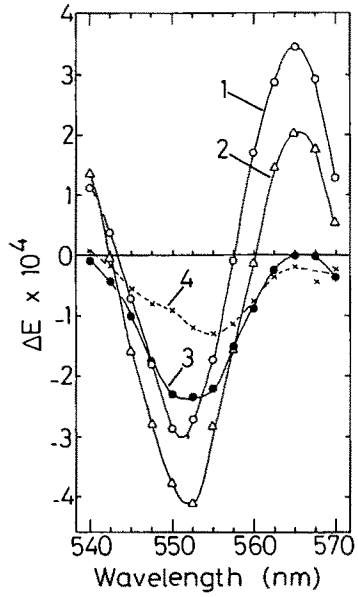

Fig. 7. Spectrum of light-induced absorbance changes obtained with thylakoids from anaerobically grown cells in the presence of 10 μM NQNO. Conditions as in Fig. 6. Curve 1: 0.15 s after light on. Curve 2: 1 s after light on. Curve 3: 0.6 s after light off. Curve 4: 1.5 s after light off.

In the presence of DBMIB, too, we observed only cytochrome *c*-553 oxidation during the first 0.15 s of illumination (Fig. 6B, curve 1). Continued illumination (up to 1 s) resulted in a considerable increase in the extent of the absorbance changes in this region (curve 2). This was due mainly to oxidation of cytochrome *f* (curve 4). After the switching off of the light, cytochrome *c*-553 was reduced first; at 0.6 s after light off, only cytochrome *f* was still oxidized (curve 3). In these experiments there was no evidence for participation of cytochrome b_6 in light-induced electron transport. The increase in the extent of photo-oxidation of cytochromes *c*-553 and *f* in the presence of DBMIB was apparently due to a decrease in the rate of re-reduction of these electron carriers.

NQNO caused an increase in the extent of cytochrome *c*-553-oxidation, as well as an increase in the extent of cytochrome b_6-reduction during the first 0.15 s of illumination (Fig. 7, curve 1). The latter effect was apparently due to a decrease in the rate of oxidation of cytochrome b_6 in the light (curve 2), although after the switching off of the light cytochrome b_6 was rapidly reoxidized (curve 3). Again, cytochrome *c*-553 was re-reduced more rapidly then cytochrome *f* after the switching off of the light (curves 3 and 4).

Fig. 8 shows a difference spectrum (obtained after 1 s of illumination) observed in thylakoids from aerobically grown cells. Similar spectra were observed at other times after switching light on or off (data not shown). In these experiments there was no evidence for participation of cytochrome b_6 in light-induced electron transport. The absorbance changes are probably due to

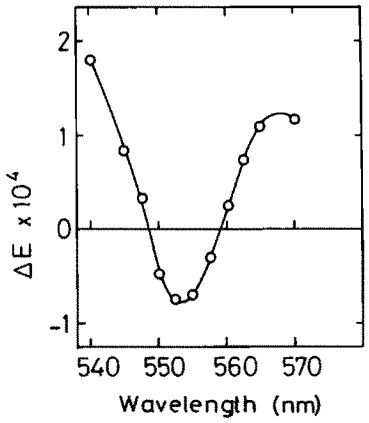

Fig. 8. Spectrum of light-induced absorbance changes obtained with thylakoids from aerobically grown cells. Conditions as in Fig. 6A, except that the sulfide concentration was 500 μM.

a mixture of cytochrome *c*-553 and *f*, since light-induced difference spectra in the Soret band region (at 5-nm intervals) showed a maximum at about 425 nm (not shown), which is characteristic of cytochrome *f* [20,21]. The sulfide concentration in these experiments was 0.5 mM, in agreement with the sulfide requirement of the rapid phases in the light-induced absorbance changes (Fig. 4A). However, this precluded the use of DBMIB as an inhibitor.

The left half of Table I shows a compilation of the amounts of the several cytochromes (relative to P-700) detected in light-induced difference spectra. This part of the table is restricted to conditions which were found to be optimal for observation of each particular cytochrome. Only small amounts of cytochrome were observed: for example, at most 0.067 mol per mol P-700 for cytochrome $_6$ in thylakoids from anaerobically grown cells, and approx. 0.10–0.12 mol per mol P-700 for cytochromes *c*-553 plus *f*. The latter cytochromes were observed in similar amounts in thylakoids from aerobically grown cells (in the absence of DBMIB) and in thylakoids from anaerobically grown cells (in the presence of DBMIB). This is reasonable, since sulfide-dependent electron transport in (thylakoids from) aerobically grown cells bypasses the DBMIB-sensitive site [9,13].

It was of interest to compare the light-induced difference spectra in the cytochrome region with chemically induced difference spectra. These are shown in Fig. 9. The main figure shows experiments performed with thylakoids from aerobically (left) and anaerobically grown cells (right). In order to ensure that the cytochromes were completely oxidized at the start of the experiments, the thylakoids of both the sample and reference cuvette were preilluminated for 10 s in the presence of 10 μM ferricyanide. Curve 1 is a baseline obtained with thylakoids pretreated this way. As expected, addition of 0.3 mM ferricyanide caused no further oxidation of cytochromes (data not shown). Addition of 25 μM sulfide (curve 2) caused in thylakoids from anaerobically grown cells reduction of mainly cytochrome *f* (with a maximum at 556 nm). However, in thylakoids from aerobically grown cells the maximum was at 557.5 nm, suggesting that a mixture of cytochrome *f* and *b*-559 was reduced by this treatment. Raising the concentration of sulfide to 500 μM caused reduction of cytochrome *b*-559 (curve 3) in both preparations. Finally, addition of 1 mM dithionite caused reduction of cytochrome b_6 (curve 4). The right half of Table I shows the amounts of the cytochromes detected in the thylakoids, relative to P-700. The numbers ranged mostly from 0.32 to 0.45 mol per mol P-700. The only exception was cytochrome *b*-559, which was less abundant in thylakoids from anaerobically grown cells than in thylakoids from aerobically grown cells.

It should be noted that these experiments revealed no evidence for reduction of cytochrome *c*-553, except perhaps in the shoulders (around 553 nm) in the dithionite-induced difference spectra (curves 4). One might argue that cytochrome *c*-553, being trapped inside the thylakoids, is not accessible for exogenous reductants. However, this is in contradiction with, for example, the sulfide dependence of the light-induced absorbance

TABLE I

Molar ratios of cytochromes and P-700 detected in thylakoids from aerobically (aer) and anaerobically (anaer) grown cells

For the cytochromes, differential extinction coefficients of 20 $mM^{-1} \cdot cm^{-1}$ were assumed. P-700 was determined from chemically induced difference spectra (cf. Fig. 1). n.d., not determined.

Cytochrome	Amount (mol per mol P-700) detected from			
	light-induced ΔE		chemically induced ΔE [9]	
	aer [1]	anaer [3]	aer	anaer
c-553	n.d.	0.069 [2,4]	n.d.	n.d.
f	n.d.	0.019 [5]	0.35 [10,11]	0.45 [10]
c-553 + *f*	0.100 [2]	0.124 [2,6]	n.d.	n.d.
b-559	0	0.019 [7]	0.12 [12]	0.32 [12]
b_6	< 0.015	0.067 [8]	0.34 [13]	0.25 [13]

[1] Data from Fig. 8.
[2] From the absorbance at 552.5 nm relative to a striaght line drawn through isosbest assumed at 542.5 and 562.5 nm.
[3] Data from Figs. 6 and 7.
[4] In the presence of 2 μM DBMIB at 0.15 s after light on.
[5] Determined as in Methods, in the presence of 2 μM DBMIB at 0.6 s after light off.
[6] In the presence of 2 μM DBMIB at 1 s after light on.
[7] Determined as in Ref. 18, at 0.6 s after light off (Fig. 6A).
[8] From the absorbance change at 565 nm in the presence of 10 μM NQNO at 0.15 s after light on, after subtraction of a contribution due to cytochromes *c*-553 and *f*, estimated at −0.2 times the absorbance change at 552.5 nm.
[9] Data from Fig. 9 (main figure); see Methods.
[10] 25 μM sulfide minus no additions.
[11] Possibly a mixture of cytochromes *f* and *b*-559.
[12] 0.5 mM sulfide minus 25 μM sulfide.
[13] 1 mM dithionite minus 0.5 mM sulfide.

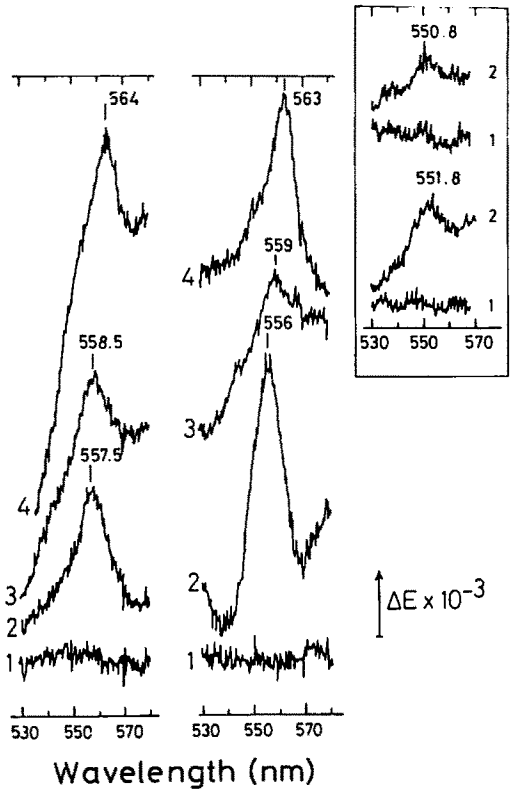

Fig. 9. Chemically induced difference spectra obtained with thylakoids (main figure) and equivalent amounts of soluble enzyme fractions (inset), in basal medium supplemented with 20 mM glucose and 10 u/ml glucose oxidase. The chlorophyll concentration was 24 μg/ml. Main figure: thylakoids from aerobically (left) and anaerobically grown cells (right). Curve 1: base line, obtained after preillumination of both the sample and reference cuvette in the presence of 10 μM ferricyanide. Curve 2 was obtained after addition of 25 μM sulfide to the sample cuvette. Curve 3, after addition of 25 μM sulfide to the reference cuvette and 500 μM sulfide (final concn.) to the sample cuvette. Curve 4, after addition of 500 μM (final concn.) sulfide to the reference cuvette and 1 mM dithionite to the sample cuvette. Inset: soluble enzyme fractions from anaerobically (top) and aerobically grown cells (bottom). Curve 1, base line; curve 2, difference spectrum 50 μM sulfide + 10 μM ferricyanide minus 10 μM ferricyanide.

changes at 415 nm, reflecting mainly cytochrome *c*-553 (Fig. 4). In addition, we have measured chemically induced difference spectra in the α-band region of the cytochromes in thylakoids in the presence of 0.1% Triton X-100. This solubilizes the membrane and should render any trapped cytochrome *c*-553 accessible for exogenous oxidants or reductants. However, neither oxidation with 0.3 mM ferricyanide nor reduction with 25 or 500 μM sulfide yielded any clear-cut evidence for the presence of cytochrome *c*-553 under those conditions; the results (not shown) were similar to those shown in Fig. 9. Yet cytochrome *c*-553 was clearly detected in light-induced difference spectra (figs. 6–8); however, the amounts were low compared with the amounts of cytochromes detected in chemically induced difference spectra (Table I). In conclusion, the amount of cytochrome *c*-553 present in the thylakoids was apparently too low (relative to the amounts of cytochrome *f*, *b*-559 and b_6) to allow its identification in chemically induced difference spectra with any certainty.

In order to check whether cytochrome *c*-553 had been released in the soluble enzyme fraction during sonication, we measured chemically induced difference spectra with this fraction as well. The results are shown in the inset of Fig. 9. The base line (curve 1) was obtained in the presence of 10 μM ferricyanide. Addition of 50 μM sulfide caused reduction of small amounts of *c*-type cytochromes with maxima at 550.8 and 551.8 nm in thylakoids from anaerobically and aerobically grown cells, respectively. Higher concentrations of sulfide or dithionite could not be added, since this caused bleaching of the phycobiliproteins present in the soluble enzyme fractions.

Discussion

In thylakoids from cyanobacteria [22,23] as well as from higher plants [24], the absorption maxima of chlorophyll associated with Photosystem II are at lower wavelengths than those of chlorophyll associated with Photosystem I. Hence the increase in the P-700-content on a chlorophyll basis, and the red-shift of the chlorophyll absorption maxima [13] indicate an enrichment of Photosystem I after growth of *O. limentica* on sulfide. The decrease in the cytochrome *b*-559/P-700-ratio after growth on sulfide points in the same direction: cytochrome *b*-559 is known to be associated with Photosystem II [25–27], although in thylakoids from anaerobically grown cells it also seems to be engaged in slow oxidation-reduction reactions during illumination in the presence of sulfide (Fig. 6A). This needs further clarification. As indicated in the Methods section, the chlorophyll concentration remained constant during the first 20 h after the transition to anaerobic growth. This lag may represent the balance of synthesis of Photosystem I–chlorophyll and breakdown of Photosystem II–chlorophyll, since sulfide utilisation started already at 2 h after the transition to anaerobic growth.

Our data seem to be at variance with those of Oren and Padan [7], who found that aerobic carbon fixation was unimpaired after a shift of the culture from anaerobic growth (with sulfide as electron donor) to aerobic growth (with water as electron donor). However, it may be pointed out that in those experiments a lag of about 15 min occurred before the onset of aerobic carbon fixation (Fig. 6 in Ref. 7).

We have shown that the amounts of cytochrome b_6, and of cytochromes *c*-553 and *f* detectable in light-induced difference spectra are much lower than the amounts of cytochromes b_6 and *f* detected in chemically induced difference spectra (Table I). In addition, the amount of cytochrome *c*-553 present within the

thylakoids was too low to allow its detection in chemically induced difference spectra (Fig. 9). Presumably, much of the cytochrome *c*-553 originally present within the thylakoids was lost during sonciation of the cells. At least some of the losses may be accounted for by the presence of *c*-type cytochromes in the soluble enzyme fraction (Fig. 9, inset). All this suggests that participation of cytochrome *f* (in thylakoids from aerobically grown cells) and of cytochromes b_6 and *f* (in thylakoids from anaerobically grown cells) was limited by the low amount of cytochrome *c*-553 present within the thylakoids. With this in mind it is possible to explain most of our data on light-induced electron transport, and to relate these data to results obtained on NADP-reduction.

We have shown that thylakoids from both aerobically and anaerobically grown cells possess a 'non-specific' pathway of electrons from sulfide to NADP. This pathway bypasses inhibitor-sensitive sites located on the cytochrome b_6/f complex; it is characterized by a low sulfide affinity (K_m on the order of millimolar), and a V_{max}/K_m ratio of 7–12 (data from Ref. 13). In thylakoids from aerobically grown cells, the rapid phase in the re-reduction kinetics of P-700 and cytochrome *c*-553 can be associated with this pathway. This appears from the sulfide-dependence of the amplitude (Fig. 4A, dashed lines) as well as of the rate constants (Fig. 3B, circles) of the rapid phases in the decay kinetics at 415 and 435 nm. Light-induced difference spectra in the α-band region (Fig. 8), which we attributed to a mixture of cytochromes *c*-553 and *f*, did not show absorbance changes attributable to cytochrome b_6. This, combined with our previous findings [13], indicates that in thylakoids from aerobically grown cells cytochrome b_6 is bypassed in electron transport from sulfide to P-700. The low sulfide affinity of this pathway may be taken as evidence that below 0.5 mM sulfide the rate of electron transport is limited by diffusion of sulfide through the membrane. By contrast, the slow phases in the re-reduction of P-700 and cytochrome *c*-553 in these thylakoids were saturated at much lower sulfide concentrations (Fig. 4B, dashed lines). This may indicate that in the slow phase electron donation from sulfide or cytochrome *f* to improperly positioned cytochrome *c*-553 was the rate-limiting step at saturating sulfide concentrations.

Only thylakoids from anaerobically grown cells possess a 'specific' pathway of electrons from sulfide to NADP. This pathway involves the cytochrome b_6/f complex; it is characterized by a high sulfide-affinity ($K_m \approx 24$ μM), and a V_{max}/K_m ratio of about 1200 (data from Ref. 13). In these thylakoids, the rapid phases in the kinetics of the dark re-reduction of P-700 and cytochrome *c*-553 ($k > 6$–7/s) can be attributed to this pathway. The high rate of electron transfer, combined with the high sulfide affinity, indicates that in this pathway sulfide is oxidized at a site which is readily accessible from the external medium. That this electron transfer proceeded via the cytochrome b_6/f complex is clear from the kinetics of the absorbance changes in the α-band region in the absence as well as in the presence of inhibitors. During illumination in the absence of inhibitors we observed reduction of cytochrome b_6 and oxidation of cytochrome *c*-553; this was followed by reduction of cytochrome *c*-553 and oxidation of cytochromes b_6 and *f*. DBMIB inhibited reduction of cytochrome b_6 during illumination, and caused an increase in the extent of photo-oxidation of cytochromes *c*-553 and *f*, apparently by slowing down the re-reduction of these compounds. That photo-oxidation of cytochrome *f* was still observed in the presence of DBMIB indicates either that the DBMIB-inhibition of electron transfer from sulfide through the b_6/f complex was not complete, or that a relatively slow electron transfer from sulfide to cytochrome *f* by-passed the DBMIB-block. The same problem remains with respect to the photo-oxidation of cytochrome *f* with presence of NQNO (Fig. 7).

In thylakoids from anaerobically grown cells, only the amplitude of the slow phase in the re-reduction of P-700 (Fig. 4B) exhibited the low sulfide affinity characteristic of the 'non-specific' pathway of electrons from sulfide to NADP [13]. This suggests that in these thylakoids direct electron transfer from sulfide to P-700 was the major route in the 'non-specific' pathway of electrons from sulfide to NADP. Again, diffusion of sulfide through the membrane (to P-700) may have been the rate-limiting step in this process at sulfide concentrations below 0.5 mM. In addition, a slow phase in the re-reduction of cytochrome *c*-553 was observed in these thylakoids; this phase was saturated at much lower sulfide concentrations (Fig. 4B), in line with the explanation given for the slow phases of electron transport in thylakoids from aerobically grown cells (above). However, our major concern in this work was with the 'specific' pathway of electron transfer, which is induced by growth of *O. limnetica* on sulfide. The absorbance changes of the cytochromes involved in this pathway, and the influence of inhibitors on these absorbance changes are in broad agreement with literature data. Thus, NQNO is thought to inhibit reoxidation of photo-reduced cytochrome b_6 [28,29], and to inhibit the re-reduction of photo-oxidized cytochrome *f* [28]. DBMIB blocks electron flow to cytochrome b_6 as well as to cytochrome *f* [19,28,30]. These finding are currently interpreted as indications for some sort of Q-cycle [31,32]. The general agreement of our results (Figs. 6–7) with the above-mentioned findings suggests that a Q-cycle is also operative during sulfide-dependent electron transport via the cytochrome b_6/f complex in *O. limnetica*.

A major conclusion from this work is that, since we

worked only with the thylakoids without soluble enzyme fraction, the highly active sulfide oxidase associated with the 'specific' electron transport pathway (involving the cytochrome b_6) is indeed membrane-bound. However, the structural basis for the difference in reactivity toward sulfide, between thylakoids from aerobically and anaerobically grown cells, remains unclear. Among the possible explanations we mention the sulfide-dependent induction of a spectroscopically silent enzyme acting as a sulfide : plastoquinone or sulfide : cytochrome b_6/f oxidoreductase; alternatively, the presence of sulfide may elicit in intact cells some structural or conformational change resulting in an increased accessibility of the cytochrome b_6/f complex for sulfide. It remains for the future to determine whether one of these hypotheses is correct.

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