CYANOBACTERIAL RESPIRATORY AND PHOTOSYNTHETIC ELECTRON TRANSFER CHAINS

Cytochrome Oxidase and Cytochrome $\underline{b_6}\underline{f}$ Complex

1989

YOSHIKO MINAMI

CONTENTS

ABBREVIATIONS

UQ_2; ubiquinone-2

UQH_2; ubiquinol-2

PS I; photosystem I

PS II; photosystem II

ISP; iron-sulfur protein

DBMIB; 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone
(dibromothymoquinone)

PMSF; phenylmethylsulfonyl fluoride

TMPD; N,N,N',N'-tetramethylphenylenediamine

MV; methyl viologen

DCMU; dichlorophenyldimethylurea

LDAO; lauryldimethylamine oxide

$C_{12}E_8$; octa-ethyleneglycol mono n-dodecyl ether

Part I: The Isolation and Characterization of a Cytochrome $\underline{b_6f}$ Complex from the Cyanobacterium *Spirulina* sp.

SUMMARY

A cytochrome $\underline{b}_6\underline{f}$ complex was isolated and purified from *Spirulina* sp. The complex was solubilized with *n*-heptyl β-D-thioglucoside and chromatographed on a DEAE-Toyopearl 650M column. The purified complex contained a small amount of chlorophyll and carotenoid. At least four polypeptides were present in the complex: cytochrome *f* (29 kDa), cytochrome $\underline{b}_6$ (23 kDa), iron-sulfur protein (ISP, 20 kDa), and a 17 kDa polypeptide. Each polypeptide was separated from the complex treated with 2-mercaptoethanol or urea. The absorption spectra of cytochrome $\underline{b}_6$ and cytochrome *f* were similar to those of *Anabaena* and spinach as expected. The complex was active in supporting ubiquinol-cytochrome *c* oxidoreductase activity. Fifty percent inhibition of the activity was accomplished by 1 μM dibromothymoquinone (DBMIB). The K_m values for ubiquinol-2 (UQH_2) and cytochrome *c* (horse heart) were 5.7 μM and 7.4 μM, respectively.

INTRODUCTION

A cytochrome b_6f complex, which corresponds to the bc_1 complex in mitochondria and bacteria, has been isolated from spinach (1,2) Anabaena variabilis (3) and Chlamydomonas reinhardtii (4). These complexes participate in electron transfer and proton translocation in photosynthesis and/or respiration (5,6). The b_6f complex transfers electrons between the two photosystems (from plastoquinol to cytochrome c_{553} or plastocyanin), and in cyclic electron flow around photosystem I (7-10).

The b_6f complex contains four major polypeptides: cytochrome f with one heme c, cytochrome b_6 with two protohemes, iron-sulfur protein with one high potential iron-sulfur center, and a 17 kDa polypeptide. This composition is similar to that of bacterial bc_1 complex and much simpler than that of mitochondrial bc_1 complex (5,11). To date, the bc_1 complex in mitochondria and bacteria has been well characterized, but further studies on the b_6f complex are necessary. Characterization of the b_6f complex and comparison between the b_6f and bc_1 complexes should bring considerable information about the mechanism of electron transfer and proton pumping. Furthermore, cyanobacterial b_6f complex was reported to link the respiratory chain to the photosynthetic electron transfer chain (12-16). The cyanobacterial b_6f complex should be involved in electron transfer from NAD(P)H dehydrogenase to cytochrome oxidase, neither of which has been isolated from cyanobacteria yet.

In this paper I report the isolation and characterization of $\underline{b_6f}$ complex from cyanobacterium Spirulina sp. as the first step in exploring the electron transfer mechanism of cyanobacterial photosynthesis in conjunction with respiration. The present experiment supports the electron flow from $\underline{b_6f}$ complex to the membrane-bound cytochrome oxidase as discussed later and more detailed studies are described in Part II.

MATERIALS AND METHODS

Organisms

Cells of the cyanobacterium Spirulina sp. were supplied by Dr. K. Kodo and stored in a frozen state at -20 °C.

Preparation

All subsequent steps of the preparation were performed at 0-4 °C. Cells were suspended in 15 mM Tricine-Tris buffer (pH 8.1) containing 0.2 M mannitol, 1 mM NaH_2PO_4 and 2 mM $MgCl_2$. The suspension was sonicated and centrifuged at 3,000 x g for 10 minutes, and the supernatant was further centrifuged at 100,000 x g for 1 hour. The resulting precipitate was washed with 2 M NaBr following the method of Hurt and Hauska(1). This precipitate was resuspended in the first buffer and recentrifuged. The membrane pellet was then suspended in 20 mM Tricine-Tris buffer (pH 8.1) containing 0.4 M ammonium sulfate, 0.2 M sucrose, 1.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 % (w/v) Na-cholate and 40 mM heptyl thioglucoside (Dojin, Kumamoto, Japan). After 30 minutes the suspension was centrifuged at 300,000 x g for 1 hour. To the supernatant was added a saturated solution of ammonium sulfate (pH 7.5) to give 35 % saturation, and the mixture was then stirred for 20 minutes. The pellet obtained after centrifugation at 27,000 x g for 10 minutes was discarded. The concentration of ammonium sulfate in the supernatant was then adjusted to 65 % saturation. After centrifugation the pellet was dissolved in a minimal volume of 20 mM Tris-HCl buffer (pH 8.1) containing 0.2 %(v/v) Triton X-100 and dialyzed against the same solution for 3 hours. The

dialysate was loaded onto a DEAE-Toyopearl 650M column (2.1 x 24.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 8.1) containing 0.1 M NaCl and 0.2 % Triton X-100. The column was washed with 20 mM Tris-HCl buffer containing 0.2 M NaCl and 1 % Na-cholate and developed with a linear gradient of NaCl from 0.2 M to 0.3 M in the same buffer. The fractions containing the $\underline{b_6f}$ complex were dialyzed against 5 mM K-phosphate buffer (pH 6.0) for 2 hours and loaded onto a hydroxylapatite column (2 x 10 cm) equilibrated with 5 mM K-phosphate buffer (pH 6.4), which was then developed with 200 mM K-phosphate buffer containing 0.05 % Triton X-100.

Separation of components

Cytochrome $\underline{f}$, cytochrome $\underline{b_6}$, and ISP were purified by a modification of the method of Hurt and Hauska (17). Two strategies were used. In the first place, 2-mercaptoethanol was added to make 15 % (v/v) to the $\underline{b_6f}$ complex (equivalent to 27 nmol cytochrome $\underline{f}$). After 1 hour, the solution was applied to a DEAE-Toyopearl 650M column (1.1 x 11 cm) equilibrated with 0.1 % 2-mercaptoethanol, 1 % Na-cholate, and 10 mM K-phosphate buffer (pH 7.4). The column was successively developed in a step-wise manner with 0, 0.2, and 0.5 M NaCl in the same buffer. Secondly, the $\underline{b_6f}$ complex (50 nmol cytochrome $\underline{f}$) was treated with 5 M urea for 30 minutes and loaded onto a hydroxylapatite column (1.3 x 5.5 cm) equilibrated with 5 mM K-phosphate buffer (pH 6.4) containing 0.5 % Triton X-100. This column was developed with the same buffer.

Assays

The concentration of protein was determined by a Lowry assay

(18) and chlorophyll *a* was measured according to the method of Mackinney (19). Electrophoresis was performed using Laemmli's system (20), by staining of the protein with Coomassie Brilliant Blue and heme staining according to the method of Thomas et al. (21).

The ISP antibody from spinach was a gift from Dr. R. Malkin (University of California, Berkeley) and Western blotting was performed using ^{125}I-protein A according to the methods of Haid and Suissa (22) and Johnson et al. (23).

DBMIB and cytochrome *c* (Type II-S) were purchased from Sigma Chemicals (St Louis, Mo., USA). UQ_2 was a gift from Eisai Co. Ltd., Tokyo. Its solutions were reduced according to the reference (24) and the concentration was estimated in ethanolic solution using an $\varepsilon_{290\ nm}$ of 4.14 $mM^{-1}{\cdot}cm^{-1}$. Oxidoreductase activity was monitored in a Hitachi 220A spectrophotometer at 550 nm for cytochrome *c* reduction ($\varepsilon_{550\ nm}$= 17.6 $mM^{-1}{\cdot}cm^{-1}$). The assay mixture was in 1 ml of 50 mM Na-phosphate buffer and 0.5 mM EDTA (pH 7.5) containing about 100 nM b_6f complex and an appropriate amount of oxidized cytochrome *c*. The reaction was started by the addition of UQH_2 to the mixture. The UQH_2 was suspended in ethanol, the concentration of which in the assay mixture was less than 0.5 %. Reaction rates were expressed as µmol cytochrome *c* reduced per nmol cytochrome *f* per hour.

RESULTS

Preparation and characterization of the complex

The extraction of the b_6f complex from thylakoid membranes was performed according to the method of Hurt and Hauska (1) except for using heptyl thioglucoside. The profiles for extraction of protein, chlorophyll, and b_6f complex from the membranes are shown in Fig. 1. The concentration of cytochrome b_6 was assumed to be parallel to that of the b_6f complex. Cytochrome b_6 was effectively solubilized with 40 mM heptyl thioglucoside, and higher concentrations did not give additional solubilization of the complex. Consequently, 40 mM heptyl thioglucoside was used in the following experiments. Fig. 2 shows the elution pattern of cytochrome b_6 from the DEAE-Toyopearl column chromatography. The column was developed with a linear gradient of 0.2-0.3 M NaCl. Orange-colored solution (possibly carotenoid protein) was first eluted in fractions from No.1 to No.12 in fig. 2, followed by yellow fractions of b_6f complex (fraction numbers from 34 to 42) eluted at about 0.25 M NaCl. The final fractions (fraction numbers from 46 to 56) contained chlorophyll. The fractions containing the b_6f complex were concentrated on a hydroxylapatite column. Table I summarizes the course of the purification. About 25 nmol of the b_6f complex was obtained from about 50 g of wet cells. Fig. 3(a) shows the absorption spectra and oxidized-reduced difference spectra for the b_6f complex. The peak at 670 nm in the spectrum was due to contaminated chlorophyll. The α peaks of cytochromes f

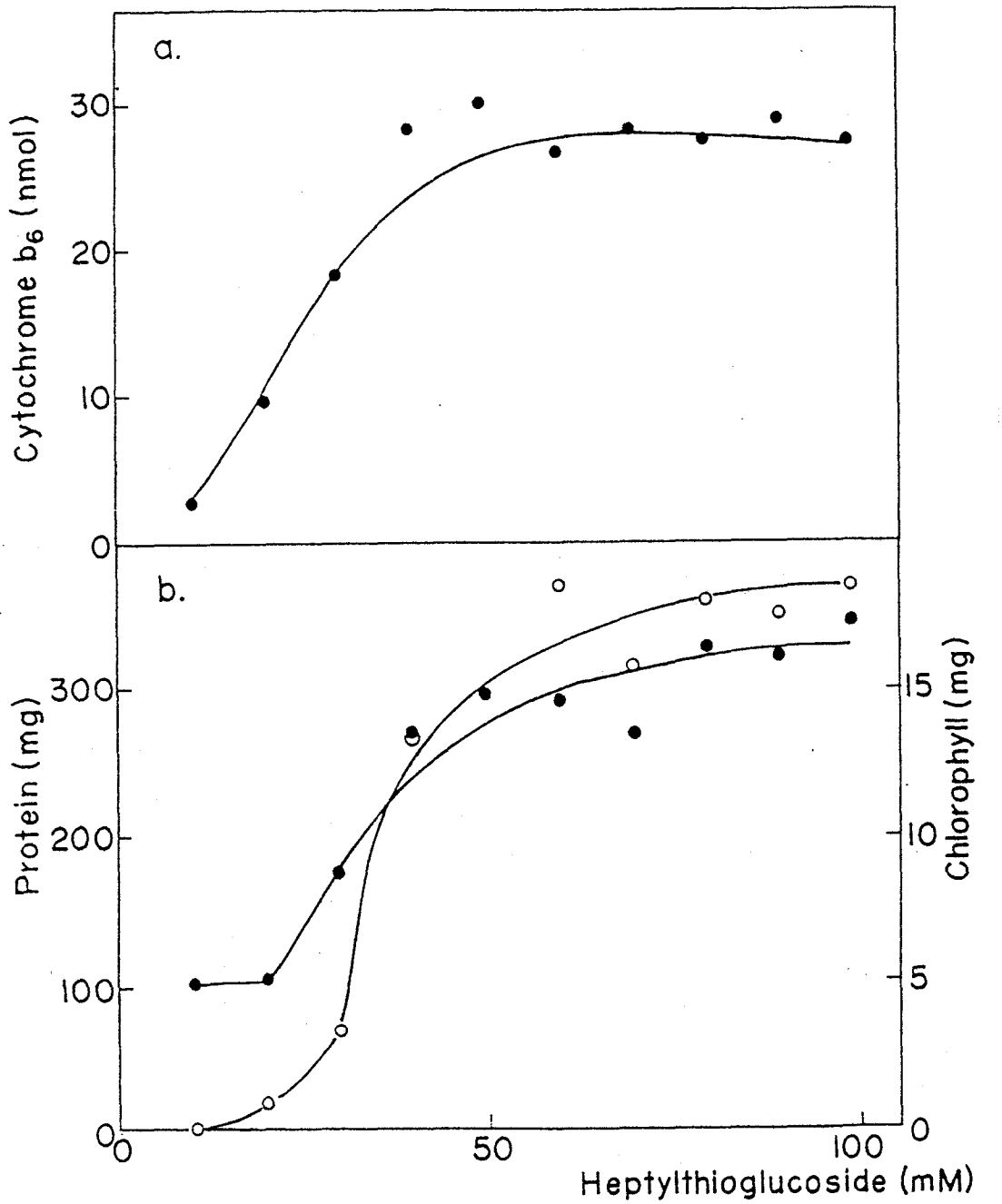

Fig. 1: Solubilization of the cytochrome $\underline{b}_6\underline{f}$ complex with heptyl thioglucoside. The membrane was suspended to be 1mg chlorophyll $\underline{a} \cdot ml^{-1}$ in Tricine-Tris buffer. (a) The complex measured as the cytochrome $\underline{b}_6$ content and (b) protein (-●-) and chlorophyll $\underline{a}$ (-○-) content of the extracts.

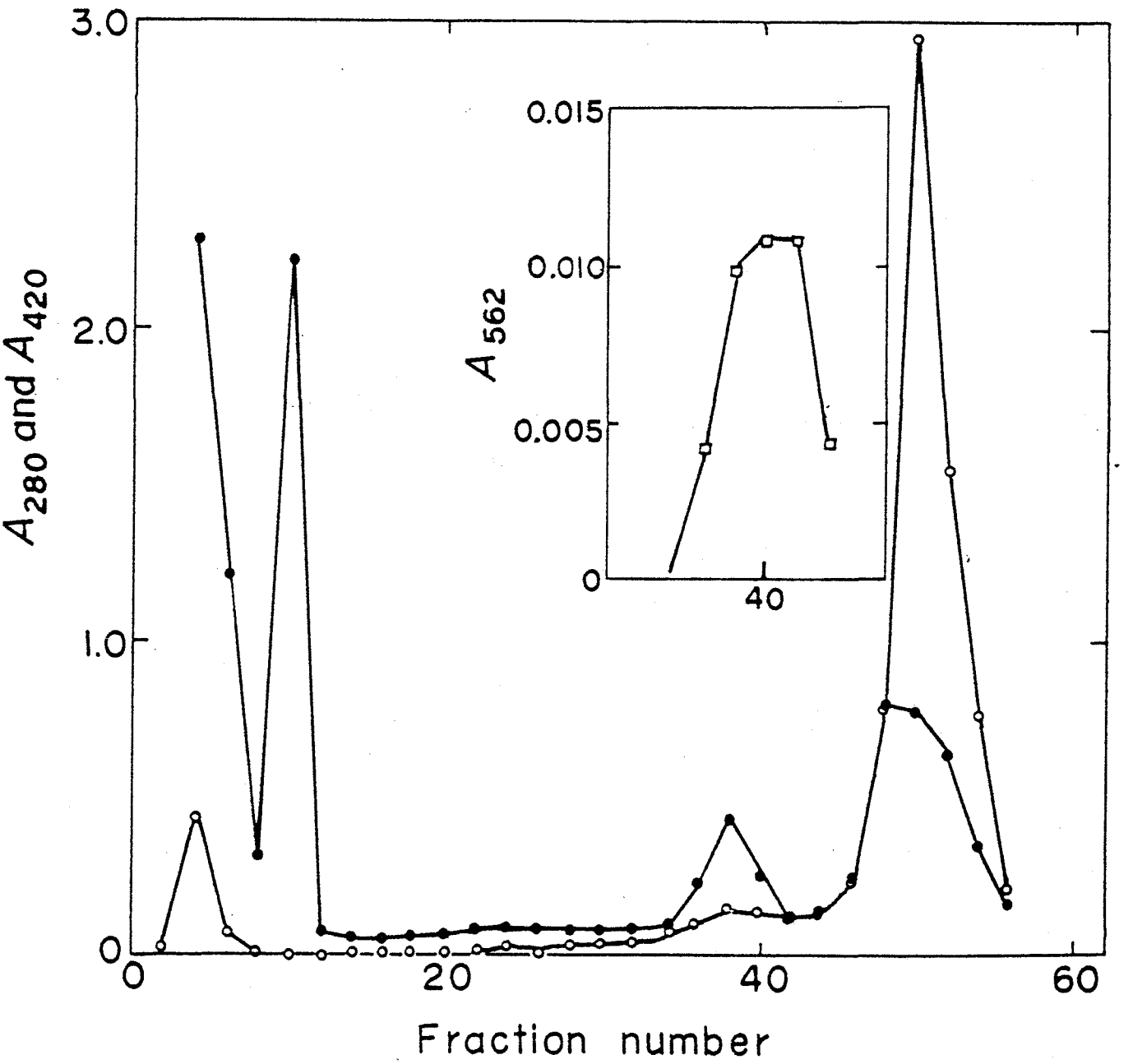

Fig. 2: Elution profile for the purification of the cytochrome $\underline{b}_6\underline{f}$ complex on a DEAE-Toyopearl column. Proteins are monitored at 280 nm (—●—), cytochromes at 420 nm (—○—), and cytochrome $\underline{b}_6$ at 562 nm (inset) after reduction (—□—), respectively.

Table I Summary of purification of the cytochrome b_6f complex

Purification step	Cyt f (nmol)	Chl a/Cyt f ($mol \cdot mol^{-1}$)	Cyt f/Protein ($nmol \cdot mg^{-1}$)	Activity[a] ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	Yield[b] (%)
Extraction	54.1	259.0	0.05	1.3	100
Solubilization	47.1	65.8	0.17	8.2	87.2
Ammonium sulfate fractionation	34.1	35.6	0.82	31.5	84.6
DEAE column chromatography	25.2	0.7	12.2	87.2	46.7

[a] µmol cytochrome c reduced·mg^{-1} protein·$hour^{-1}$

[b] Recovery of cytochrome f

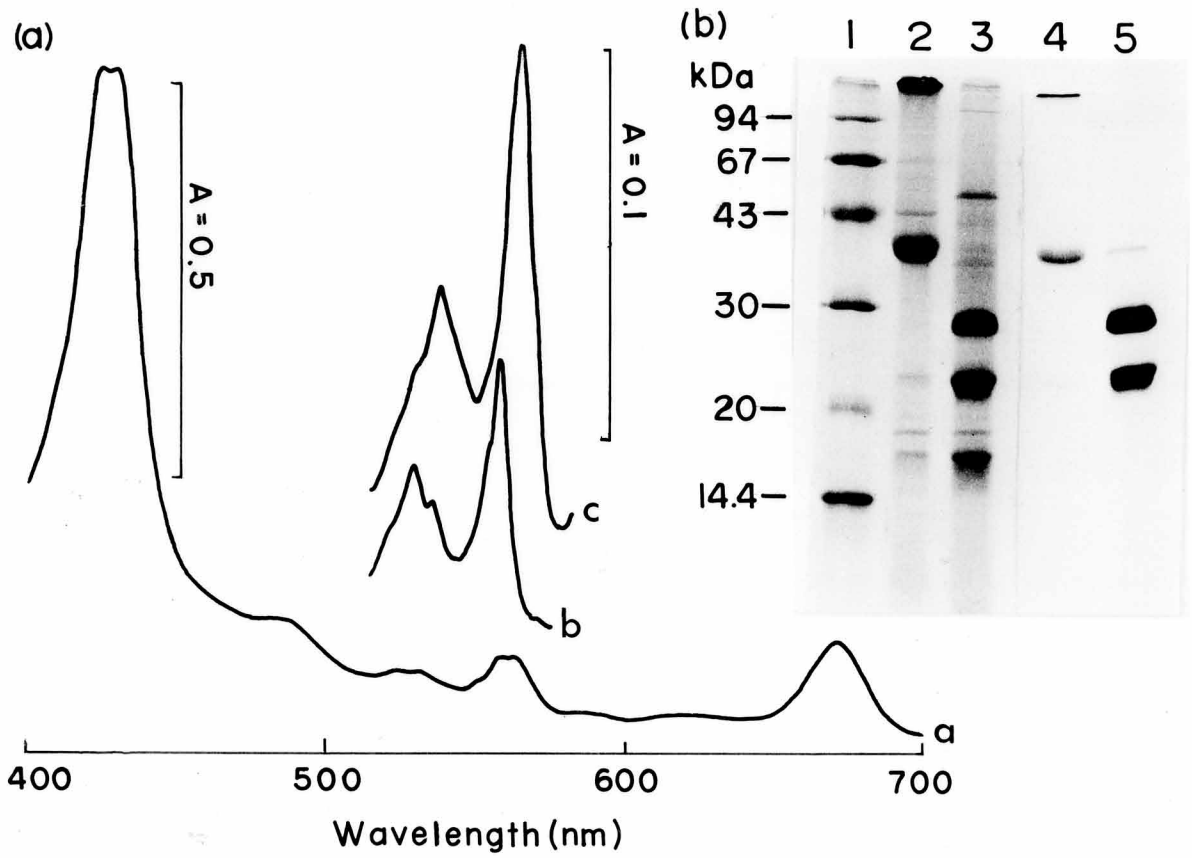

Fig. 3: Characterization of the isolated $\underline{b}_6\underline{f}$ complex. (a) Absorption spectra: a, dithionite reduced; b, ascorbated-reduced minus ferricyanide-oxidized difference spectrum; c, dithionite-reduced minus ascorbate-reduced difference spectrum (b) SDS-polyacrylamide gel electrophoretic pattern. Lanes 1-3 are stained with Coomassie Brilliant Blue and lane 4 and 5 are by heme-staining. Lane 1 shows standard proteins of 94, 67, 43, 30, 20, and 14.4 kDa. Lanes 2 and 4 show heated complex at 100 °C for 5 min before application to the gel and lanes 3 and 5 are the complexe without heating.

and b_6 were at 554 and 562 nm, respectively, and the γ peaks were at 420-430 nm. SDS-polyacrylamide gel electrophoresis of the b_6f complex is shown in Fig. 3(b). The b_6f complex contained three major bands of 29, 23, and 17 kDa and two minor bands of about 20 kDa. Two of these (29 and 23 kDa) were also detected by heme staining (Fig. 3b, lane 5). The 29 kDa polypeptide was shifted to about the 40 kDa position by heat treatment, as reported by Krinner et al. (3) and Guikema and Sherman (25). Based on molecular weight, this band was identified as cytochrome f and the 23 kDa polypeptide as cytochrome b_6. The band corresponding to ISP was at two minor bands of about 23 kDa. This will be discussed later.

Separation and characterization of each component

The b_6f complex treated with 15 % 2-mercaptoethanol was applied to a DEAE-Toyopearl column equilibrated with 0.1 % 2-mercaptoethanol, 1 % Na-cholate and 10 mM K-phosphate buffer (pH 7.4). The column was washed with the same buffer to elute ISP, and the pink cytochrome f was eluted with the same buffer containing 0.2 M NaCl. Finally, the yellow band, comprised of cytochrome b_6 and 17 kDa polypeptide, which remained on the top of the column was eluted at 0.5 M NaCl. The spectra of the isolated cytochrome f and ISP are shown in Fig. 4(a). The spectrum of ISP had a broad maximum at around 415 nm, but by the addition of ascorbate this absorbance increased and a small peak at about 550 nm appeared. Therefore, this was probably due to the cytochrome contaminated as discussed later. The spectrum of cytochrome f was measured in the presence of 0.1 %(v/v) 2-mercaptoethanol and was unchanged by the addition of dithionite.

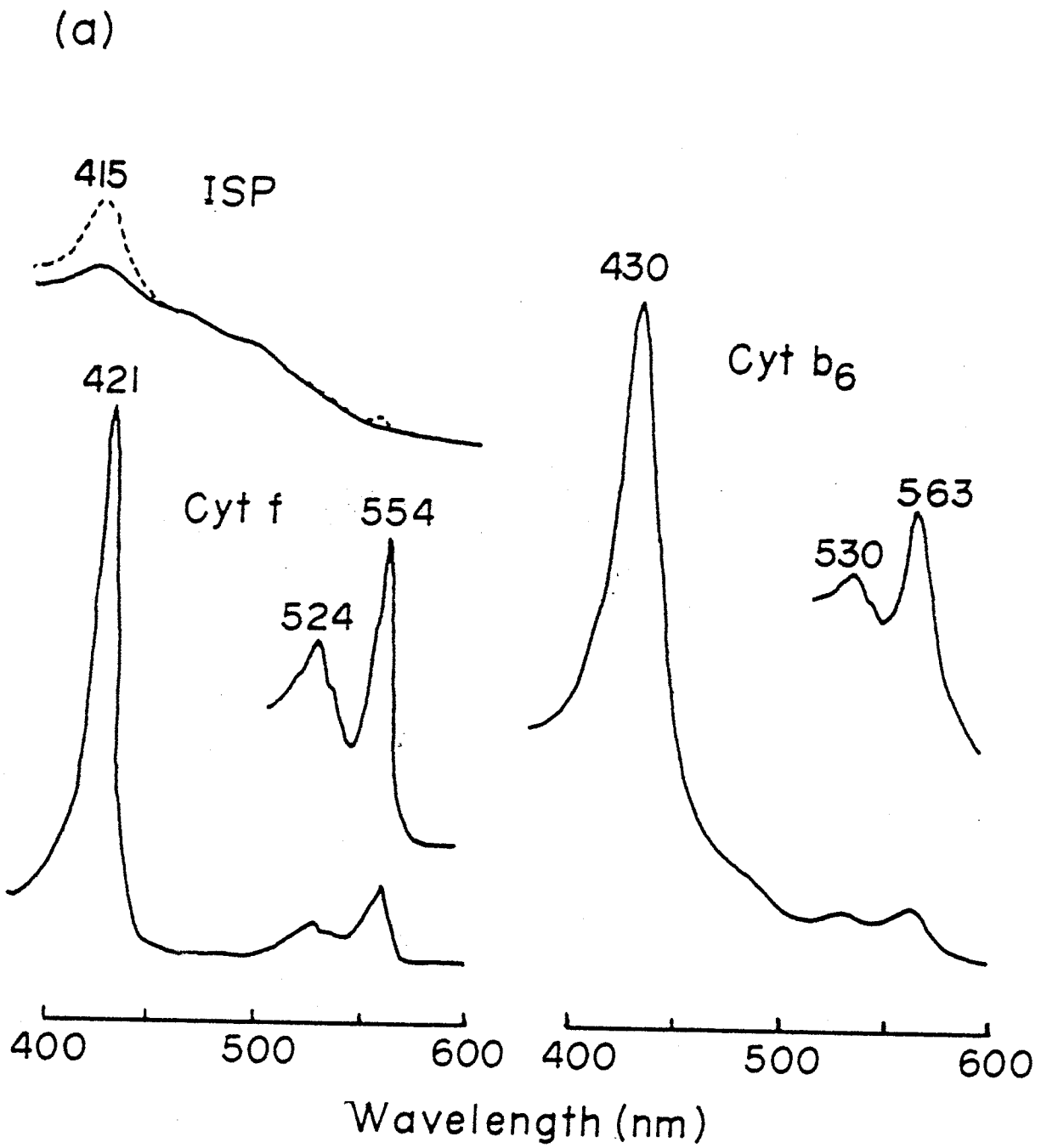

Fig. 4: Characterization of the separated components of the $\underline{b_6f}$ complex. (a) Absorption spectra of the separated components of the cytochrome $\underline{b_6f}$ complex. ISP, the broken line, is shown in the presence of ascorbate and cytochrome $\underline{b_6}$ in the presence of dithionite.

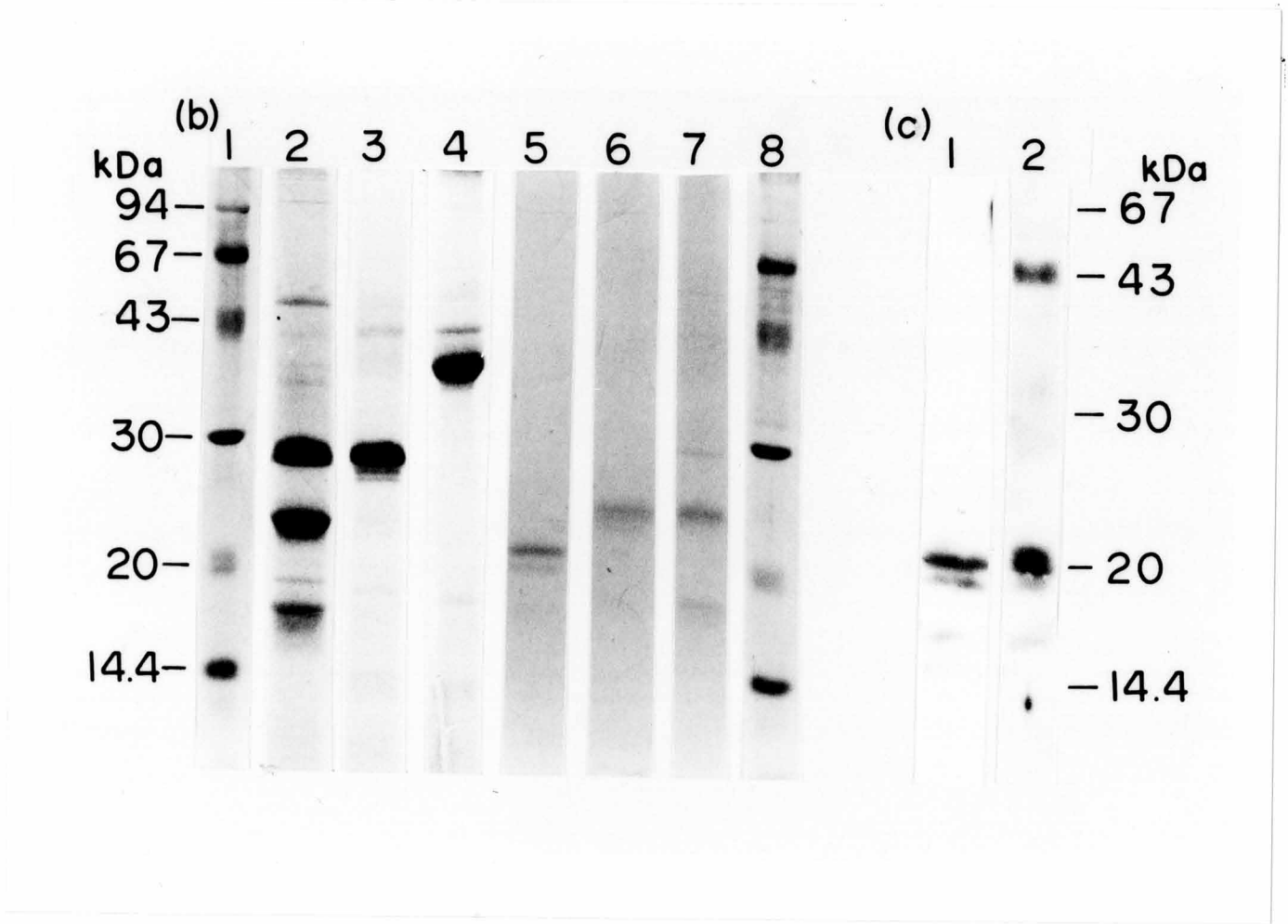

Fig. 4 (b) SDS-polyacrylamide gel electrophoretic patterns. Lanes 1 and 8, molecular weight markers; lane 2, the $\underline{b}_6\underline{f}$ complex; lane 3, cytochrome $\underline{f}$; lane 4, cytochrome $\underline{f}$ heated at 100 °C for 5 min; lane 5, ISP ; lane 6, cytochrome $\underline{b}_6$; lane 7, cytochrome $\underline{b}_6$ and 17 kDa polypeptide released by 2-mercaptoethanol treatment. (c) The pattern for ISP (lane 1) and $\underline{b}_6\underline{f}$ complex (lane 2) by immunoblotting using anti-ISP.

Cytochrome f had its α, β, and γ peaks at 554, 524, and 421 nm, respectively. The cytochrome b_6 polypeptide isolated in the presence of 2-mercaptoethanol was not reduced by dithionite and was not stained by heme staining on SDS-polyacrylamide gel electrophoresis. The heme of the isolated cytochrome b_6 had probably been lost on chromatography after 2-mercaptoethanol treatment. The b_6f complex was also treated with 5 M urea and adsorbed on a hydroxylapatite column equilibrated with 5 mM K-phosphate buffer (pH 6.4) containing 0.5 % Triton X-100. Some of the cytochrome b_6 was not adsorbed and passed directly through the column. The spectrum of cytochrome b_6 thus obtained and reduced with dithionite is also shown in Fig. 4(a). The α, β, and γ peaks of cytochrome b_6 were at 563, 530, and 430 nm, respectively. Fig. 4(b) shows the SDS-polyacrylamide gel electrophoretic pattern for each fraction as described above. A native b_6f complex was applied to lane 2. The isolated cytochrome f had a molecular mass of 29 kDa (lane 3) and the mass was shifted to about 40 kDa by heat treatment (lane 4). The isolated cytochrome b_6 gave a band of 23 kDa (lane 6). Lane 7 shows the cytochrome b_6 and 17 kDa polypeptide fractions from the column chromatography on DEAE-Toyopearl. The ISP isolated in the presence of 2-mercaptoethanol had two bands of about 20 kDa (Fig. 4 (b), lane 5 and Fig. 4 (c), lane 1). The presence of ISP was confirmed by Western blotting. The spinach ISP antibody reacted with the isolated ISP (Fig. 4 (C), lane 1) and also with two corresponding bands of about 20 kDa of the b_6f complex, together with a band at about 43 kDa (Fig. 4 (c), lane 2).

Oxidoreductase activity

The oxidoreductase activity from UQH_2 to cytochrome *c* was measured by a spectrophotometric method. In all assays, the background activity was determined by the addition of UQH_2 to the reaction mixture without the b_6f complex. Fig. 5 shows the kinetic data obtained with 15 µM UQH_2 in the reaction mixture. The K_m value for cytochrome *c* was 5.7 µM and V_{max} was 7.4 µmol cytochrome *c* reduced·nmol^{-1} cytochrome *f*·hour^{-1}. With 20 µM cytochrome *c* the K_m for UQH_2 was 7.4 µM and V_{max} was 10.0 µmol cytochrome *c* reduced·nmol^{-1} cytochrome *f*·hour^{-1}. The effect of inhibition by DBMIB, an inhibitor acting at the ISP site, is shown in Fig. 6. About 1 µM of DBMIB was required to inhibit half the activity. Thus, low concentrations of DBMIB effectively inhibited the oxidoreductase activity.

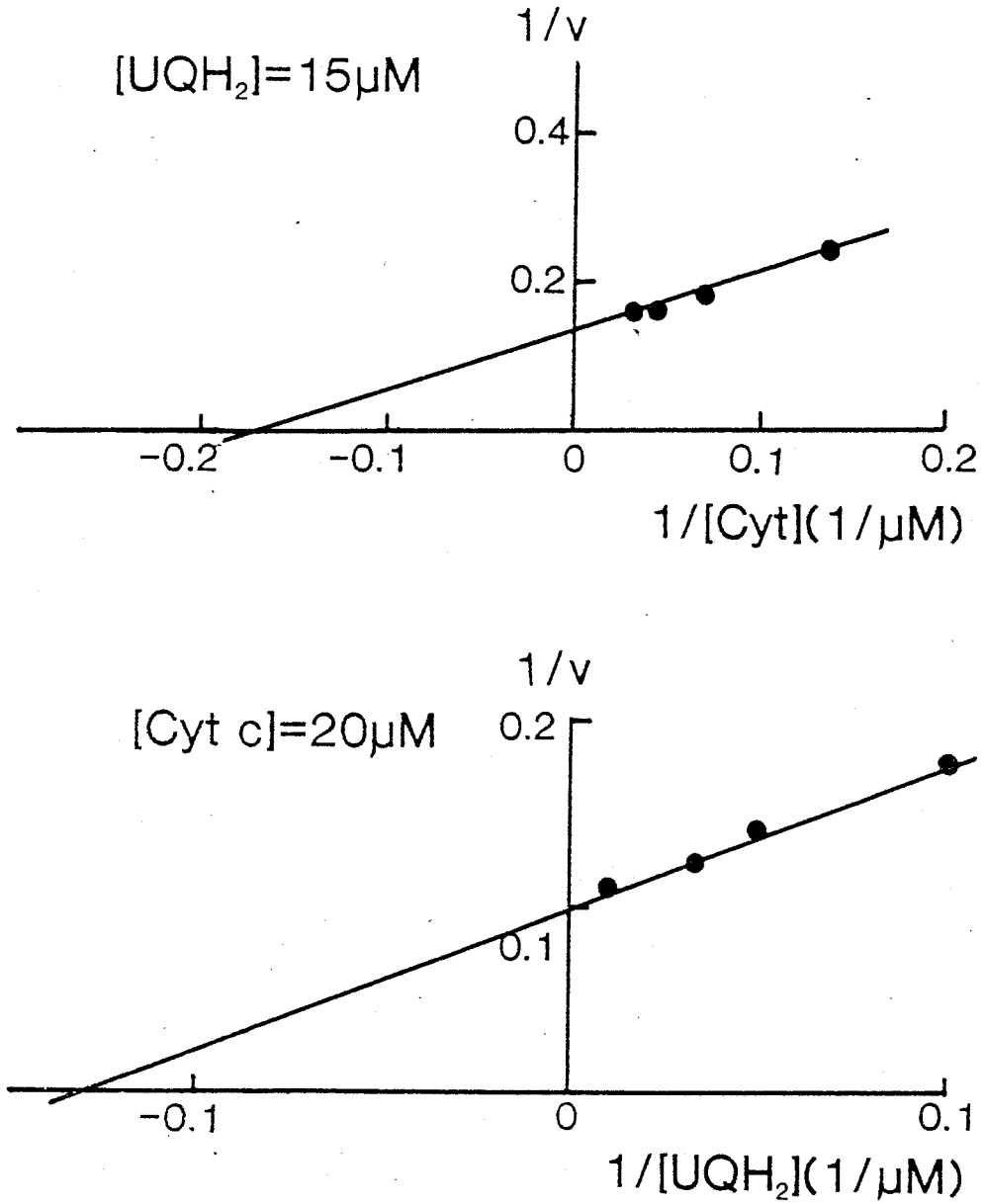

Fig. 5: Kinetic data for the cytochrome $\underline{b}_6\underline{f}$ complex. Experimental details are given in the text. v = μmol cytochrome $\underline{c}$ reduced·nmol^{-1} cytochrome $\underline{f}$·hour^{-1}

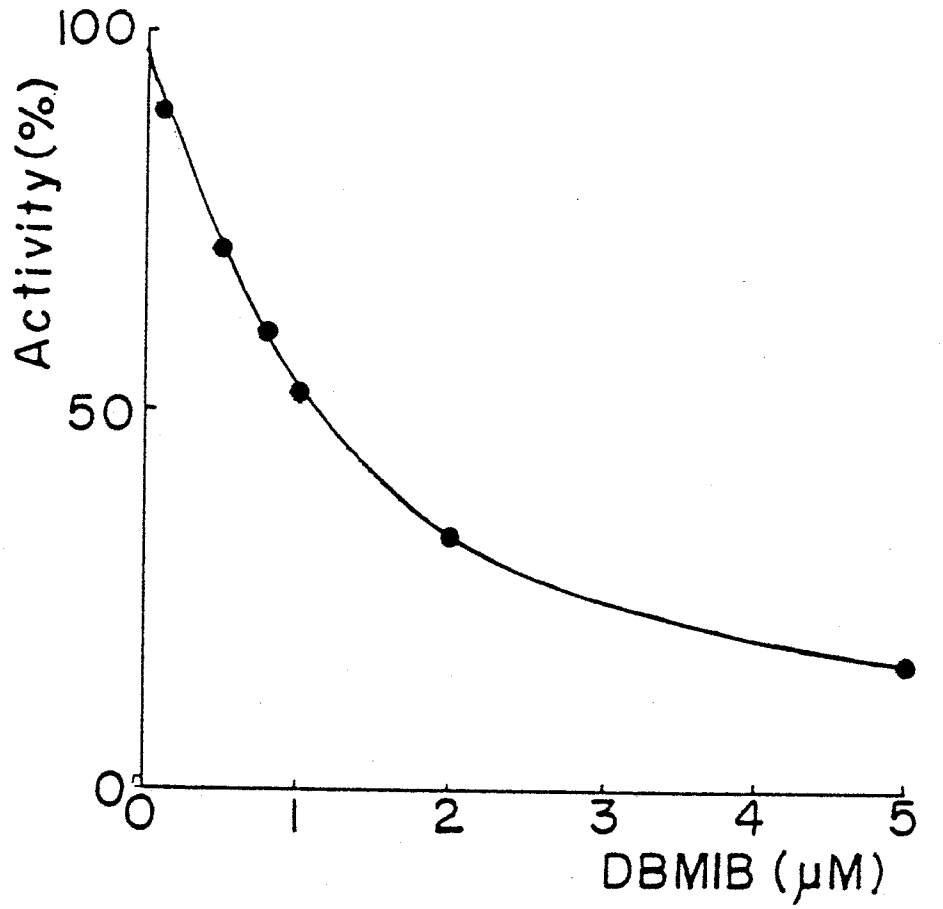

Fig. 6: Inhibition of the oxidoreductase activity of the cytochrome $\underline{b}_6\underline{f}$ complex by DBMIB. DBMIB is suspended to be a suitable concentration in ethanol. Experimental details are given in the text.

DISCUSSION

In the present work heptyl thioglucoside was effectively used for the solubilization of the thylakoid membrane due to its low cost and stability compared to octylglucoside (26,27). I succeeded in solubilizing the $\underline{b_6f}$ complex with 40 mM heptyl thioglucoside, and a successive DEAE-Toyopearl column chromatography was used for the purification of the $\underline{b_6f}$ complex. By this method, the $\underline{b_6f}$ complex was obtained in high recovery and this process provided considerable advantage as compared with the conventional purification method by sucrose density gradient (1,2).

The $\underline{b_6f}$ complex consisted of protoheme, heme $\underline{c}$, non-heme irons and sulfurs and four polypeptides. The 29 kDa polypeptide was found to be cytochrome $\underline{f}$ from its absorption characteristics, its N-terminal sequence consistent with the report by Ho and Krogmann (28)(data not shown). It was also observed that the 29 kDa polypeptide shifted on electrophoresis to about 40 kDa by heat treatment, as reported by Krinner et al.(3). The apparent molecular mass (29 kDa) of Spirulina cytochrome $\underline{f}$ on SDS gel was slightly lower than that from Anabaena (31 kDa)(3). It should be noted that Ho and Krogmann (28) reported that the molecular mass of cytochrome $\underline{f}$ from Spirulina maxima was 38 kDa, which interestingly corresponds to the molecular mass of the heated cytochrome $\underline{f}$. Heme staining of the $\underline{b_6f}$ complex showed only two bands of 29 and 23 kDa, the latter of which was cytochrome $\underline{b_6}$. Therefore, it is not plausible that cytochrome $\underline{f}$ in this sample

was digested by proteases.

Use of 2-mercaptoethanol allowed separation of three fractions from the $\underline{b}_6\underline{f}$ complex. Cytochrome $\underline{f}$ and ISP were isolated by DEAE-Toyopearl column chromatography, but this treatment denatured cytochrome $\underline{b}_6$, as shown by the loss of the characteristic spectrum and a lack of heme staining. However, cytochrome $\underline{b}_6$ could be isolated using a hydroxylapatite column after urea treatment. The spectra of isolated cytochrome $\underline{f}$ and $\underline{b}_6$ agreed with the oxidation-reduction difference spectral data obtained for the $\underline{b}_6\underline{f}$ complex. These isolated polypeptides consistently migrated to the same molecular mass on SDS-polyacrylamide gel electrophoresis as they did in the $\underline{b}_6\underline{f}$ complex. The isolated ISP gave two bands of 20 and 19 kDa as found in the $\underline{b}_6\underline{f}$ complex. It is presently unknown why ISP has two bands in the $\underline{b}_6\underline{f}$ complex. Since the digestion by protease could not be ruled out, some protease inhibitors, such as PMSF and EDTA, were added during the preparation, but they were not effective. I have no idea at present about the phenomenon observed here. That two bands of ISP were weakly stained as compared with the other components may indicate that ISP was not stoichiometrically recovered in the present preparation methods. Furthermore, the spectrum of ISP showed a peak at 415 nm as reported by Hurt et al.(29) and by the addition of ascorbate increase of the absorbance at 415 nm and appearance of a small 550 nm band were observed. This was presumably due to a contamination of denatured cytochrome $\underline{f}$ in addition to the absorption of ISP itself, because the heme staining in SDS-polyacrylamide gel electrophoresis of this fraction showed a

faint band corresponding to 29 kDa (data not shown). However, the appearance of a small peak at 550 nm and the increase at 415 nm may be due to a contamination of another low potential C-type cytochrome.

The oxidoreductase activity was 6.0 μmol cytochrome c reduced · $nmol^{-1}$ cytochrome f · $hour^{-1}$ for the purified b_6f complex, and was effectively inhibited by DBMIB, a plastoquinone antagonist which blocks electron transfer at the oxidation site of plastoquinone (30-32). This activity was lower than that of Anabaena and spinach (1,2) but it is impossible to strictly compare this with this activity, since different electron donors were used in the former studies.

In any case, this sample showed normal activity in electron transfer acting as a quinol-cytochrome c oxidoreductase. The b_6f and mitochondrial bc_1 complexes have a similar function in the photosynthetic and respiratory electron pathway, respectively, but the composition of the former complex is simpler (5). The b_6f complex should, therefore, be valuable in clarifying the mechanisms of electron transfer and proton translocation. Although further improvement of preparation of Spirulina cytochrome b_6f complex will be needed, the preparation is, as a first step, useful in the study of the electron transfer mechanism.

My interest in the cyanobacterial b_6f complex arises from the possibilty that this complex may participate in the electron transfer in both respiratory and photosynthetic electron pathways. In cyanobacteria, the b_6f complex may also serve as an

enzyme complex with the same function as the $\underline{bc}_1$ complex in mitochondria. Actually I could observe the O_2 consumption by the cyanobacterial membrane preparation on the addition of UQH_2 in Part 2. I am further trying to isolate cytochrome oxidase from the membrane. Knowledge of the structure and function of the $\underline{b}_6\underline{f}$ complex are also important in tracing the development of respiration and photosynthesis during evolution.

REFERENCE

1. Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117: 591-599.
2. Doyle, M.F. and Yu, C.-A. (1985) Biochem. Biophys. Res. Commum. 131: 700- 706.
3. Krinner, M., Hauska, G., Hurt, E. and Lockau, W. (1982) Biochim. Biophys. Acta 681: 110-117.
4. Lemaire, C., Girard-Bascou, J., Wollman, F.-A. and Bennoun, P. (1986) Biochim. Biophys. Acta 851: 229-238.
5. Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta 726: 97-133.
6. Rich, P.R. (1984) Biochim. Biophys. Acta 768: 53-79.
7. Bendall, D.S. (1982) Biochim. Biophys. Acta 683: 119-151.
8. Allred, D.R. and Staehelin, L.A. (1986) J. Bioenerg. Biomembr. 18: 419-436.
9. Matsuura, K. and Fujita, Y. (1986) Plant Cell Physiol. 27: 685-691.
10. Joliot, P. and Joliot, A. (1986) Biochim. Biophys. Acta 849: 211-222.
11. Ljungdahl, P.O., Jeffrey, D.P. and Trumpower, B.L. (1986) Methods Enzymol. 126: 181-191
12. Peschek, G.A. and Schmetterer, G. (1982) Biochem. Biophys. Res. Commun. 108: 1188-1195.
13. Sandmann, G. and Malkin, R. (1984) Arch. Biochem. Biophys. 234: 105-111.
14. Binder, A. (1982) J. Bioenerg. Biomembr. 14: 271-286.
15. Houchins, J.P. and Hind, G. (1982) Biochim. Biophys. Acta

682: 86-96.16.

16. Myers, J. (1986) Photosynthesis Res. 9: 135-147.

17. Hurt, E. and Hauska, G. (1982) J. Bioenerg. Biomembr. 14: 405-424.

18. Lowry, O.H., Rosebrough, L. N., Farr, A. L. and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.

19. Mackinney, G. (1941) J. Biol. Chem. 140: 315-322.

20. Laemmli, U.K. (1970) Nature 227: 680-685.

21. Thomas, P.E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75: 168-176.

22. Haid, A. and Suissa, M. (1983) Methods Enzymol. 96: 192-205.

23. Johnson, D.A., Gautsch, J.R., Sportsmann, J.R., and Elder, J.H. (1984) Gene Anal. Tech. 7: 3-8.

24. Rieske, J.S. (1967) Methods Enzymol. 10: 239-245.

25. Guikema, J.A. and Sherman, L.A. (1980) Biochim. Biophys. Acta 637: 189-201.

26. Saito, S. and Tsuchiya, T. (1984) Biochem. J. 222: 829-832.

27. Tsuchiya, T. and Saito, S. (1984) J. Biochem. 96: 1593-1597.

28. Ho, K.K. and Krogmann, D.W. (1980) J. Biol. Chem. 255: 3855-3861.

29. Hurt, E., Hauska, G. and Malkin, R. (1981) FEBS Lett. 134: 1-5

30. Nanba, M. and Katoh, S. (1986) Biochim. Biophys. Acta 851: 484-490.

31. Bergström, J., Andréasson, L-E. and Vänngård, T. (1986) Biochim. Biophys. Acta 852: 112-118.

32. Bose, S. (1985) Biochem. Biophys. Res. Commun. 130: 16-21.

Part II: Kinetic Studies on Cytochrome oxidase of Membrane Preparation from *Synechocystis* and its Partial Purification

SUMMARY

Cytochrome oxidase activity (oxygen uptake) of the membrane preparation from Synechocystis PCC 6714 was stimulated by cytochrome $\underline{c}_{553}$ prepared from this alga or cytochrome $\underline{c}$ from horse heart, whereas plastocyanin from this alga did not stimulate the activity. Km values of the cytochrome oxidase activity for ubiquinol-2 (UQH_2) and for cytochrome $\underline{c}$ were 26.7 μM and 14.1 μM, respectively. And these values were in good agreement with those determined with PS I activity. Cytochrome oxidase consumed a molecule of oxygen by four electrons arising from cytochrome $\underline{c}$ and was effectively inhibited by cyanide, azide, or dibromothymoquinone (DBMIB).

Synechocystis cytochrome oxidase was solubilized from total membrane with 0.2 % Na-cholate and 1.0 % lauryldimethylamine oxide (LDAO) followed by DEAE column chromatography. Cytochrome oxidase was inactivated by heat or protease treatment, and its activity was increased by the addition of lipids.

These results indicated that the oxygen uptake in dark was due to cytochrome oxidase and that cytochrome $\underline{c}_{553}$ reduced by cytochrome $\underline{b}_6\underline{f}$ complex transferred electrons to the cytochrome oxidase.

INTRODUCTION

Cyanobacteria are ubiquitous prokaryotes which carry out photosynthesis with the production of oxygen, and are assumed to be the origin of chloroplasts in the symbiotic theory (1). They can adapt to a broad spectrum of environmental conditions. Some of cyanobacteria can also carry out respiration (2-4). Photosynthetic electron transfer chain is known to be located on the thylakoid membrane, whereas location of the respiratory chain is still controversial. However, many observations support that respiratory chain is located on both of plasma and thylakoid membranes sharing a common electron transfer chain with that of photosynthesis (5-9).

All the respiratory and photosynthetic electron transfer chains have basically the same components of similar structures: quinone pool, cytochrome $\underline{b_6f}$ or $\underline{bc_1}$ complex, and soluble cytochrome $\underline{c}$ or plastocyanin (10). It has been conceived that the cyanobacterial respiratory chain might contain the three components mentioned above and these might be shared in common with photosynthetic electron transfer chain. Moreover, it has been assumed that the terminal electron transferring enzymes in respiration of cyanobacteria are NAD(P)H dehydrogenase and cytochrome oxidase, the same constitution as in mitochondrial respiratory chain. Actually, the purification of NAD(P)H dehydrogenase from Aphanocapsa 6714 was reported by Sandmann and Malkin (11). However, no conclusive evidence has been obtained on the components of cyanobacterial respiratory chain and many

problems remain to be resolved.

In recent years, several groups have described that cyanobacterial cytochrome oxidase might be an $\underline{aa_3}$-type enzyme as mitochondrial terminal oxidase. Their supposition has been based on the results obtained by spectrometry (12-14), Cu^{2+}-EPR spectrometry(15), immunoblotting experiments using an antibody against Paracoccus cytochrome $\underline{aa_3}$ (16-18). However, the low content of the cytochrome oxidase and the high contents of photosynthetic pigments such as phycobilin, carotenoid, and chlorophyll, made it difficult to purity the enzyme and gave insufficient evidence. Pesheck et al.(17) and Böger et al.(18) reported the purification of the enzyme from plasma membrane of Synechocystis 6714 and from the membrane prepared from heterocysts of Anabaena variabilis, respectively. However, the enzyme has not been isolated in pure form so as to be characterized in detail and further, there is no report on the purification of the oxidase from the thylakoid of vegetative cells.

In this experiments, I tried to elucidate the relationship between photosynthetic electron transfer chain and respiratory chain and to characterize the cytochrome oxidase of Synechocystis PCC 6714.

MATERIALS AND METHODS

Organism and Growth Conditions

Synechocystis PCC 6714 gifted from Dr. Y. Fujita, National Institute for Basic Biology, Okazaki, Japan, was basically grown in MDM medium (20) containing a half concentration of K_2HPO_4 and one-third of $NaNO_3$ and supplemented with 0.5μM $CuSO_4$. Further, in order to achieve a photoheterotropic growth, cells were grown in the existence of 0.5 % (w/v) glucose under light of fluorescent lamps. A cell suspension (500 ml) of the culture grown for 4 days in 2 l of the medium was transferred for inoculation to four flasks of each 10 l containing 9 l of the medium. Cells grown for 4 days were used in the following experiments.

Preparation of the Membrane

Cells of about 50 g harvested from 40 l of culture were washed twice with 10 mM Hepes-NaOH buffer (pH 7.5) containing 5 mM NaCl and 2 mM EDTA, and suspended in the same buffer. Lysozyme (Sigma Chemicals, St Louis, Mo., U.S.A.) was added to give a final concentration of 0.2 %(w/v) and the suspension was incubated at 37°C with shaking for 3 hours. The suspension was centrifuged at 12,000 x g for 5 minutes and washed twice with 15mM Tricine-NaOH buffer (pH 8.0) containing 0.2 M mannitol, 1 mM NaH_2PO_4, and 5 mM $MgCl_2$. The pellet was resuspended in the same buffer and passed through a French Press (model 5615, Ohtake) at 1,000 kgf/cm^2. This suspension was further treated with sonication, although the sonication was omitted in some experiments. The disrupted cells were centrifuged at 3,000 x g

for 10 minutes and the supernatant was recentrifuged at 200,000 x g for 1 hour. The supernatant was used for the preparation of cytochrome $\underline{c}_{550}$, cytochrome $\underline{c}_{553}$, and plastocyanin. The membrane pellet was suspended in the same buffer and recentrifuged. The membrane obtained was used for the measurements of respiratory and photosynthetic activity. The intact chloroplasts prepared from spinach leaves were prepared according to the method of Joy and Mills (21) and gifted from Mr. Y. Takahashi.

Preparation of Plastocyanin and C-type Cytochromes

cytochrome $\underline{c}_{553}$, cytochrome $\underline{c}_{550}$, and plastocyanin were prepared from the cell extracts obtained as described above according to the method of Kang et al.(21) with some modifications. Ammonium sulfate was added to the extract to 30 % saturation and centrifuged at 12,000 x g for 30 minutes. Ammonium sulfate was further added to the light blue supernatant to 55 % saturation and centrifuged. The supernatant was loaded onto a butyl-Toyopearl column (2.6 x 15 cm) equilibrated with 50 % saturated solution of ammonium sulfate containing 50 mM Tris-HCl (pH 7.5) and was then developed with a decreasing linear gradient of ammonium sulfate from 50 to 15 % saturation. The fraction containing cytochrome $\underline{c}_{550}$ was dialyzed against 10 mM Hepes-NaOH (pH 7.5), and used in the following experiments. The fraction containing cytochrome $\underline{c}_{553}$ and plastocyanin was dialyzed against 10 mM Tris-HCl (pH 8.0) and loaded onto a DEAE-Toyopearl column (2 x 10 cm) equilibrated with 10 mM Tris-HCl (pH 8.0). Cytochrome $\underline{c}_{553}$ which passed the column and plastocyanin was eluted from the column with 10 mM Tris-HCl (pH 7.4). They were used in the following experiments. Cytochrome $\underline{c}_2$ from

Rhodospirillum rubrum (photosynthesis bacteria) was gifted from Dr. K. Saeki, and a plastocyanin from Ulva arasaki was given by Dr. Y. Sugimura (Toho University, Chiba). Cytochrome c from horse heart (Type VI) was purchased from Sigma Chemicals. Cytochrome c_{553} from Bryopsis maxima (green alga) was prepared according to method described in the previous paper (23).

Assay Methods

Oxygen uptake was measured with an oxygen electrode (Clark-type) at 25 ℃ in the dark or light. The assay mixture was composed of 1.8 ml of 25 mM Hepes-NaOH and 0.2 M mannitol (pH 7.5) containing membrane (55.6 μg chlorophyll · ml^{-1} for the measurement of the cytochrome oxidase activity or 5.6 μg chlorophyll · ml^{-1} for that of PS I activity) and an appropriate amount of C-type cytochrome or plastocyanin. For the measurement of PS I activity, 0.1 mM MV, 20 μM DCMU, and 5 μM KCN were added to the assay mixture, and the mixture was illuminated with a 750 W lamp. The reaction was started by the addition of UQH_2 and/or a solution containing ascorbate and TMPD to the mixture. The UQH_2 was prepared from UQ_2 (a gift from Eizai Co. Ltd., Tokyo) by reducing it with dithionite (24) and the concentration was estimated in ethanolic solution using an $\varepsilon_{290\ nm}$ of 4.14 $mM^{-1} \cdot cm^{-1}$ for the reduced form. The concentration of the dissolved oxygen in the assay mixture was calculated using 252μM, the concentration of saturated oxygen in the solution. This value was determined according to the method of Robinson (25). The reaction rates were expressed as μmol O_2 uptake·mg $chlorophyll^{-1} \cdot hour^{-1}$.

Cytochrome oxidase activity was monitored in a Hitachi 220A

spectrophotometer at 550 nm as the rate of oxidation of cytochrome c . The assay mixture was composed of 1 ml of 25mM Hepes-NaOH and 0.2 M mannitol containing membrane preparation, and the reaction was started by the addition of reduced cytochrome c to the mixture. The reaction rates were expressed as μmol cytochrome c oxidized·mg chlorophyll^{-1}·hour^{-1}.

KCN, NaN_3, DBMIB, and diphenylamine were used for the inhibition of the activity. DBMIB and diphenylamine were dissolved in ethanol. The concentration of ethanol in the assay mixture was less than 0.5 %.

Examination of Solubilization

The membrane preparation was treated with 2 M NaBr according to the method described for preparation of cytochrome b_6f complex (Part I). The membranes were suspended in 20 mM Tris-HCl buffer (pH 8.0) containing a solubilizing agent. The agents used were LDAO (Hoechst, Co. Ltd.), heptyl thioglucoside (Dojin, kumamoto, Japan), Na-cholate (Sigma Chemicals), Triton X-100, urea, and NaSCN. The suspensions were centrifuged at 572,000 x g for 1.5 hours. Oxygen uptake of the supernatants were measured after dialysis and some samples were chromatographed on a DEAE-Toyopearl column.

Preparation of the Cytochrome Oxidase

Cytochrome oxidase was solubilized in a mixture containing 1.0 % LDAO and 0.2 % Na-cholate. The extract was applied to a DEAE-Toyopearl column (2.1 x 15 cm) equilibrated with 10 mM Tris-HCl (pH7.5) containing 0.2 % Na-cholate. The column was washed with the same buffer until the effluent became colorless. The column was further washed with 10 mM Tris-HCl (pH 7.5) containing

0.02 % $C_{12}E_8$ (Nikko Chemical Co. Ltd., Tokyo, Japan), followed by the same buffer containing 0.1 M NaCl. The column was developed in a step-wise manner with 0.15 and 0.3 M NaCl. The each fraction was load onto a hydroxylapatite column (2 x 10 cm) equilibrated with 5 mM Na-phosphate buffer (pH 6.0) containing 0.2 % Na-cholate. The column was washed with a 10-fold column volume of the same buffer, and was developed with 0.2 M Na-phosphate buffer (pH 6.0) containing 0.01 % $C_{12}E_8$. The fractions containing cytochrome oxidase activity or cytochrome $\underline{b_6f}$ complex were concentrated with an ultrafiltration membrane (Ultra filter UK-200, Advantic Toyo, Tokyo, Japan.)

Other Methods

Electrophoresis was performed using Laemmli's system (26) by staining of the proteins with Coomassie Brilliant Blue and heme staining according to the method of Thomas et al.(27). Protein concentration was determined by a Lowry assay (28) and chlorophyll $\underline{a}$ was measured according to the method of Mackinney (29).

L-α-phosphatidylcholine (Type II-S) was purchased from Sigma Chemicals. It was suspended in 25 mM Hepes-NaOH (pH 7.5) to give a final concentration of 1 % (w/v). An appropriate volume of the lipid suspension was added to cytochrome oxidase.

Cytochrome oxidase concentrated with an ultrafiltration membrane as described above was treated with heat or digested with chymotrypsin (enzyme/substrate = 1/50, w/w) at 25 °C. Samples purified except a hydroxylapatite column step were digested with thermolysin (enzyme/substrate = 1/50, w/w) under

the existence of 10 mM $CaCl_2$ at 25 °C.

RESULTS

Growth of Synechocystis PCC 6714

Fig. 1 shows the relation of cell growth with the activity of oxygen uptake. The activity decreased contrary to the increasing growth of the cells. An investigation on the effect of Cu^{2+} was carried out as shown in Fig. 2, the activity of the culture grown in a medium containing 0.5μM Cu^{2+} was accelerated, and increased 35 % in comparison with the culture without the addition of Cu. Thus, the cells grown in the medium containing 0.5 μM Cu^{2+} and 5g glucose/ 1 for 4 days were used in the following experiments.

Oxygen Uptake in the Dark and Light

On the basis of the studies previously reported, a hypothetical schemes of the electron flows are constructed and shown in Fig. 3 and an experimental strategy was constructed as follows. Oxygen uptake of cytochrome oxidase and of PS I (Mehler reaction) were measured using UQH_2 (scheme 1) and ascorbate+TMPD (scheme 2) as electron donors. When an excess amount of electron donor and a certain amount of horse cytochrome c were added to the reaction mixture, the cytochrome oxidase activity was 15.7 μmol O_2 uptake·mg chlorophyll^{-1}·hour^{-1} in scheme 2 and was 16.1 μmol O_2 uptake·mg chlorophyll^{-1}· hour^{-1} in scheme 1.

Fig. 4 shows the oxygen uptake by cytochrome oxidase and by PS I in scheme 2, respectively. The membrane from Synechocystis PCC 6714 containing cytochrome c_{553} or plastocyanin consumed oxygen in the dark (Fig. 4 (A)). On the contrary,

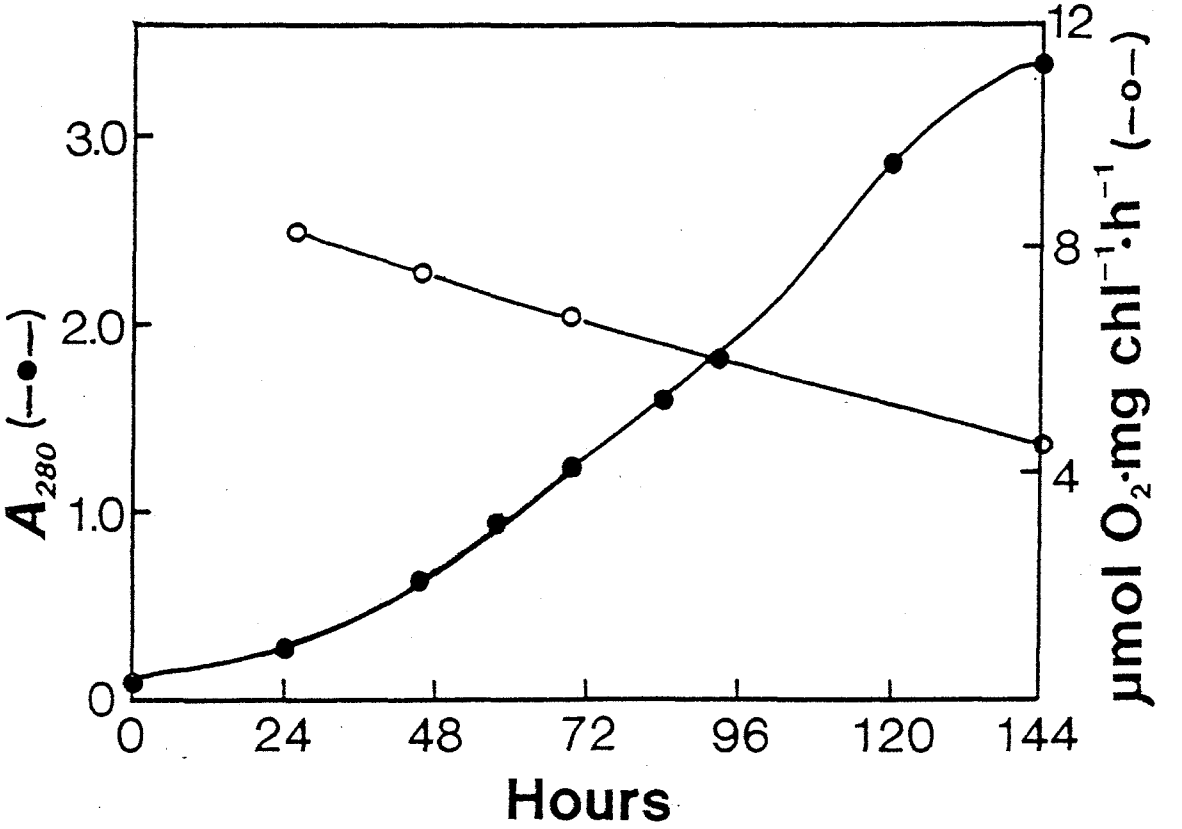

Fig. 1 Relation between the cell growth and oxygen uptake in the dark.

Cells were grown in 2 l of medium. At indicated time, absorbance at 680 nm of the culture medium was measured and the cells were harvested. To measure the activity, an appropriate volume of the culture medium was harvested. Oxygen concentration was measured with a Clark type oxygen electrode. Assay mixture contained the membrane preparation at 100 μg chlorophyll·ml^{-1}, 20 μM horse heart cytochrome c, and 50 μM UQH_2. Preparation of the membranes and details of assay method are given in the text.

—●—, absorbance at 680 nm

—o—, oxygen uptake

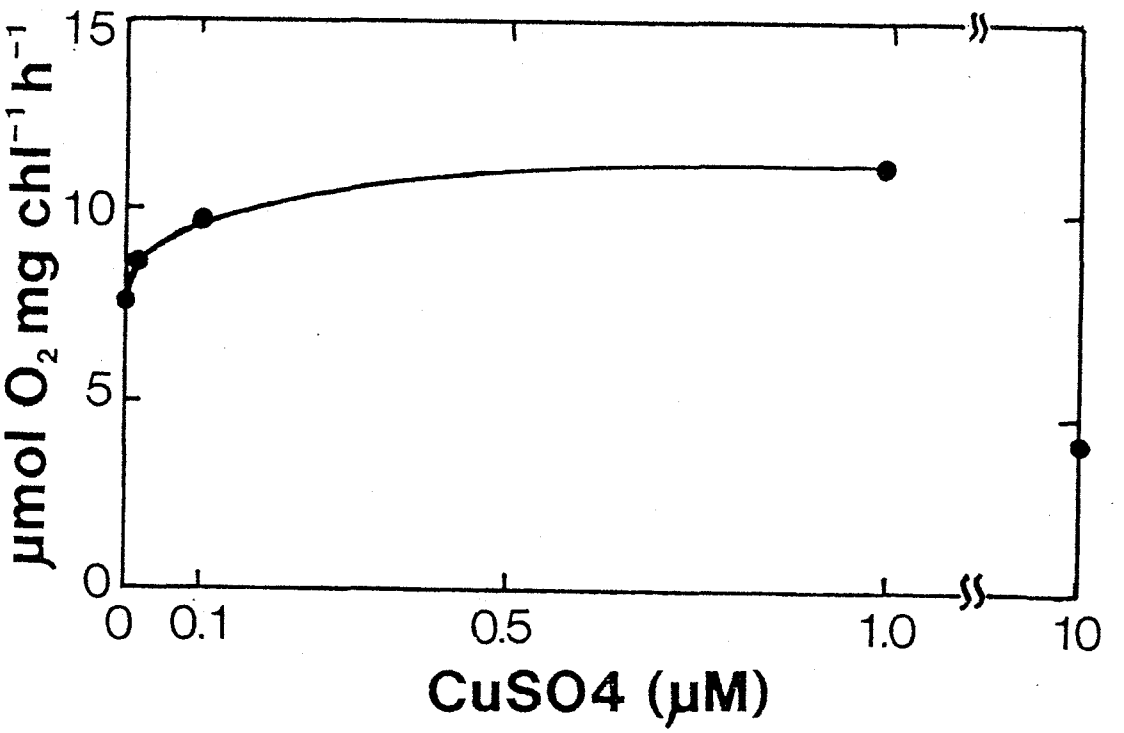

Fig. 2 Effect of $CuSO_4$ on oxygen uptake in the dark

Cells were grown in 500ml of the medium containing an appropriate concentration of $CuSO_4$ and were harvested after 4 days. Assay conditions were the same as those in Fig. 1.

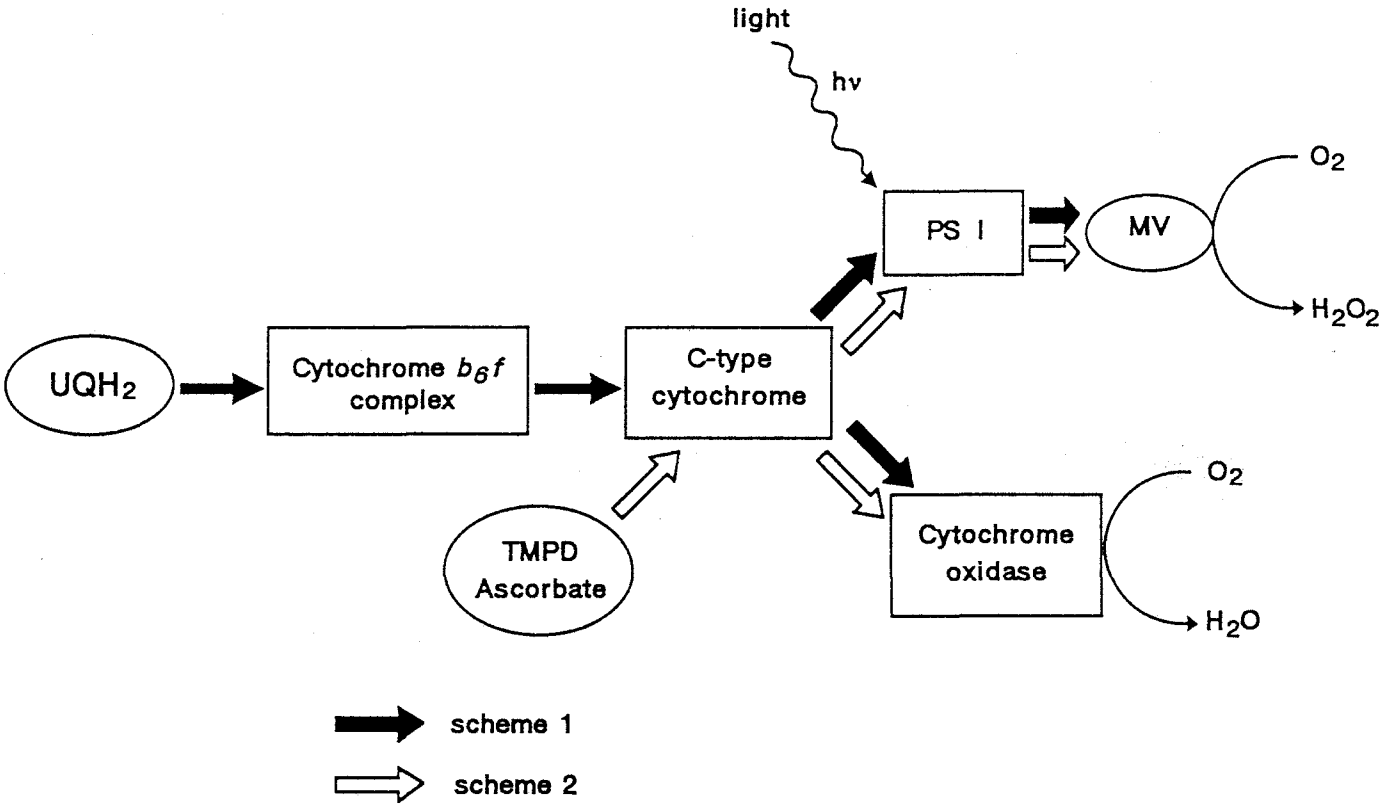

Fig. 3 A hypothetical schemes of the electron transfer chain in cyanobacterial membrane

UQH_2, TMPD and ascorbate, or MV are added to the reaction mixture containing membranes and C-type cytochrome or plastocyanin. PS I activity was measured in reaction mixture containing KCN to inhibit cytochrome oxidase and DCMU to inhibit PS II reaction. PS I activity was measured in the light, and the cytochrome oxidase activity was measured in the dark. The background activity was determined by the addition of electron donor to the reaction mixture without membranes. Solid arrows (➡) indicate the electron flow from UQH_2 to O_2, which is called scheme 1 in the text. Open arrows (⇨) indicate the electron flow from ascorbate to O_2, which is called scheme 2 in the text.

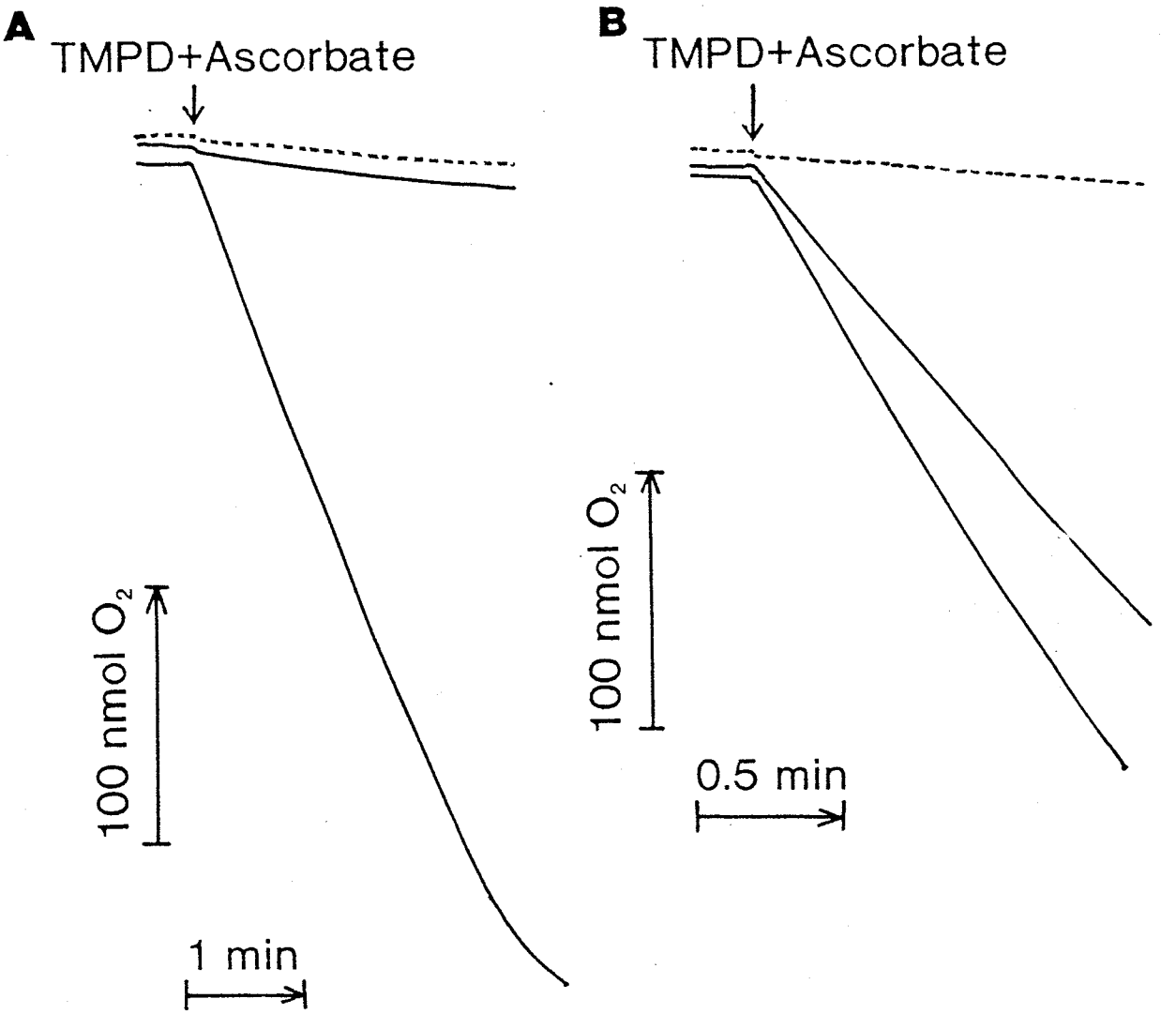

Fig. 4 Comparison of oxygen uptake between spinach chloroplasts and the membranes from Synechocystis PCC 6714

Synechocystis membranes were prepared without sonication. Since the membranes and chloroplasts contain soluble cytochrome c_{553} and plastocyanin, the redox proteins were not added to the reaction mixture. The reactions were assayed in scheme 2.

A: Oxygen uptake in the dark

Assay mixture contained membranes corresponding to 100 μg chlorophyll·ml^{-1}

B: Oxygen uptake in the light

Assay mixture contained membranes corresponding to 10 μg chlorophyll·ml^{-1}

intact chloroplast from spinach leaves were unable to uptake oxygen under the same conditions. However, as shown in Fig. 4 (B), both preparations showed the oxygen uptake in the light to nearly the same extent.

The stimulations of both cytochrome oxidase activity and PS I activity by the redox proteins are shown in Fig. 5 and Table I. UQH_2 was used as an electron donor to assay the respiratory activity via cytochrome $\underline{b_6f}$ complex (see Fig. 3, scheme 1). As a control experiment, the activity was determined in every assay system by the addition of UQH_2 to the assay mixture without the membranes. The cytochrome oxidase activity was stimulated with cytochrome $\underline{c}_{553}$ either from Synechocystis PCC 6714, or from B. maxima, and horse cytochrome $\underline{c}$ as shown in Fig. 5 (A). Little or no stimulation of the activity was observed with cytochrome $\underline{c}_2$ from R. rubrum, cytochrome $\underline{c}_{550}$ or plastocyanin from Synechocystis PCC 6714, or plastocyanin from U. arasaki. While PS I activity was not stimulated with horse cytochrome $\underline{c}$, cytochrome $\underline{c}_2$ from R. rubrum, cytochrome $\underline{c}_{550}$ from Synechocystis PCC 6714, cytochrome $\underline{c}_{553}$ from B. maxima, and plastocyanin from U. arasaki (Fig. 5 (B)), it was activated with cytochrome $\underline{c}_{553}$ and plastocyanin from Synechocystis PCC 6714. Table I summarizes the values of Km and Vmax of PS I and cytochrome oxidase, respectively, for each redox protein. In the cytochrome oxidase activity, similar Km values for horse cytochrome $\underline{c}$ and cytochrome $\underline{c}_{553}$ from Synechocystis PCC 6714 were obtained. However, cytochrome $\underline{c}_{553}$ from B. maxima did not efficiently react with cytochrome oxidase and Km value for this cytochrome $\underline{c}_{553}$ was determined to be about 300 μM. Still, in scheme 2, Km value for

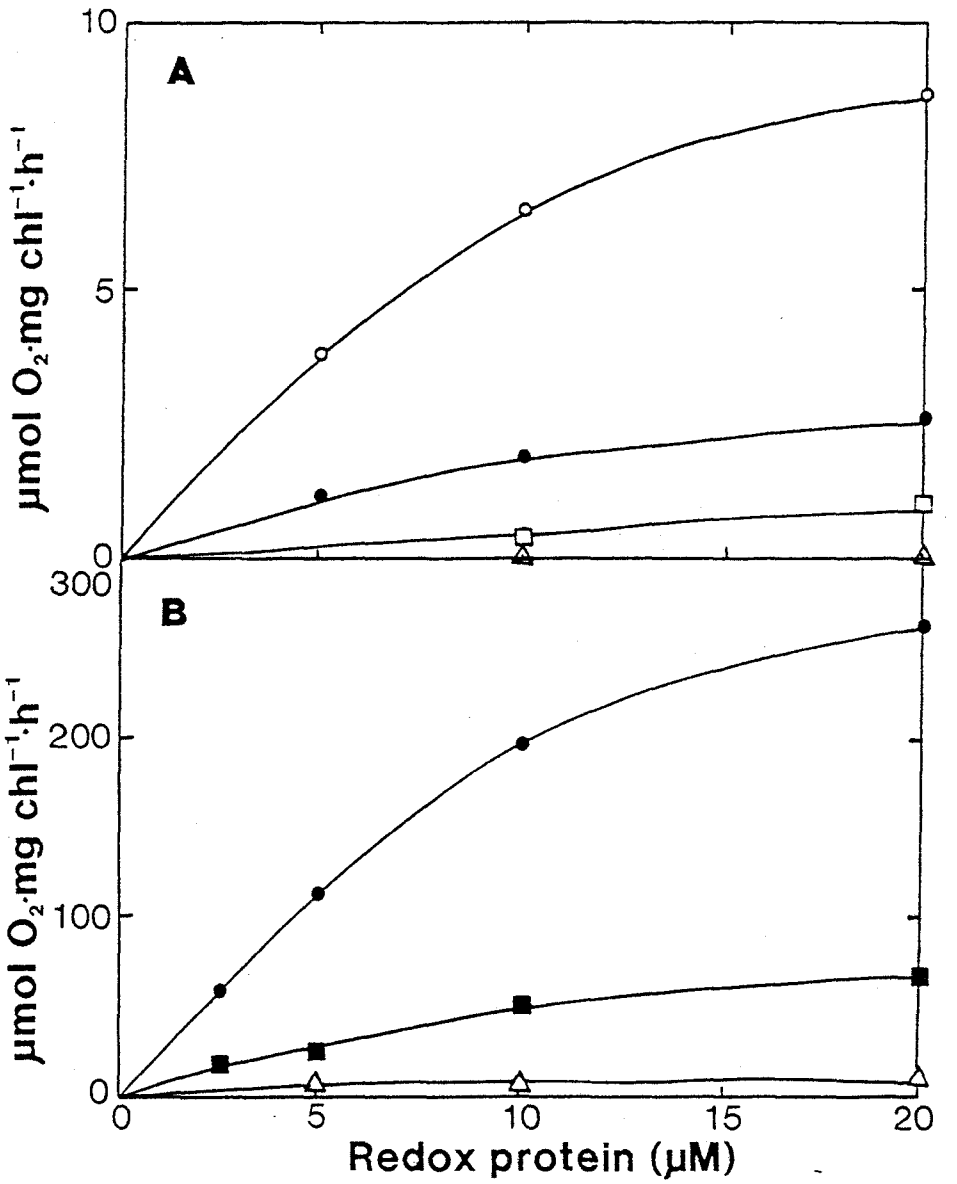

Fig. 5 Stimulation of oxygen uptake by the redox proteins
These assays were performed in scheme 1.
A: Oxygen uptake in the dark
—o—, horse cytochrome c; —●—, cytochrome c_{553} from Synechocystis PCC 6714; —□—, cytochrome c_{553} from Bryopsis maxima; —△—, cytochrome c_2 from Rhodospirillum rubrum, cytochrome c_{550} from Synechocystis PCC 6714, or plastocyanin from Ulva arasaki or Synechocystis PCC 6714.
B: Oxygen uptake in the light
—●—, cytochrome c_{553} from Synechocystis PCC 6714; —■—, plastocyanin from Synechocystis PCC 6714; —△—, cytochrome c_2 from Rhodospirillum rubrum, horse cytochrome c, cytochrome c_{550} from Synechocystis PCC 6714, plastocyanin from Ulva arasaki, or cytochrome c_{553} from Bryopsis maxima.
Assay conditions were the same as those in Fig. 4 except addition of redox proteins

Table I Comparison of Kinetic parameters for Various Redox Proteins in PS I and Cytochrome Oxidase Assays

Redox protein	PS I			Cytochrome oxidase		
	Activity	Km[a]	Vmax[b]	Activity	Km[a]	Vmax[b]
Horse heart Cytochrome *c*	-	——	——	+	14.5	15.4
Rhodospirillum rubrum Cytochrome c_2	-	——	——	-	——	——
Synechocystis PCC 6714 Cytochrome c_{553}	+	16.1	500	+	14.7	4.5
Synechocystis PCC 6714 Cytochrome c_{550}	-	——	——	-	——	——
Bryopsis maxima Cytochrome c_{553}	-	——	——	+	ca.300	ca.20
Synechocystis PCC 6714 Plastocyanin	+	18.2	166.7	-	——	——
Ulva arasaki Plastocyanin	-	——	——	-	——	——

[a] μM, concentration of a redox protein giving half maximal velocity.

[b] μmol O_2 uptake·mg chlorophyll^{-1}·hour^{-1}

horse cytochrome was 12.5 μM and Vmax was 10.4 μmol O_2 uptake·mg chlorophyll^{-1}·hour^{-1} (data not shown). These values were in good agreement with those in scheme 1 (Table I). In the following experiments, horse cytochrome c was used as the redox protein. Fig. 6 shows kinetic data using UQH_2 for the cytochrome oxidase activity: Km values for UQH_2 was 26.7 μM and Vmax was 13.6 μmol O_2 uptake · mg chlorophyll^{-1}·hour^{-1} (Fig. 6 (A)). And Km value using UQH_2 for PS I activity was 28.6 μM and Vmax 363.6 μmol O_2 uptake·mg chlorophyll^{-1}·hour^{-1} (Fig. 6 (B)). These Km values were in good agreement between both activities.

Effects of Several Inhibitors

Cytochrome oxidase activity was effectively inhibited by KCN and NaN_3 in both scheme 1 and 2 (Fig. 7 (A) and (B)). Fifty percent inhibition of the activity was accomplished by 0.15 μM KCN and 2 mM NaN_3. Little activity remained by higher concentration of inhibitors. Diphenylamine, an inhibitor of cyanide-insensitive oxidase (30), inhibited 36 % of the activity in scheme 1, but no inhibition was found in scheme 2 (Fig. 7 (c)). DBMIB, an inhibitor of cytochrome b_6f complex (31-33), inhibited both of the cytochrome oxidase activity and PS I activity in scheme 1, whereas it did not inhibit in scheme 2 (Fig. 8). Both activities were inhibited 50 % with about 1 μM DBMIB.

Relation of Oxygen Uptake with Cytochrome c Oxidation

Oxygen uptake and oxidation of cytochrome c were measured by the addition of the reduced cytochrome c to the reaction mixture containing the membranes. When cytochrome c was oxidized at a rate of 4.5 μmol·mg chlorophyll^{-1} · hour^{-1}, oxygen was consumed

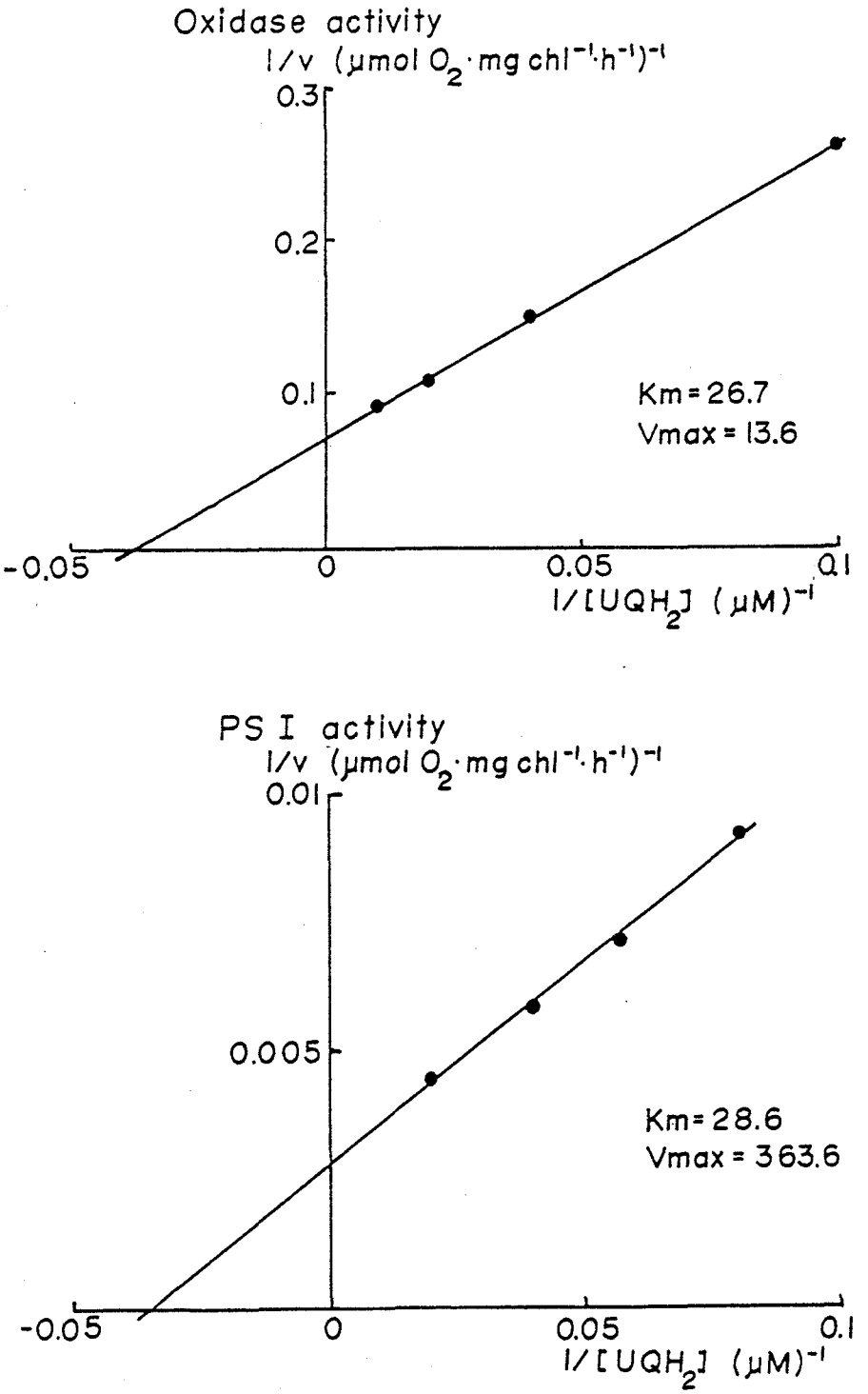

Fig. 6 Kinetic data for the cytochrome oxidase and PS I activities.

Synechocystis membranes were prepared without sonication. Reactions were performed without the addition of cytochrome c. Reaction conditions were the same as those in Fig. 4.

A: Oxygen uptake in the dark

B: Oxygen uptake in the light

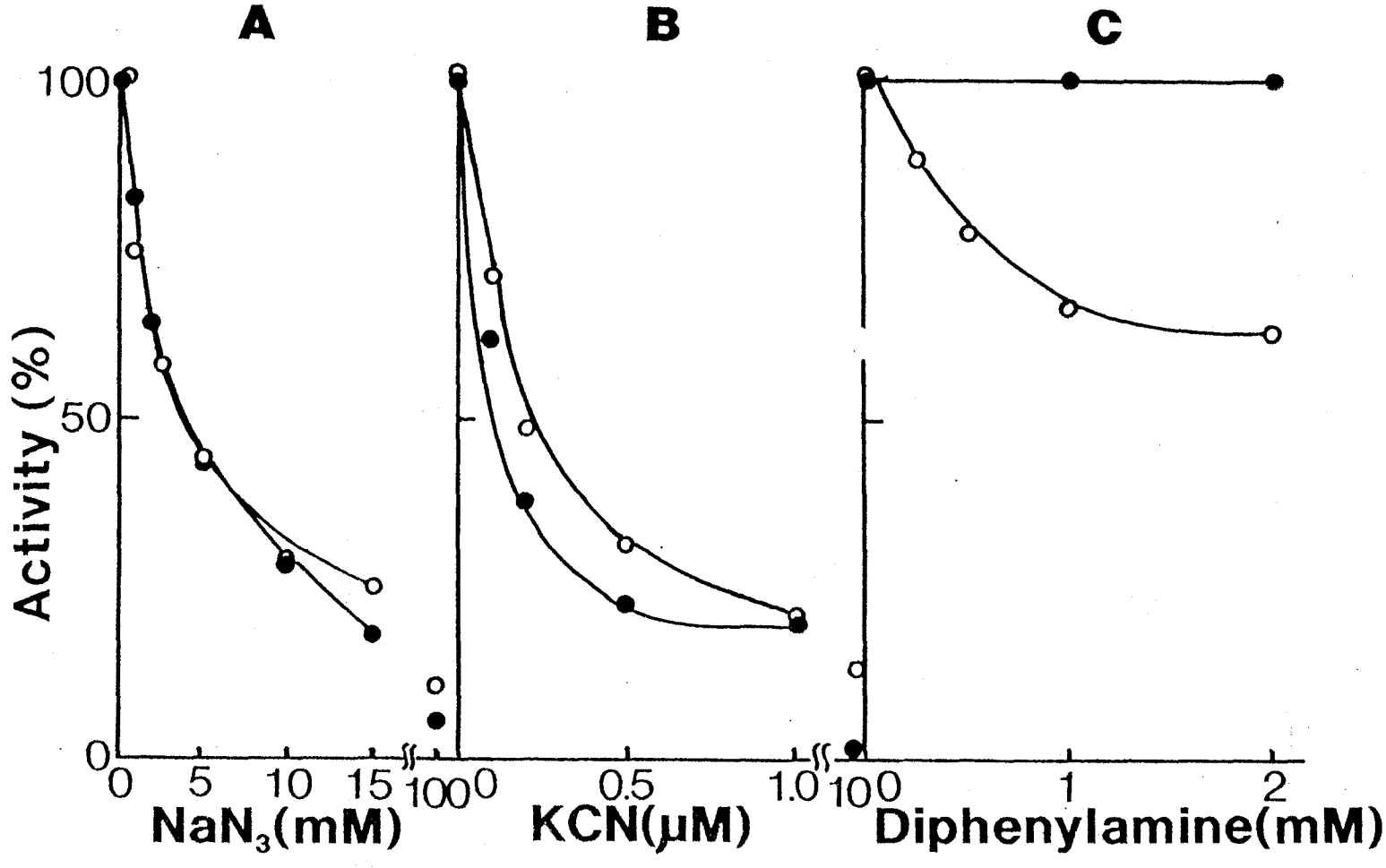

Fig. 7 Inhibition of Cytochrome Oxidase Activities (oxygen uptake) were measured in scheme 1 (—o—) and in scheme 2 (—•—) in the dark. Assay conditions were the same as those in Fig. 1. The background activities without membranes were determined in the reaction mixture containing each inhibitor.

A: inhibition by NaN_3, B: inhibition by KCN, C: inhibition by diphenylamine.

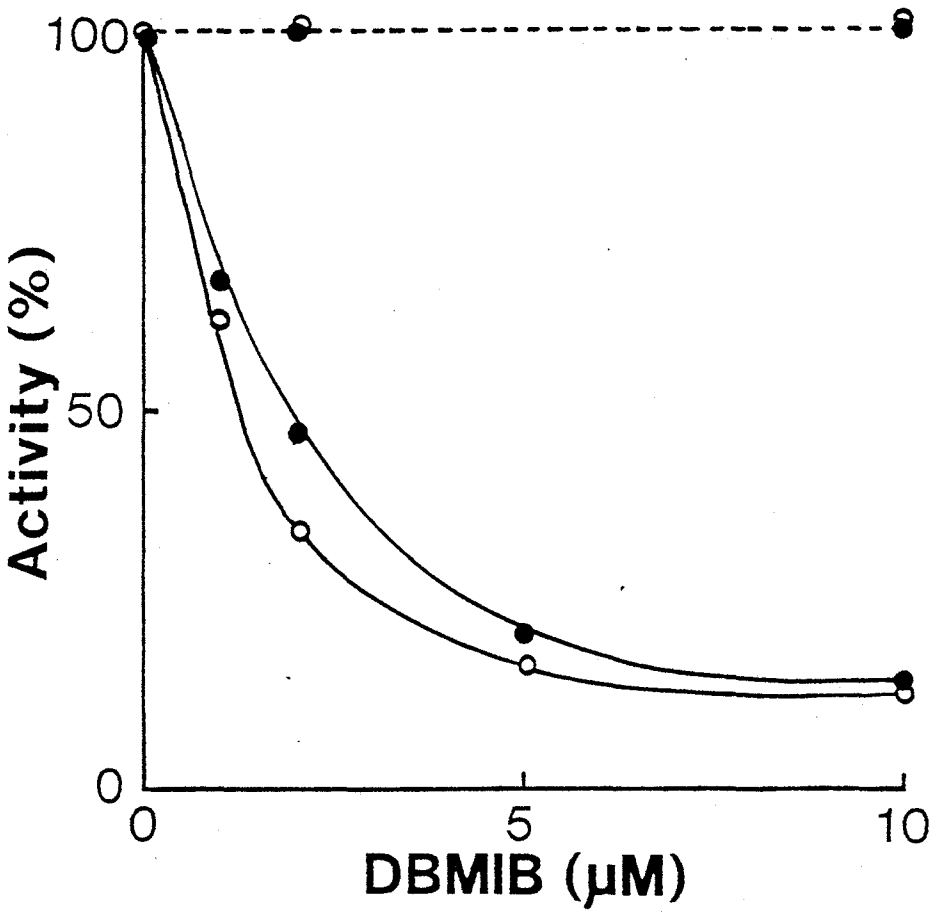

Fig. 8 Inhibition of cytochrome oxidase activity and PS I activity by DBMIB

Solid lines show the activities in scheme 1 and dotted lines in scheme 2. Symbols: —●—, cytochrome oxidase activity; —o—, PS I activity. Assay conditions were the same as those in Fig. 4.

at a rate of 1.1 μmol·mg chlorophyll^{-1}·hour^{-1}. Thus, cytochrome oxidase reduced one mol of oxygen with 4 electrons from 4 mol of cytochrome c.

Solubilization of the Membranes

Table II shows the results of solubilization with several solubilizing agents. When nonionic detergents such as heptyl thioglucoside and LDAO were independently used, proteins were effectively solubilized, but became insoluble in the following processes. Cytochrome oxidase was not solubilized with Na-cholate , and the activity decreased with Triton X-100. Treatments with urea and NaSCN inactivated the cytochrome oxidase activity. Since LDAO solubilized the membrane completely, the solubilization with 0.8 % LDAO was found to yield only a small amount of precipitate by ultracentrifugation. Na-Cholate was added to the solubilizing buffer containing LDAO on purpose to suppress the extraction of chlorophyll protein (34). Cytochrome oxidase activity was solubilized effectively with a mixture of LDAO and Na-cholate.

Preparation of the Cytochrome Oxidase

Fig. 9 shows extraction profiles of proteins, chlorophyll, and cytochrome oxidase activity from the Synechocystis membrane preparation. The activity was effectively solubilized with 1.0 % LDAO and 0.2 % Na-cholate, and higher concentration of LDAO was decreased the specific activity. Thus, 1.0 % LDAO was used in the following experiments. After ultracentrifugation, the supernatant was loaded onto a DEAE-Toyopearl column. Then, the detergent in the column buffer was replaced with $C_{12}E_8$. The column was developed with 0.15 M NaCl to elute cytochrome b_6f complex. Cytochrome oxidase was eluted with 0.3 M NaCl. The each fraction

Table II Effect of Various Agents in Solubilizing Synechocystis Cytochrome Oxidase

Agents	Concentration range	Properties	Cytochrome oxidase
LDAO	0.5-1.5 %(v/v)	non-charged	solubilized
Heptyl thioglucoside	20-100mM	non-charged	solubilized
Na-Cholate	0.5-3.0 %(w/v)	anionic	not solubilized
Triton X-100	1.0-1.5 %(v/v)	non-charged	solubilized
Urea	7M	denaturing agent	inactivated
NaSCN	7M	denaturing agent	inactivated
LDAO + Na-Cholate	LDAO 0.2-2.0%(v/v) Cholate 0.2% (w/v)	———	solubilized

Assay mixture contained an appropriate amount of sample and 20 μM cytochrome (horse heart).

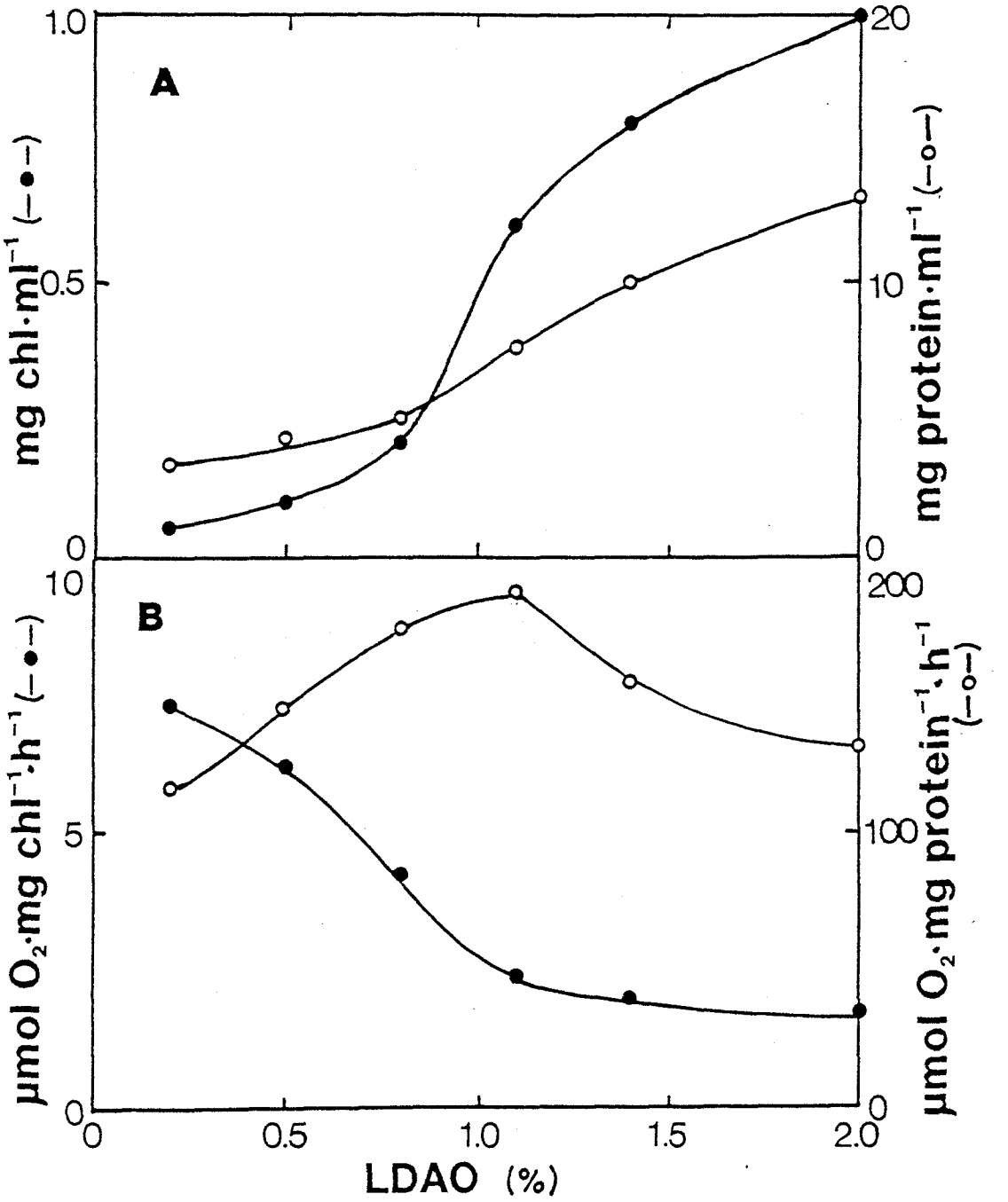

Fig. 9 Solubilization of cytochrome oxidase with LDAO

The membranes were extracted in a mixture containing 0.2 % Na-cholate and 0 to 2 % LDAO. The assay mixture contained 20 µM cytochrome c and the reaction was performed as given in the text.

A: Total proteins (—o—) and chlorophyll a (—●—) content of the extracts

B: Solubilization of cytochrome oxidase activity expressed in µmol O_2 uptake·mg chlorophyll^{-1}·hour^{-1} (—●—) and µmol O_2 uptake·mg protein^{-1}·hour^{-1} (—o—).

was collected and applied to a hydroxylapatite column. Cytochrome oxidase was eluted with 0.2 M Na-phosphate buffer (pH 6.0) containing 0.01 % $C_{12}E_8$. Fractions of cytochrome oxidase or cytochrome $\underline{b_6f}$ complex were concentrated by ultrafiltration.

SDS-polyacrylamide gel electrophoresis analyzing partially purified oxidase and cytochrome $\underline{b_6f}$ complex is shown in Fig. 10. Synechocystis cytochrome $\underline{b_6f}$ complex consisted of three major bands of 29, 23, and 17 kDa (lane 2), which showed the same molecular masses as those of the enzyme complex from Spirulina. The 29 and 23 kDa polypeptides, probably cytochrome $\underline{f}$ and $\underline{b_6}$, were also detected on SDS gels stained for heme (lane 5). Lane 4 shows the fraction containing cytochrome oxidase activity. Table III summarized the course of the purification. The partially purified cytochrome oxidase was largely inactivated during the purification process.

Properties of the Cytochrome Oxidase

Although the oxidase activity decreased gradually during the purification process given above, it could be recovered by the addition of lipids as shown in Fig. 11. Maximum activity was obtained by the addition of 2 mg lipid per mg protein. However, cytochrome oxidase activity was not remarkably recovered by addition of lipids.

Effects of temperature and proteolytic enzyme on partially purified cytochrome oxidase was examined as shown in Fig. 12 and 13. Incubation at 90 °C for 10 minutes inactivated completely cytochrome oxidase (Fig. 12 (A)). About 50 % inactivation was attained by the incubation at 40 °C for 15 minutes and completely after 4 hours (Fig. 12 (B)). About 50 % of inactivation was

observed by chymotryptic or thermolytic digestion for about 1.5 hours and the activity was completely lost after incubation for 24 hours (Fig. 13).

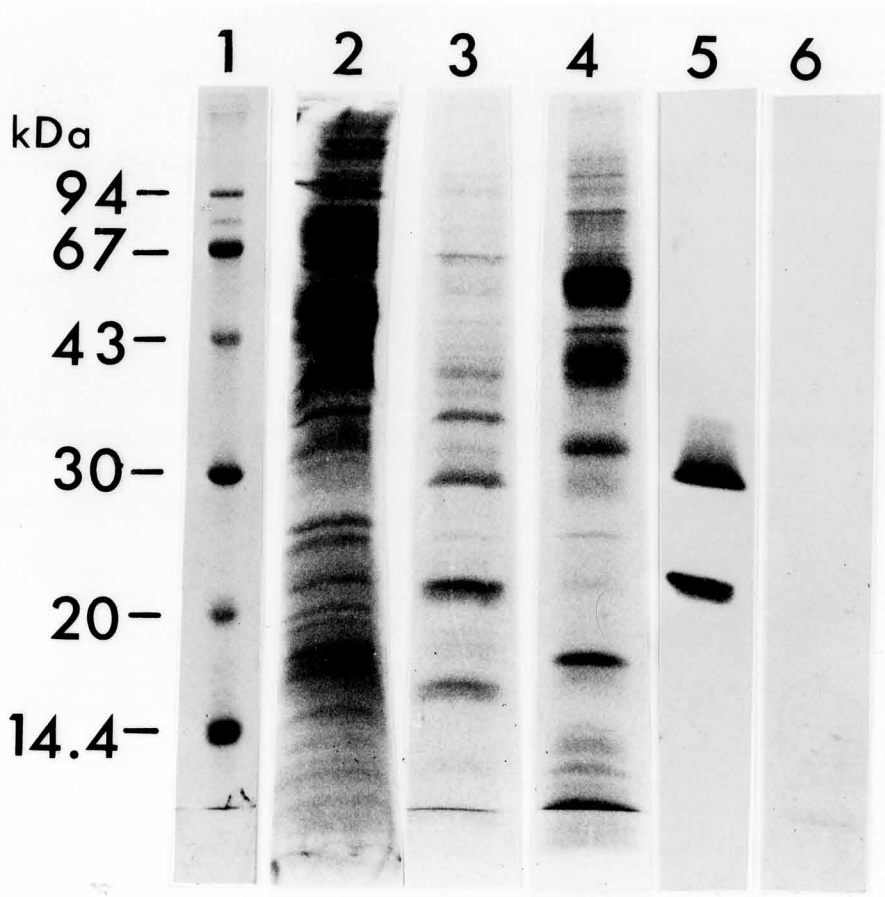

Fig. 10 SDS-polyacrylamide gel electrophoresis pattern lane 1-4 are stained with Coomassie Brilliant Blue and lane 5 and 6 are by heme staining. Lane 1 shows standard proteins of 94, 67, 43, 30, 20, and 14.4 kDa. Lane 2, extract; lane 3 and 5, cytochrome $\underline{b}_6\underline{f}$ complex fraction from DEAE-Toyopearl; lane 4 and 6, cytochrome oxidase fraction from DEAE-Toyopearl

Table III Summary of Purification of Cytochrome Oxidase

Step	Chlorophyll (mg)	Protein (mg)	Specific activity[a]
Membranes	70	917	0.34
LDAO-Extract	28	304	8.13
Chromatography	18	114	3.04

All activity were measured under the existence of lipids (lipid/protein = 2/1(w/w)). Other assay conditions were the same as those in Fig. 9.

[a] μmol O_2 uptake·mg protein^{-1}·hour^{-1}

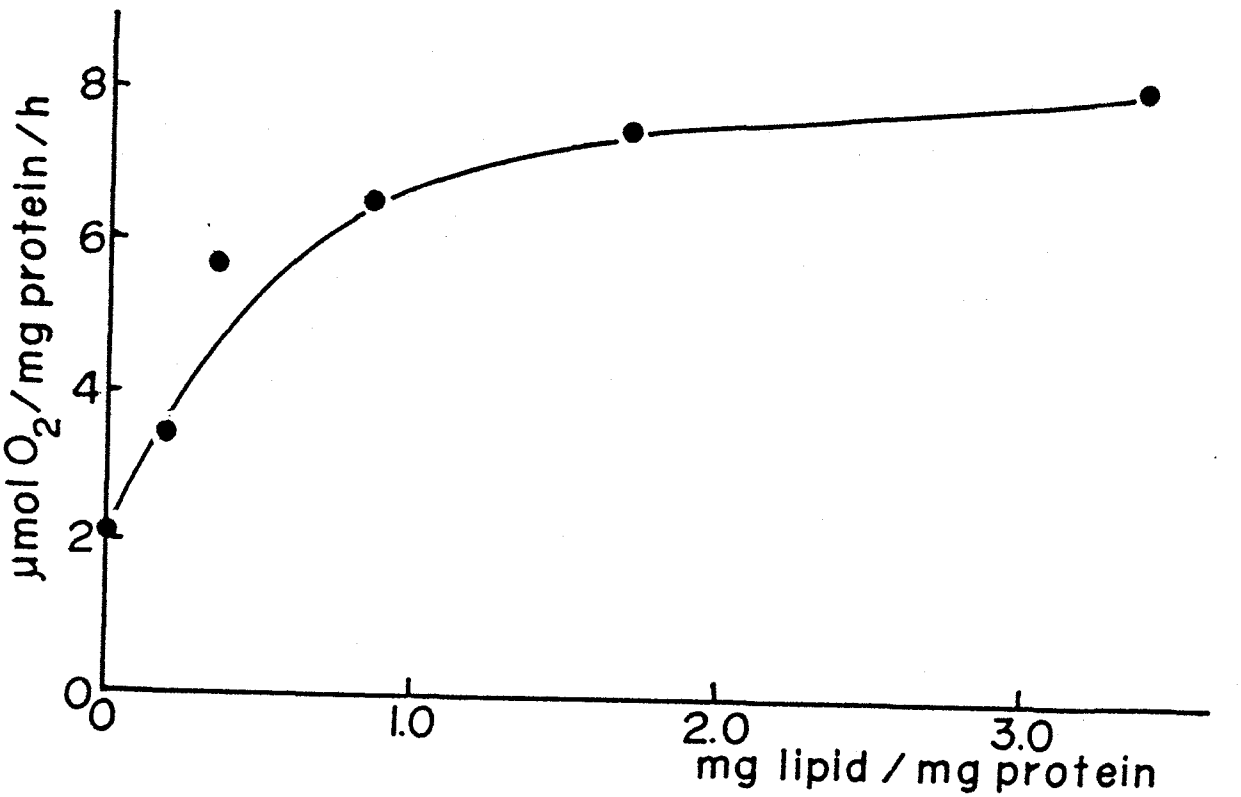

Fig. 11 Effect of lipid concentration on the activity of the partially purified cytochrome oxidase.

The assay conditions were the same as those in Fig. 9.

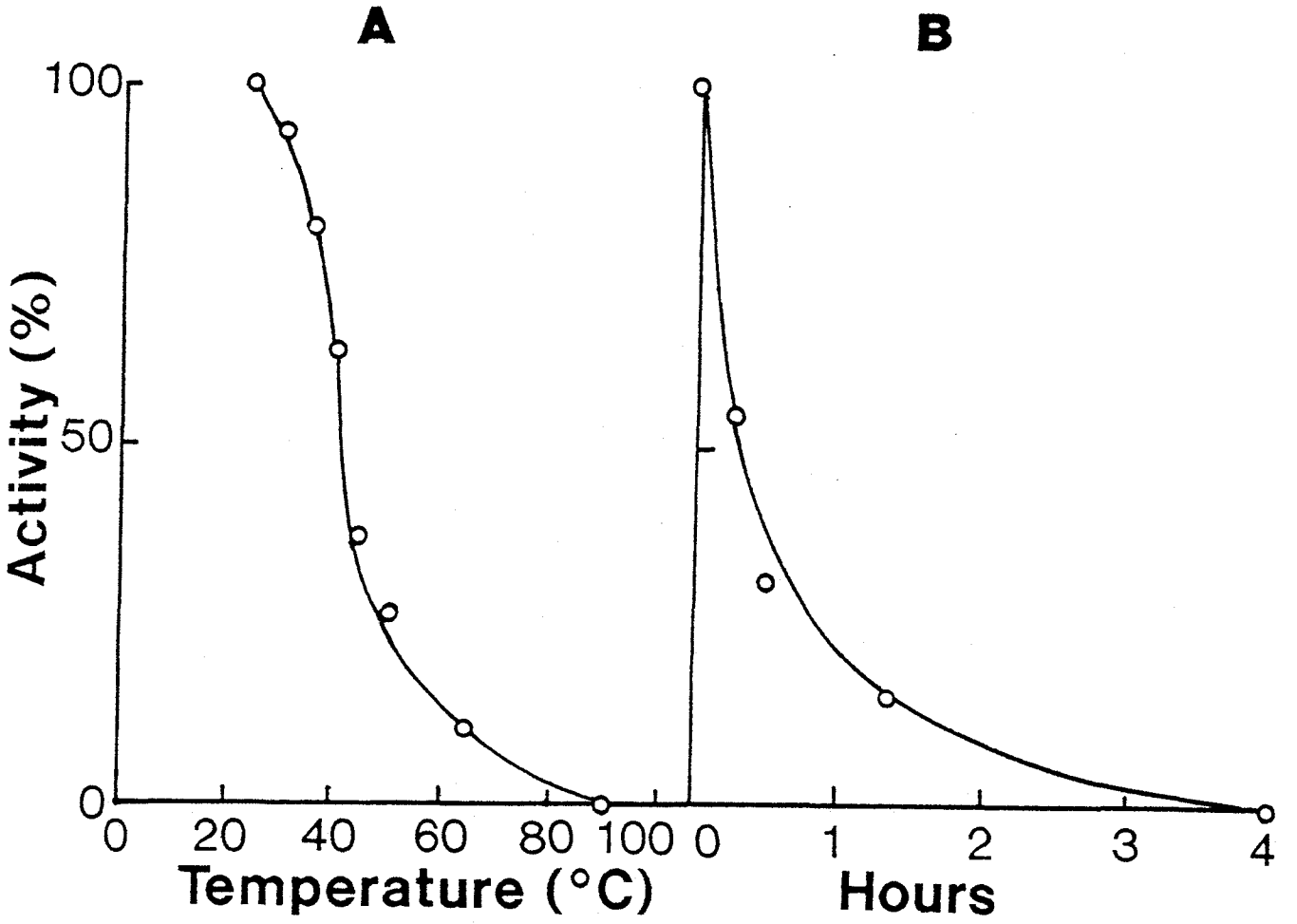

Fig. 12 Inactivation of the partially purified cytochrome oxidase by heat

A: Incubation for 10 minutes at temperatures indicated

B: Incubation at 40 °C

The assay conditions were the same as those in Fig. 9.

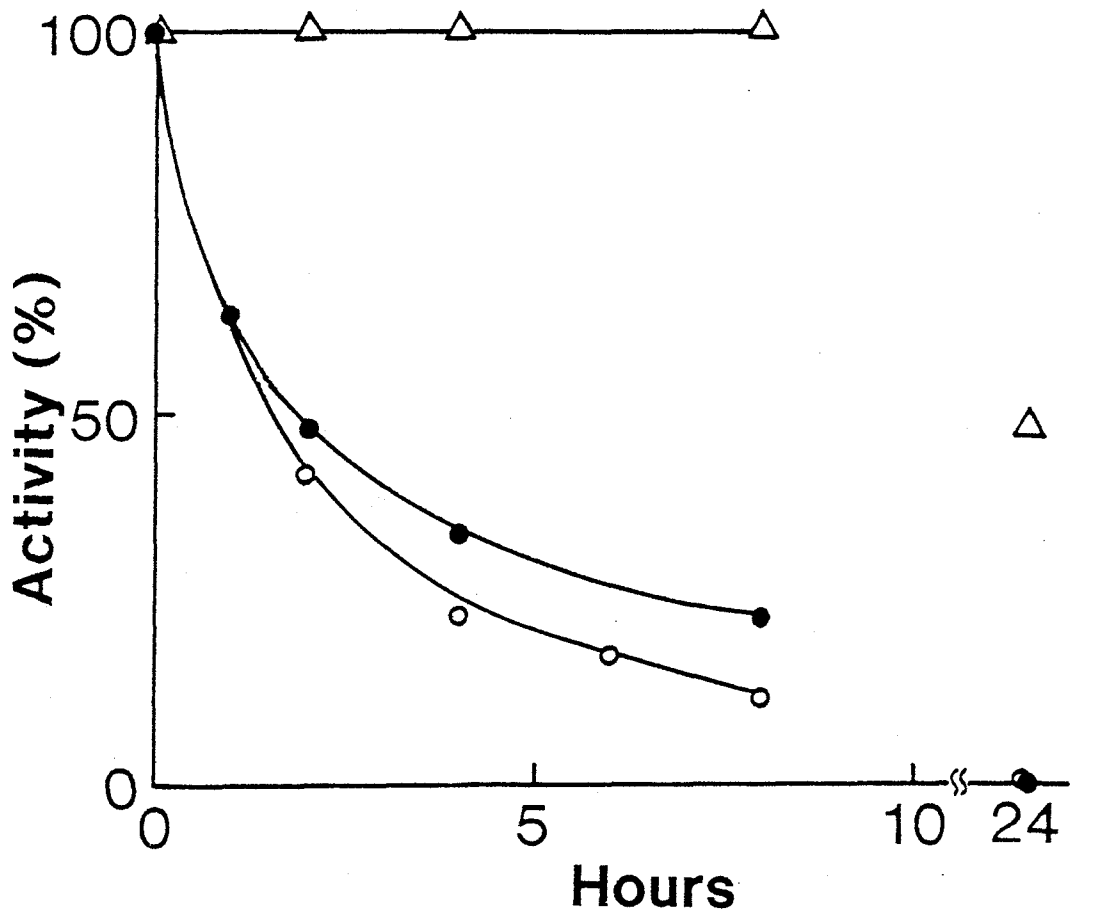

Fig. 13 Inactivation of cytochrome oxidase by protease treatments

The partially purified cytochrome oxidase was digested with chymotrypsin (—●—) or thermolysin (—○—) at 25 °C.

—△—, without protease

DISCUSSION

Cyanobacterial respiratory chain may share a common part of electron transfer machinery with the photosynthetic chain, and may transfer electrons from NAD(P)H dehydrogenase to cytochrome $\underline{aa_3}$. I had previously examined whether the membranes prepared from Spirulina, a cyanobacterium used in part I, showed a respiratory activity or not. Since the culture had been contaminated by many bacteria, the oxidase activity gained was doubtful. The isolation of Spirulina from other bacteria had been difficult. Synechocystis PCC 6714 used in the present experiment was free from any bacterial contaminant. Thus, cyanobacterial cytochrome oxidase was investigated with Synechocystis PCC 6714 grown for 4 days. The addition of $CuSO_4$ to the culture medium increased the content of plastocyanin increased (data not shown) as well as the cytochrome oxidase activity (Figs. 2 and 5) although it's own plastocyanin did not stimulate the oxygen uptake activity. The observation of increase of cytochrome oxidase activity and plastocyanin was consistent with the report by Sandmann (35). The increase of activity with the supplement of copper indicated the involvement of a copper protein similar to mitochondrial cytochrome $\underline{aa_3}$. I also tried to prepare the membranes from the cells grown heterotropically in the dark and compare the activity with that of the membranes prepared from autoheterotropically grown cells. However, no difference was found in the activity between these two preparations (data not shown), suggesting that biosynthesis of cytochrome oxidase might

occur without regard to the illumination of the cells with light.

Chlororespiration of Chlamydomonas reinhardtii was reported (36) and this phenomenon might be applicable to the respiration of cyanobacteria, although spinach chloroplasts did not show any respiration (Fig. 4). This might be the fundamental difference between algae such as Chlamydomonas and cyanobacteria and higher plants, or cyanobacterial cytochrome oxidase activity might be different from that observed in chlororespiration. The result that cytochrome oxidase activity was not stimulated by its own plastocyanin but was stimulated by cytochrome c_{553} from B. maxima might indicate that algae having cytochrome c_{553} carry out respiration as commonly found in mitochondria having cytochrome c. Then, the respiratory activity need to be measured in other plants, particularly algae.

The stimulation of oxygen uptake in scheme 2 was also described by Lokau (37), and in this content I would like to discuss on the properties of the respiratory electron transfer via cytochrome b_6f complex which has solely been considered to be an important electron transfer complex residing between PS I and PS II. As shown in Fig. 3, most of the experiments were performed in scheme 1. It has been known that plastocyanin and soluble C-type cytochrome such as cytochrome c_{553} function as the mobile electron transfer proteins between cytochrome b_6f complex and PS I. Cytochrome oxidase requires also some of C-type cytochromes to show its full activity (Fig. 5). Horse cytochrome c, a basic protein, activated the cyanobacterial cytochrome oxidase but did not PS I. Cytochrome c_2, another basic protein, from R. rubrum

and the acidic plastocyanin from U. arasaki did not activate either PS I or cytochrome oxidase. Furthermore, the acidic cytochrome c_{553} from B. maxima stimulated cytochrome oxidase a little but did not PS I. Therefore, both activities are regardless of pI of the electron carriers.

Cyanobacteria have plastocyanin and several soluble C-type cytochromes including cytochrome c_{550} and cytochrome c_{553}, although not all of these are reported to be present in some cyanobacterial species (38,39). Cyanobacterial cytochrome c_{550}, which has a low redox potential (-260 mV) and is so far functionally unknown, did not stimulated both of PS I and cytochrome oxidase. Some recent reports suggested this cytochrome to be involved in sulfur metabolism (40) or in the cyclic electron transport system (41). Cytochrome c_{553} stimulated both PS I and oxidase activities, whereas plastocyanin activated PS I only. While the reaction between cytochrome oxidase and plastocyanin was thought to depend upon pH of the assay mixture (23), cytochrome oxidase was not activated with plastocyanin in the assay mixture with pH range from 6.0 to 8.5. Nevertheless, plastocyanin transferred electrons to cytochrome oxidase in scheme 2, which was consistent with the previous report by Lokau (23). In scheme 2, Km value for plastocyanin was 7.8 μM, which was lower than that for cytochrome c_{553}, and the Vmax was 8.3 μmol O_2 uptake · mg chlorophyll^{-1} · hour^{-1}. This reaction in scheme 2 might be an artificial bypass reaction from plastocyanin to cytochrome oxidase. These results suggest that cytochrome c_{553} is the physiological electron donor for cytochrome oxidase. In

vivo, therefore, plastocyanin transfers preferentially electrons to PS I, and cytochrome $\underline{c}_{553}$ transfers electrons to both of PS I and cytochrome oxidase. Some experimental results indicate that cytochrome $\underline{b}_6\underline{f}$ complex is a common intermediary constituent functioning in both electron transfer chains:

1) Km values for UQH_2 were in good agreement between both activities. Therefore, the electron carriers between UQH_2 and cytochrome oxidase can assume to be shared with the photosynthetic electron transfer chain.

2) An inhibitor of cytochrome $\underline{b}_6\underline{f}$ complex, DBMIB, inhibited both activities in the same manner. Both activities measured in scheme 1 were not inhibited by DBMIB (Fig. 8).

Cytochrome oxidase activity was inhibited by cyanide and azide. Since a half of oxidase activity in scheme 1 and 2 was inhibited by 0.15-0.2 μM KCN, the same electron carrier, cytochrome oxidase, is involved commonly in both systems. However, the extent of inhibition in scheme 1 was lower than that in scheme 2 with the same inhibitor concentrations and 15 % of activity in scheme 1 remained even at 10 μM KCN. Furthermore, diphenylamine known as an inhibitor of cyanide-insensitive oxidase inhibited about 30 % of the activity in scheme 1, but did not inhibit in scheme 2. Since cyanide-insensitive oxidase activity is known to be affected by a high concentration of KCN (10), the cyanide insensitive activity observed and diphenylamine-sensitive activity may be due to the same oxidase. This results suggest the existence of an other oxidase, which is not stimulated by cytochrome $\underline{c}$. This might be the oxidase described in previous papers (42,43). Comparison of the oxidation

rate of reduced cytochrome c with the rate of oxygen consumption indicated that this cytochrome oxidase converted molecular oxygen into water.

Solubilization of cytochrome oxidase was examined by using several agents. Most of the detergents used except Na-cholate, solubilized the cytochrome oxidase together with chlorophyll proteins and the solubilization effect was not specific for cytochrome oxidase. Na-cholate suppresses unspecific membrane solubilization by partially coating the surface (34). And in fact, the combination of LDAO with Na-cholate solubilized effectively cytochrome oxidase. However, cytochrome oxidase could neither be isolated by the following chromatography on a CM-Toyopearl column to which cytochrome oxidase was not adsorbed, gel-filtration columns, an affinity column (cytochrome c-Sepharose 4B), a hydroxylapatite column, and a DEAE-Toyopearl, nor by sucrose density gradient centrifugation. These procedures could not separate the cytochrome oxidase from chlorophyll. Fractionation with ammonium sulfate resulted in aggregation. Although I also examined the preparation method reported by Böger et al. using the solubilization with a mixture of deoxycholate and octyl-β-D-thioglucoside and the purification method using QAE column chromatography (19), the purity of the cytochrome oxidase did not increase. Among the various chromatographic procedures adopted, however, DEAE-Toyopearl column chromatography was simpler and more effective than the others to give a higher extent of purification. Cytochrome oxidase purified by a DEAE-Toyopearl column still contained a

large amount of chlorophyll and proteins and gave many bands in SDS-polyacrylamide gel electrophoresis. Polypeptides consisting cytochrome oxidase could not be assigned on the SDS gels. Further, the spectral characterization of this sample did not detect cytochrome oxidase owing to high content of chlorophyll.

Thus, some of the chlorophyll proteins were always accompanying together with the cytochrome oxidase even after several rigorous purification processes. Cytochrome oxidase form a complex in a micelle of the detergent and was assumed to be located in the vicinity of photosynthetic apparatus including chlorophyll protein. Therefore, in order to improve the purification of cytochrome oxidase care must be taken in the solubilization step in future. Although any cationic detergent was not tried in this experiment, it might be useful in selective solubilization.

The cytochrome oxidase eluted from a DEAE-Toyopearl column consumed one mol of oxygen with the concomitant reduction of 4 moles of cytochrome c, and was inactivated by heat or protease treatments. The increase in the activity was found by addition of lipids as found in general for membrane bound proteins (44,45). These results may indicate that this cytochrome oxidase is the oxidase described by Peschek et al., probably a cytochrome aa_3 (4,12-19). The effluent passing through the DEAE-Toyopearl column consumed oxygen without regard to the addition of cytochrome c. This activity was purified with a molecular mass of 10 kDa on an SDS gel (data not shown). The small molecular mass and inhibition of the activity by KCN suggested it to be laccase or ascorbate oxidase (3).

The conclusions based on the results described above are as follows. The oxygen uptake in the dark is due to cytochrome oxidase; electrons are transferred to cytochrome oxidase from cytochrome $\underline{b_6f}$ complex; cytochrome $\underline{c_{553}}$ function as the electron transfer protein between cytochrome $\underline{b_6f}$ complex and cytochrome oxidase; the respiratory chain exists in close connection with and shares the components with photosynthetic electron transfer chain.

Though cyanobacteria can grow under photoautotoropic conditions (1-3), there is a problem why they need the respiratory chain. Since cyanobacteria are primitive organisms, it is not surprising to have a unique respiratory chain which is not fully separated from photosynthetic electron transfer chain as exemplified in photosynthetic bacteria. If chloroplasts had evolved from one of cyanobacteria, only the photosynthetic electron transfer chain has remained in chloroplasts, but the components involved in respiration had been lost from chloroplasts. Cyanobacteria gained during evolution the ability to respire for the maintenance of a energy-acquiring metabolic system in order to survive in the dark.

Although the preparation of cytochrome oxidase needs to be further improved, a clue of the isolation procedure was obtained. Studies on the respiration of cyanobacteria is important to know the evolution of plant as well as that of the electron transfer chain.

REFERENCE

1. Margulis, L. (1970) Origin of Eukaryotic Cell Yale University Press
2. Carr, N. G. and Whitton, B. A. eds. (1973) The Biology of blue-green algae University of California Press, Berkeley and Los angeles
3. Fogg G. E., Stewart, W. D. P., Fay, P. and Walsby, A. E. eds. (1973) The blue-green algae Academic Press, London and New York
4. Fay,P. and Baalen, C. V. eds. (1987) The cyanobacteria Elsevier, Amsterdom, New York, and Oxfold
5. Binder, A. (1982) J. Bioenerg. Biomembr. 14: 271-286
6. Peschek, G. A. and Schmetterer, G. (1982) Biochem. Biophys. Res. Commun. 108: 1188-1195
7. Houchins, J. P. and Hind, G. (1982) Biochim. Biophys. Acta. 682: 86-96
8. Sandmann, G. and Malkin, R. (1984) Arch. Biochem. Biophys. 234: 105-111
9. Myers, J. (1986) Photosynthesis Res. 9: 135-147
10. Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1983) Biochim. Biophys. Acta. 726: 97-133
11. Sandmann, G. and Malkin, R. (1983) Arch. Microbiol. 136: 49-53
12. Pesheck, G. A. (1981) Biochim. Biophys. Acta. 635: 470-475
13. Peschek, G. A. (1981) Biochem. Biophys. Res. Commun. 98: 72-79

14. Peschek, G. A., Schmetterer, G., Lauritsch, G., Nitschmann, W. H., Kienzl, P. F. and Muchl, R. (1982) Arch. Microbiol. 131: 261-265

15. Fry, I. V., Peschek, G. A., Huflejt, M. and Packer, L. (1985) Biochem. Biophys. Res. Commun. 129: 109-116

16. Trnka, M. and Peschek, G. A. (1986) Biochem. Biophys. Res. Commun. 136: 235-241

17. Wastyn, M., Achatz, A., Trnk, M. and Peschek, G. A. (1987) Biochem. Biophys. Res. Commun. 149: 102-111

18. Molitor, V., Trnka, M. and Peschek, G. A. (1987) Current Microbilogy 14: 263-268

19. Hafele, U., Scherer, S. and Boger, P. (1988) Biochim. Biophys. Acta. 934: 186-196

20. Watanabe, A. (1960) J. Gen. Appl. Microbiol. 6: 283-292

21. Joy, K. W. and Mills, W. R. (1987) Methods. Enzymol. 148: 179-188

22. Kang, B.H., Tan, S. and Ho, K-K. (1988) Arch. Biochem. Biophys. 263: 387-393

23. Okamoto, Y., Minami, Y., Matsubara, H. and Sugimura, Y. (1987) J. Biochem. 102: 1251-1260

24. Rieske, J. S. (1967) Methods. Enzymol. 10: 239-245

25. Robinson, J. and Cooper, J. M. (1970) Anal. Biochem. 33: 390-399

26. Laemmli, U. K. (1970) Nature 227: 680-685

27. Thomas, P. E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75: 168-176

28. Lowry, O. H., Rosebrough, L. N., Farr, A. L. and Randall, R.

J. (1951) J. Biol. Chem. 193: 265-275

29. Mackinney, G. (1941) J. Biol. Chem. 140: 315-322

30. Sharpless, T. K. and Butom, R. A. (1970) J. Biol. Chem. 245: 58-70

31. Bose, S. (1985) Biochem. Biophys. Res. Commun. 130: 16-21

32. Bergström, J., Andréasson, L-E. and Vänngård, T. (1986) Biochim. Biophys. Acta. 852: 112-118

33. Nanba, M. and Katoh, S. (1986) Biochim. Biophys. Acta. 851: 484-490

34. Hauska, G. (1986) Methods. Enzymol. 126: 271-285

35. Sandmann, G. (1985) Physiol. Plant. 65: 481-486

36. Bennoun, P. (1982) Proc. Natl. Acad. Sci. USA 79: 4352-4356

37. Lockau, W. (1981) Arch. Microbiol. 128: 336-340

38. Matsubara, H. and Wada, K. (1988) Methods. Enzymol. 167: 387-410

39. Sandmann, G. (1986) Arch. Microbiol. 145: 76-79

40. Ho, K. K., Ulrich, E. L., Krogmann, D. W. and Gomez-Lojero, C. (1976) Biochim. Biophys. Acta. 545: 236-248

41. Kienzl, P. F. and Peschek, G. A. (1983) FEBS Lett. 162: 76-80

42. Peschek, G. A. (1980) Arch. Microbiol. 125: 123-131

43. Scherer, S., Hafele, U., Kruger, G. H. and Boger, P. (1988) Physiol. Plant. 72: 379-384

44. Epstein, N. and Racker, E. (1978) J. Biol. Chem. 253: 6660-6662

45. Doyle, M. F. and Yu, C-A. (1985) Biochem. Biophys. Res. Commun. 131: 700-706

ACKNOWLEDGEMENTS

I would like to express my thanks to Prof. H. Matsubara for his support through this study. I thanks Prof. K. Wada (Kanazawa University) for his guidance and valuable discussions through this work. I also thanks Prof. S. Yoshikawa (Himeji Institute of Technology) for his advices and discussions for cytochrome oxidase.

I express my thanks to Dr. Kodo for the generous gift of Spirulina cells, Dr. R. Malkin (Univ. of California at Berkeley, U.S.A.) for anti-ISP, Prof. Y. Fujita (National Institute of Basic Biology, Okazaki, Japan) for Synechocystis PCC 6714, Prof. Y. Sugimura (Toho University) for plastocyanin from Ulva arasaki, Dr. K. Saeki for cytochrome c_2 from Rhodospirillum rubrum, Mr. Y. Takahashi for spinach chloroplast, and Eizai Co. Ltd. for UQ_2. I also wish to thank Nakamura Laboratory (Osaka University) for my use of French Press.

I am my thanks to Dr. A. Murakami (National Institute for Basic Biology) for his technical advices for the cultivation of Synechocystis PCC 6714. I also thank Mr. Y. Takahashi for his technical advices and various discussions and Mr. K. Mukai, Mr. S. Tanaka, Mr. Y. Fujita, Mr. F. Shounai, Mr. H. Toyosaki, and Mr. T. Kubota for their technical assistances and discussion in this study. I wish to thank my colleagues of this laboratory for their interests. And I am grateful for the JSPS Fellowships for Japanese Junior Scientists.

PUBLICATIONS

I. Publications related to the thesis.

1. Yosiko MINAMI, Keishiro WADA, Hiroshi MATSUBARA (1988)
   The Isolation and Characterization of a Cytochrome $\underline{b_6f}$ Complex from the Cyanobacterium Spirulina sp.
   Plant Cell Physiol. in press

2. Yoshiko Minami, Keishiro Wada, Hiroshi Matsubara (1989)
   Kinetic Studies on Cytochrome Oxidase of Membrane Preparation from Synechocystis and its Partial Purification
   in preparation

II. other publications.

1. Yoshiko MINAMI, Sadao WAKABAYASHI, Fumihiro YAMADA, Keishiro WADA, Walter G. ZUMFT, and Hiroshi MATSUBARA (1984)
   Ferredoxins from the Photosynthetic Purple Non-Sulfur Bacterium Rhodopseudomonas palustris. Isolation and Amino Acid Sequence of Ferredoxin I
   J. Biochem. 96, 585-592

2. Yoshiko MINAMI, Sadao WAKABAYASHI, Keishiro WADA, Hiroshi MATSUBARA, Lorenz KERSHER, and Dieter OESTERHELT (1985)
   Amino Acid Sequence of a Ferredxin from Thermoacidophilic Archaebacterium, Sulfolobus acidocaldarius. Presence of an N^6-Monomethyllysine and Phyletic Consideration of Archaebacteria
   J. Biochem. 97, 745-753

3. Yoshiko MINAMI, Sadao WAKABAYASHI, Setsuko IMOTO, Yoshimoto OHTA, and Hiroshi MATSUBARA (1985)
Ferredoxin from a Liverwort, Marchantia polymorpha. Purification and Amino Acid Sequence
J. Biochem. 98, 649-655

4. Y. Minami, F. Yamada, T. Hase, H. Matsubara, A. Murakami, Y. Fujita, T. Takao and Y. Shimonishi (1985)
Amino acid sequences of allophycocyanin α- and β-subunits isolated from Anabaena cylindrica Presence of an unkown derivative of aspartic acid in β-subunit
FEBS LETTERS 191, 216-220

5. Yoshiko MINAMI, Yasutomo SUGIMURA, Sadao WAKABAYASHI, Keishiro WADA, Yasuhiro TAKAHASHI and Hiroshi MATSUBARA (1985)
Isolation, properties, and amino acid sequence of a ferredoxin from a multinuclear, unicellular green alga, Bryopsis maxima
Physiol. Veg. 23, 669-678

6. Yoko OKAMOTO, Yoshiko MINAMI, Hiroshi MATSUBARA, and Yasutomo SUGIMURA (1987)
Studies on Algal Cytochromes VI: Some Properties and Amino Acid Sequence of Cytochrome c_6 from a Green Alga, Bryopsis maxima
J. Biochem. 102, 1251-1260