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RESPIRATION IN ENERGY-TRANSDUCING MEMBRANES OF THE THERMOPHILIC CYANOBACTERIUM *MASTIGOCLADUS LAMINOSUS*

II. OXIDATIVE PHOSPHORYLATION

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Oxidative phosphorylation was measured in isolated energy-transducing membranes of the thermophilic cyanobacterium *Mastigocladus laminosus* with NADH-mediated electron transport. This dark phosphorylation was similar to photophosphorylation in its sensitivity to uncouplers and energy-transfer inhibitors. However, photophosphorylation was 20- to 50-times more active than oxidative phosphorylation. The P/O ratio of oxidative phosphorylation was about 0.2. Besides oxidative phosphorylation, adenylate kinase- and ADP-P_i exchange activity were measured in the dark. The ADP-P_i exchange reaction was identified as polynucleotide phosphorylase.

Introduction

Cyanobacteria like eucaryotic algae and higher plants mainly satisfy their need for energy in the light with photosynthesis through photophosphorylation. In dark periods, these organisms survive with respiration, i.e., they produce ATP through oxidative phosphorylation. Photophosphorylation in cell-free systems of cyanobacteria has been characterized in the thermophilic *Mastigocladus laminosus* [1] as well as in many other species (for a review, see Ref. 2). The characterization of the cyanobacterial coupling factor ATPase (AF_1) from *M. laminosus* was described earlier [1,3,4].

Much less is known about oxidative phosphorylation in cyanobacteria. Although it was clearly demonstrated in whole cells [5], spheroplasts and heterocysts [6], there is only one report of oxidative phosphorylation measured in isolated membranes [7]. This lack of data is probably due to the difficulty in preparing intact membranes which are able to build up by a respiratory electron transport a protonmotive force high enough to drive phosphorylation. However, the use of a thermophilic cyanobacterium could yield more stable membranes with higher activities.

In the present work we describe oxidative phosphorylation in isolated energy-transducing membranes of the thermophilic cyanobacterium *M. laminosus*. The characterization of the respiratory electron transport as the driving force for oxidative phosphorylation is published in the preceding paper [8]. Besides the energy-dependent ATP synthetase/ATPase reaction, an adenylate kinase activity as well as a pronounced ADP-P_i exchange activity is measured in the dark. The latter activity is further characterized and identified.

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Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; CF_1, coupling factor isolated from spinach; DCCD, *N,N'*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EDTA, ethylenediamine tetraacetic acid; P/O, ATP synthetized per two electrons transported; PMS, phenazine methosulphate.

Materials and Methods

Cell growth favouring respiration (0.4‰ CO_2 at 40°C), membrane preparation and measurement of electron transport was performed as described in the preceding paper [8]. If not stated otherwise, the reaction mixture contained 20 mM Tricine-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM ADP and 1mM P_i with or without 5 mM NADH. Cyclic photophosphorylation was mediated by 50 μM PMS and non-cyclic photophosphorylation by 1 mM ferricyanide. For the measurement of the ADP-P_i exchange and phosphorylation reaction, carrier-free $^{32}P_i$ ($1 \cdot 10^5$ cpm per sample) and for the measurement of the adenylate kinase reaction, carrier-free [^{14}C]ADP ($1 \cdot 10^3$ cpm per sample) were added. If not stated otherwise, the reactions were carried out for 5 min at 20°C, either in the dark or in the light, and were stopped by adding 5% trichloroacetic acid.

Nucleotides and P_i were separated on charcoal and Dowex columns as described by Vinkler et al. [9]. Total esterified $^{32}P_i$ was measured according to the method of Avron [10]. ^{32}P was measured by Cerenkov radiation in water and ^{14}C in an organic scintillation cocktail.

Mg- and Ca-dependent ATPase was measured as described earlier [4] and ATP-P_i exchange as reported by Pick [11]. Chlorophyll and protein determinations were done as described in the preceding paper [8].

The cyanobacterial coupling factor from *M. laminosus* was extracted with EDTA or chloroform and isolated as described earlier [4]. The polynucleotide phosphorylase (E.C. 3.1.3.33) in this preparation was separated from the ATPase activity by sucrose gradient centrifugation [4] or on a Poly(U)-Sepharose 4B column (3 mm × 7 cm). The column was equilibrated and eluted with Tris-HCl (pH 7.5). For the latter experiments glassware and solutions were autoclaved prior to use in order to avoid RNase activity.

All activities are expressed in nmol O_2 or P_i per mg protein per min. They represent an average of at least three experiments. The chlorophyll-to-protein ratio is 1 : 20, as was determined in the preceding paper [8].

Results

Oxidative phosphorylation

In the presence of ADP and P_i, adenylate kinase and ADP-P_i exchange reactions can be measured in the dark with isolated membranes independent of the electron transport: for oxidative phosphorylation, however, an electron donor is necessary (Table I). The adenylate kinase activity alone is measured by the production of [^{14}C]AMP from [^{14}C]ADP. This activity is inhibited to 75% by the adenylate kinase inhibitor diadenosine pentaphosphate. The ADP-P_i exchange activity alone is monitored by the appearance of [^{32}P]ADP from ADP and $^{32}P_i$. This activity increases 20–25% with the addition of NADH, i.e., when an electron

TABLE I
REACTIONS OF ADENYL NUCLEOTIDES IN ISOLATED MEMBRANES IN THE DARK
All activities were measured as described in Materials and Methods.

Reaction	Analyzed product	Activity (nmol per mg prot. per min)	
		−NADH	+NADH
Adenylate kinase:	[^{14}C]AMP		
2[^{14}C]ADP = [^{14}C]AMP + [^{14}C]ATP		24.2	24.8
+ 5 μM diadenosine pentaphosphate		6.1	–
ADP-P_i exchange:	[^{32}P]ADP		
ADP + $^{32}P_i$ = [^{32}P]ADP + P_i		5.2	6.4
All reactions involved:	[^{32}P]ATP		
ADP-P_i exchange + adenylate kinase:		0.5	3.4
ADP + $^{32}P_i$ = [^{32}P]ADP + P_i			
[^{32}P]ADP + ADP = [^{32}P]ATP + AMP		0.5	0.6
Oxidative phosphorylation:			
ADP + $^{32}P_i$ = [^{32}P]ATP		0	2.8

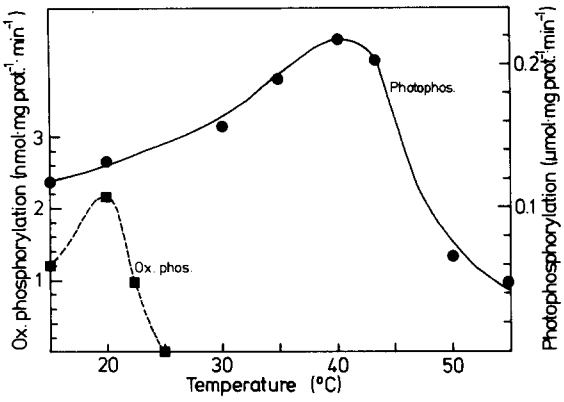

Fig. 1. Temperature dependence of phosphorylation and proton uptake. Cyclic phosphorylation (●———●), oxidative phosphorylation (■-----■).

transport is induced in the membranes. This increase is due to the activity of the adenylate kinase producing more [^{32}P]ADP from the increased level of [^{32}P]ATP originating from oxidative phosphorylation. The total $^{32}P_i$ esterified found in ATP is thus composed of all three activities as follows. (i) Under the conditions where no electron transport occurs (no NADH), the [^{32}P]ATP derives from the combination of ADP-P_i exchange and adenylate kinase activity, whereas oxidative phosphorylation is zero. (ii) When the dark electron transport is activated with NADH, the 7-fold increase of [^{32}P]ATP can clearly be attributed to oxidative phosphorylation. The ATPase and ATP-P_i exchange activity were also measured but were found to be negligible under the present conditions (results not shown).

Oxidative phosphorylation and photophosphorylation show quite a different temperature dependence (Fig. 1): the optimal temperature for oxidative phosphorylation is 20°C, whereas the one of the PMS mediated cyclic photophosphorylation is 40°C. As was discussed in the preceding paper, the temperature optimum of the electron transport in the dark as well as in the light is about 45°C.

The P/O ratios of the dark and the light reactions are summarized in Table II. At low electron transport activities, both in respiration and in photosynthesis – e.g., at low light intensities – the P/O ratios are low (about 0.2). Yet, at high photosynthetic electron transport activites (high light intensities), the P/O ratio increases to 0.8.

Oxidative phosphorylation in membranes of *M. laminosus* can further be characterized by the sensitivity towards uncouplers and energy-transfer inhibitors (Table III). The uncouplers CCCP and ammoniumchloride as well as the energy transfer inhibitor DCCD inhibit phosphorylation completely. The uncoupler nigericin and the ATPase

TABLE II
P/O RATIOS IN ISOLATED MEMBRANES IN THE DARK AND IN THE LIGHT
All reactions were performed as described in Materials and Methods.

Phosphorylation (nmol ATP per mg prot. per min)	Electron transport (nmol O_2 per mg prot. per min)	P/O ratios
Respiration (NADH to O_2)		
4.2	12.4	0.17
3.5	9.2	0.19
4.3	8.5	0.25
Photosynthesis (H_2O to ferricyanide)		
high light (50 W/m^2)		
88.8	52.0	0.80
low light (2 W/m^2)		
7.7	15.0	0.25

TABLE III
INHIBITOR SENSITIVITY OF OXIDATIVE AND LIGHT-DRIVEN PHOSPHORYLATION IN ISOLATED MEMBRANES
Oxidative phosphorylation: 100% = 3.5 nmol ATP per mg protein per min; light-driven cyclic phosphorylation: 100% = 240 nmol ATP per mg protein per min.

Inhibitors	Phosphorylation (%)	
	oxidative	light driven
Control	100	100
5 mM NH_4Cl	33	21
10 mM NH_4Cl	0	0
10 µM CCCP	0	0
40 µM nigericin	31	42
5 mM phlorizin	24	30
50 µM DCCD	0	0

TABLE IV
Mg-DEPENDENT DARK REACTIONS BETWEEN NUCLEOTIDES AND P_i IN ISOLATED MEMBRANES IN THE ABSENCE OF ELECTRON TRANSPORT
All measurements were done as described in Materials and Methods in the absence of NADH but at 50°C. Activities are given in nmol P_i per mg protein per min.

Treatment	ADP-P_i exchange	ATP-P_i exchange	ATPase
Control	27	11	120
50 μM CCCP	26	2	130
5 μM DCCD	25	4	35
1 mM EDTA (after centrifugation and washing)	2	0	10

inhibitor phlorizine only inhibit 60–70%. Photophosphorylation reacts similarly towards these inhibitors.

ADP-P_i exchange activity

In contrast to most other energy-transducing membranes, the isolated membranes of *M. laminosus* have a pronounced ADP-P_i exchange activity. The question arises whether this activity is related to the phosphorylation reaction. In isolated membranes it can be shown that the ADP-P_i exchange activity is sensitive neither to uncouplers nor to energy-transfer inhibitors (Table IV). The

TABLE V
CHARACTERIZATION OF THE ADP-P_i EXCHANGE REACTION IN AN AF_1-COUPLING-FACTOR PREPARATION OF *M. LAMINOSUS*
All reactions were done as described in Materials and Methods, but at 50°C. The Ca-dependent reactions were done in the presence of 15 mM $CaCl_2$. Activities are given in nmol P_i per mg protein per min.

Additions and treatment	ADP-P_i exchange	ATPase
Ca-dependent		
Control	10	1700
Trypsin activated	8	22000
Mg-dependent		
Control	210	1450
5 mM ATP added	200	–
5 mM AMP added	210	–
Adenylate kinase added	70	–
Afer Poly(U)-Sepharose column	50	1650

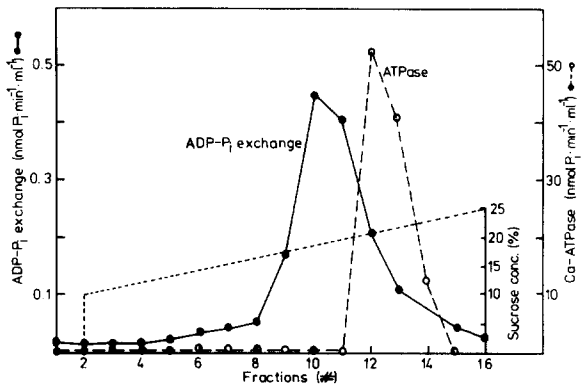

Fig. 2. Separation of the ADP-P_i exchange from the ATPase activity by sucrose gradient centrifugation. Centrifugation: 4 h at 50000 rpm and 25°C in a Beckman rotor 60Ti (sucrose gradient, 10–25%; sample volume, 1 ml; fraction volume, 2 ml).

comparison with the ATPase and the ATP-P_i exchange activity shows that the ADP-P_i exchange reaction behaves very differently. Yet, with EDTA all three activities can be extracted from the membranes. In such an extract and even in a purified coupling factor preparation, the ADP-P_i exchange activity can be demonstrated, (Table V). Unlike the ATPase activity, the exchange reaction is strictly Mg-dependent and is not influenced by trypsin, ATP or AMP, but it is inhibited by added adenylate kinase. This shows that one product of the reaction with ADP and $^{32}P_i$ is indeed [^{32}P]ADP.

The ATPase- and ADP-P_i activities in a purified coupling factor preparation can clearly be separated by sucrose density gradient centrifugation (Fig. 2). This indicates that the ADP-P_i exchange activity is not carried out by the coupling factor but by a different enzyme. In order to identify this enzyme, the coupling factor extract was loaded on a Poly(U)-Sepharose 4B affinity column. The results show that the ADP-P_i exchange activity is retained to 75% on the column whereas the ATPase activity is not absorbed (Table V). This separation suggests that the ADP-P_i exchange reaction is an activity of a polynucleotide phosphorylase contaminating the coupling factor preparation.

Discussion

Respiration in cyanobacteria is in general very low – just high enough to maintain a minimal

energy level during dark periods. In vivo respiration and respiratory electron transport in isolated membranes are 10–20 times lower than the corresponding photosynthetic reactions, as was discussed in the preceding paper. The activity for oxidative phosphorylation in isolated membranes is even 20–50 times lower than the one for photophosphorylation. This may be due to a denaturing process during membrane preparation, i.e., membranes are more leaky in the cell-free system than in vivo in the cell. Thus, higher electron transport activities are necessary to create a protonmotive force, large enough to drive phosphorylation.

Oxidative phosphorylation has a lower temperature optimum (20°C) than the growth temperature of the cells (40–50°C), whereas photophosphorylation in strong light (high electron transport rates) shows its optimum at temperatures close to the growth temperature. A similar dependence in thylakoids of higher plants was shown by Admon et al. [12] and Graan and Ort [13]. This phenomenon can be explained as follows: membranes are more leaky at higher temperatures, but these leaks are compensated for by higher electron-transport activities resulting in higher phosphorylation rates. This is observed with the PMS-mediated reaction in the light but not with the low respiratory activities. The same holds for low P/O ratios at low electron transport activities and high ratios at high activities, i.e., only high electron transport activities are able to compensate for the leaks.

Although the reactions with adenine nucleotides are very complex and may mask net ATP synthesis, oxidative phosphorylation has been clearly demonstrated: (i) by the susceptibility of the phosphorylation towards uncouplers and energy-transfer inhibitors and (ii) by the correlation of the oxidative phosphorylation with the respiratory electron transport. Non-respiring cells grown at high CO_2 concentrations (see preceding paper, Ref. 8) never show oxidative phosphorylation.

Uncouplers and energy-transfer inhibitors block both light- and substrate-driven phosphorylation reactions to the same extent, which shows that oxidative phosphorylation takes place at the coupling factor ATPase complex (AF_1) as does photophosphorylation. Depletion and reconstitution experiments with oxidative phosphorylation are difficult to perform with membranes of *M. laminosus* because of the very low activities. Yet, a slight reconstitution of oxidative phosphorylation of about 20% could be achieved with the addition of a coupling factor extract from the same species to partly depleted membranes.

The ADP-P_i exchange activity in these isolated membranes is an intriguing reaction, which complicates the interpretation of other reactions of adenine nucleotides with phosphate, as well as the determination of the proper phosphorylation activity. Yet, our experiments clearly show that the ADP-P_i exchange activity is attributed to a membrane bound polynucleotide phosphorylase, which is extracted together with the coupling factor. This phosphorylase has a slightly lower molecular weight than the ATPase. A similar polynucleotide phosphorylase was identified in membranes and F_1-ATPase extracts of *Escherichia coli* (Heppel, L.A., personal communication).

In the present paper, oxidative phosphorylation in isolated membranes of *M. laminosus* is demonstrated as had been predicted from respiration measurements in whole cells. It is also evident that the coupling factor F_1, which was described in many respiratory as well as photosynthetic systems, does obviously function for both systems in these cyanobacterial energy-transducing membranes.

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