ISOLATION AND CHARACTERIZATION OF A COUPLING FACTOR I ATPase OF THE THERMOPHILIC BLUE–GREEN ALGA (CYANOBACTERIUM) *MASTIGOCLADUS LAMINOSUS*

Andres BINDER and Reinhard BACHOFEN
Institut für Pflanzenbiologie, Universität Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland

Received 11 May 1979

1. Introduction

Coupling factor I ATPases have been isolated from a variety of energy transducing membranes, i.e., from mitochondria [1], mesophilic and thermophilic bacteria [2,3] showing oxidative phosphorylation as well as from chloroplasts [4] and bacteria [5] able to synthesize ATP in the light. All these ATPases are very similar: they have mol. wt ~ 350 000 and consist of 4–5 subunits with mol. wt 12 000–60 000. In all cases the coupling factor complex is easily extractable from the membrane and is water soluble. In solution the enzyme shows a Ca^{2+}-dependent ATPase activity which is latent in some cases [4]. Such a coupling factor ATPase has not yet been described from cyanobacterial membranes.

Cell-free membranes from cyanobacteria with high activity in light-driven electron transport and photophosphorylation are delicate to prepare and unstable [6]. However we succeeded [7] in isolating photosynthetically active and stable membranes from the thermophilic cyanobacterium *Mastigocladus laminosus*. We report here the isolation and characterization of a coupling factor ATPase from this thermophilic organism.

2. Materials and methods

2.1. *Culture*

Mastigocladus laminosus was obtained from a hot spring in Sudur Reykir, Iceland as in [8]. The cultures were grown as 9 l batches in 10 l bottles in medium D of [9] at pH 8.2, stirred with a magnetic stirrer in a temperature-controlled room at 45°C. The cultures were gassed with air which was saturated with water and supplemented with 5% CO_2. Illumination was with Osram Universal-White lamps at ~ 50 000 lux. Growth was stopped in the exponential phase after 4–5 days by transferring the culture bottles to 4°C. After 24 h, the sedimented cells were harvested by centrifugation (2 min at 2000 × *g*). The cells were then resuspended in a buffer containing 20 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 5 mM Na-K-phosphate (pH 7.8) and 0.5 M mannitol.

2.2. *Membrane preparation*

Lysozyme was added to the cell suspension (0.3–0.5 mg chl/ml) to give 0.5% (w/v) final conc. The mixture was homogenized and incubated at 50°C for 90 min in the dark. The reaction was stopped in ice and centrifuged for 2 min at 2000 × *g*. The pellet was resuspended and diluted to 5–10 μg chl/ml in a buffer containing 20 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$ and 5 mM Na-K-phosphate. The suspension was kept at 4°C for 12–15 h, centrifuged and washed 5 times in the same buffer until the supernatant was free of the blue phycocyanin.

2.3. *Coupling factor preparation*

For the extraction of the coupling factor with chloroform the method in [10] was slightly modified: The washed membranes were resuspended in 20 mM Tris–HCl buffer (pH 7.8) containing 2 mM EDTA

Abbreviations: DTT, dithiothreitol; CF_1, chloroplast coupling factor; chl, chlorophyll; AF_1, cyanobacterial (algal) coupling factor

Elsevier/North-Holland Biomedical Press

and 2 mM ATP to 0.5 mg chl/ml. Twice the volume of chloroform was added to the aqueous membrane suspension and the mixture shaken for 5 s. The two phases were separated by low speed centrifugation (300 × *g*) and the aqueous phase was removed and centrifuged at 12 000 × *g* for 30 min. The whole procedure was performed at room temperature. The slightly blue supernatant contained the latent ATPase activity.

For further purification, the supernatant was dialyzed against 50 mM Tris–HCl buffer (pH 7.8), 1 mM ATP and 2 mM EDTA, centrifuged at 150 000 × *g* for 30 min to remove remaining membrane particles and concentrated to a minimal volume in an Amicon ultrafiltration apparatus with a Diaflo XM-50 membrane. The clear protein solution (1 ml) containing 2–5 mg protein was layered on 34 ml of a 10–25% (w/v) sucrose gradient in 20 mM Tris–HCl (pH 7.8), 2 mM EDTA. The gradients were prepared as in [11]. The samples were centrifuged for 180 min at 55 000 rev./min and 25°C in a Beckman Ti-60 rotor (density gradient centrifugation I). After fractionation of the gradient, ATPase activity was checked and the purity of the preparation was estimated from the pattern of protein bands in slab gel electrophoresis with and without SDS. When desired, the purification on sucrose gradients was repeated (density gradient centrifugation II).

2.4. *Analytical methods*

If not stated otherwise, the latent ATPase of the coupling factor was activated with trypsin (Fluka, 3 × cryst.) at 50 μg/ml for 12 min at room temperature in 20 mM Tris–HCl buffer (pH 8.0) and 2 mM EDTA (total vol. 125 μl). The ATPase activity was assayed by adding 50 mM Tris–HCl (pH 9.0), bovine serum albumin 0.1 mg/ml, $CaCl_2$ 20 mM and ATP 5 mM (total vol. 1 ml). After 10 min incubation at 50°C, the reaction was stopped by adding 2 ml molybdate reagent for phosphate determination as described [12] in the presence of 2% SDS. The P_i complex was measured at 740 nm.

Protein concentration was determined according to [13] using the modification [14]. Chlorophyll-*a* was extracted in 80% acetone and measured at 665 nm according to [15].

Polyacrylamide gel electrophoreses with and without 0.1% SDS was performed on a vertical slab gel apparatus as in [11] with the buffer system as in [16].

3. Results and discussion

Table 1 shows a typical purification experiment starting with 11.2 g alga and giving 1.2 mg pure algal coupling factor (AF_1) with spec. act. 12.1 μmol P_i .mg protein^{-1} .min^{-1}. The profile of the sucrose gradient I is given in fig.1. The protein peak collected in fraction number 1–4 is blue and contains mostly C-phycocyanin with mol. wt 96 000. The ATPase activity coincides with a protein peak in fractions 6–10 but according to polyacrylamide gel electrophoreses without SDS some impurities are still present (not shown). The active fractions are further

Table 1
Purification of AF_1

Purification step	Total protein (mg)	ATPase activity		
		Total units (μmol P_i · min^{-1})	(%)	Spec. act. (μmol P_i .min^{-1} .mg protein^{-1})
Chloroform extraction	12.8	51.6	100	4.0
Density gradient centrifugation I	2.1	22.1	42	10.5
Density gradient centrifugation II	1.2	14.5	28	12.1

From 11.2 g wet wt cells, giving washed membranes with 27.5 mg chl. The extraction and purification was performed as described in section 2

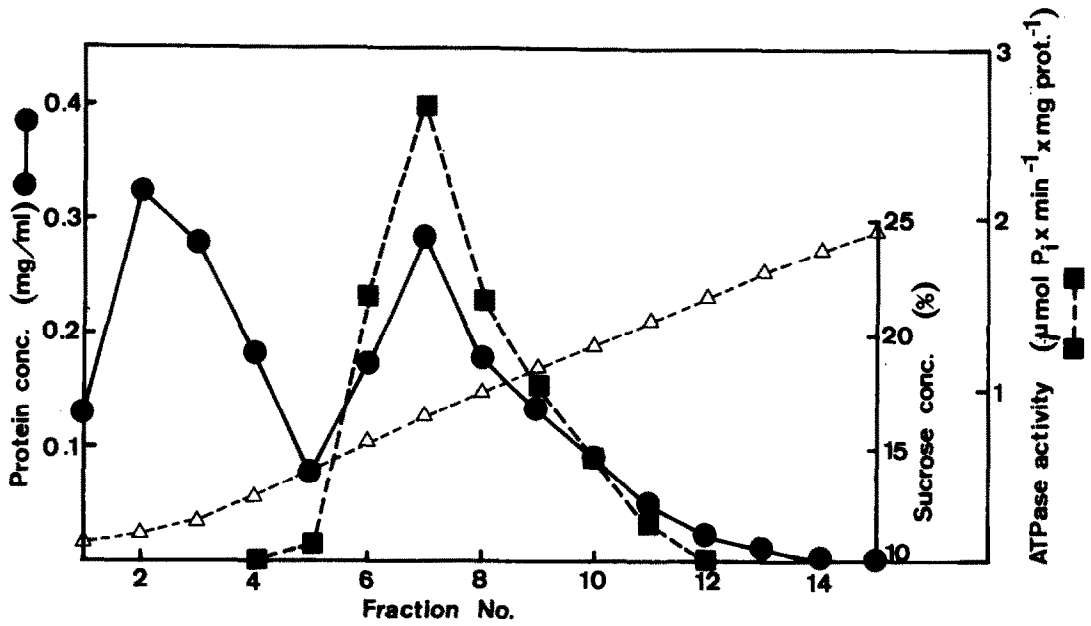

Fig.1. Density gradient centrifugation I. Crude extract (1 ml) containing 6 mg protein was loaded on a sucrose gradient as in section 2. Density was measured spectrophotometrically by adding bromphenol blue to the dense solution prior to formation of the gradient. Fractions (2 ml) were collected.

purified on a second identical sucrose gradient (density gradient centrifugation II). After this step AF_1 is considered pure giving only one band in gel electrophoresis without SDS (fig.2A). Spinach coupling factor (CF_1) with mol. wt 320 000 centrifuged on the same sucrose gradient is eluted at the same density as AF_1 (not shown). From this we can conclude that the molecular weight of AF_1 is similar to the one of CF_1.

The subunit pattern of AF_1 on SDS–gel electrophoresis is shown in fig.2B. The 4 subunits are similar to the subunits α, β, γ and ϵ of CF_1. When AF_1 is extracted with chloroform as described here, no δ is seen. The lack of δ was also observed with CF_1 when extracted with chloroform [17]. The EDTA extraction reported earlier for the preparation of CF_1 [11] can also be used to obtain active AF_1 (not shown).

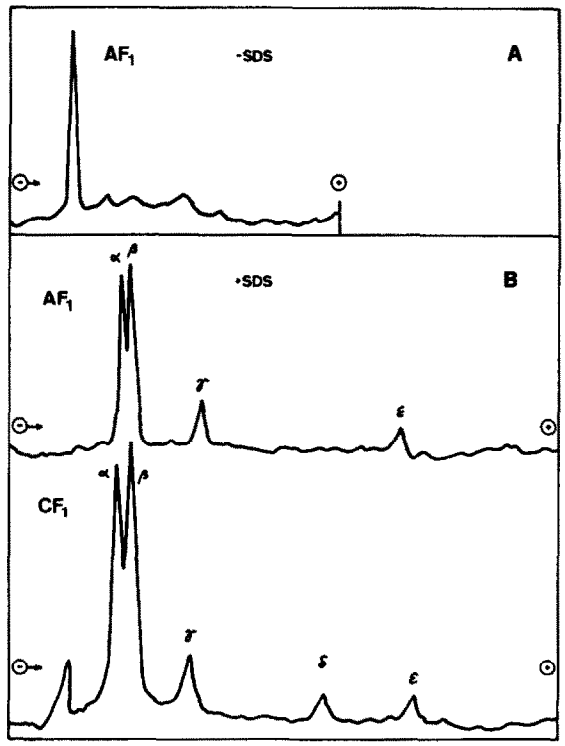

Fig.2. Polyacrylamide gel electrophoresis of AF_1 after density gradient centrifugation II. (A) 7.5% slab gel without SDS; (B) 12.5% slab gel with 0.1% SDS. Spinach CF_1 was prepared as in [11]. Further details are given in section 2.

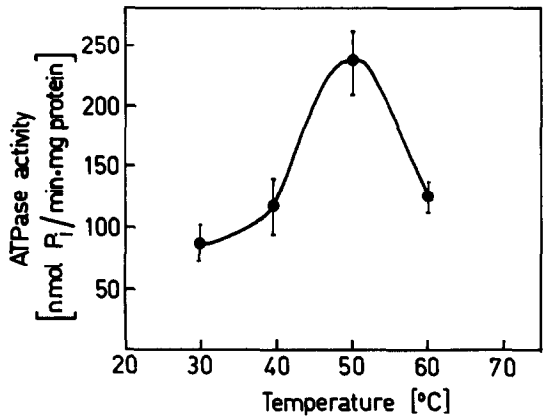

Fig.3. Temperature dependence of AF_1-ATPase activity. An AF_1 extract was assayed for 10 min at the given temperature and as in section 2.

But also under these milder conditions where CF_1 usually has all 5 subunits (fig.2B), AF_1 does not show any δ subunit.

Figure 3 shows the temperature dependence of the ATPase activity. The optimum lies at 50°C, which is similar to the optimal growth temperature of the alga.

Figure 4 demonstrates the dependence of the ATPase activity on trypsin activation. It shows that, as in CF_1, the AF_1 ATPase activity is latent and has to be activated. The optimal concentration with 12 min activation lies at 0.5 mg trypsin/ml. In CF_1 the optimal conditions are 0.25 mg/ml for 6 min. From these results we can conclude that the inhibitor of AF_1 is not digested by trypsin as effective as in CF_1. CF_1 can also be activated by heat and DTT [18]. In AF_1 this activation yields only 30–40% of the activity as compared with trypsin activation (not shown). Temperatures > 60°C do not improve the activation.

Figure 5 shows that a minimal concentration of 20 mM $CaCl_2$ is necessary in order to get optimal ATPase activity in AF_1 at 50°C; whereas for CF_1 only 5 mM $CaCl_2$ is sufficient. Mg^{2+} gives about 25% of the activity when compared with Ca^{2+} (not shown). This behaviour is similar to the one found in CF_1.

As presented in table 2, AF_1 can be stored at room temperature without significant loss in activity. Similar to the other coupling factors, AF_1 is cold labile in the absence of ATP. The best storage conditions for AF_1 tested, were found to be 50% glycerol containing 1 mM ATP at –25°C. A slightly increased activity is due to the stimulating effect of glycerol. After freezing without glycerol or after

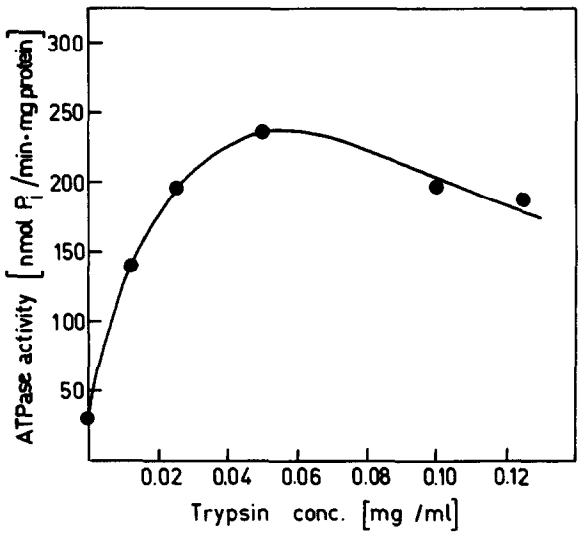

Fig.4. Trypsin activation of AF_1. An AF_1 extract was activated for 12 min with the given concentrations of trypsin. The ATPase assay was done as in section 2.

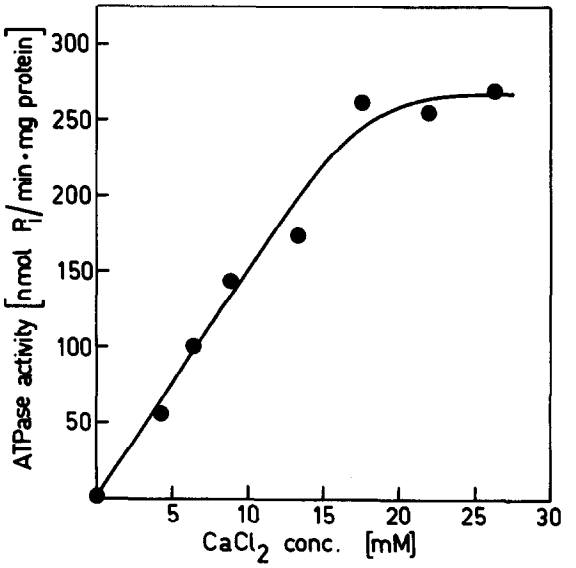

Fig.5. Dependence of AF_1-ATPase activity on $CaCl_2$. An AF_1 extract was assayed with the given concentration of $CaCl_2$ and as in section 2.

Table 2
Storage of AF_1

Storage conditions	ATPase act. (%)
With 1 mM ATP at 20°C	95
With 1 mM ATP at 4°C	92
Without ATP at 4°C	13
With 1 mM ATP and 50% glycerol at −25°C	113
With 1 mM ATP at −25°C	0
Ammoniumsulfate precipitation (80% sat.) at 4°C	0

AF_1 was stored for 1 week in 50 mM Tris–HCl buffer (pH 7.8), 2 mM EDTA under the conditions described. The activity is given in % of the original activity (12.1 μmol P_i .min^{-1} .mg $protein^{-1}$) measured in the above buffer

ammoniumsulfate precipitation the enzyme activity is lost.

Although AF_1 has some unique properties compared with other coupling factor ATPases, i.e., thermostability, high Ca^{2+} requirement for activity and high trypsin requirement for activation, we can conclude from these data, that AF_1 resembles spinach CF_1 best. Moreover we showed [7], that CF_1 is indeed closely related to AF_1 being able to plug the proton channels of F_0 in the algal membrane in the place of AF_1. However the cross reconstitution of the ATP synthase activity with CF_1 and algal membranes was not possible. The resemblance between CF_1 and AF_1 would support the postulated close relationship between cyanobacteria and chloroplasts.

AF_1 is thermostable as is TF_1, the coupling factor of the thermophilic bacterium PS3 [3]. However many properties of TF_1 are quite different from AF_1, e.g., TF_1 need not be activated and its stability is not dependent on ATP.

Further characterization of the thermostability and activity of AF_1 including the reconstitution to the membranes is currently under study.

Acknowledgements

Parts of the experiments were performed by Markus Wolf. The work was supported by the Swiss National Foundation grant no. 3.041.76.

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