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A rapid, simple method for the isolation and characterization of the photoreceptor of *Dictyostelium discoideum* amoebae

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Abstract A membrane-bound 45.5 kDa protein has been isolated from *Dictyostelium discoideum* amoebae. It shows an absorption spectrum, which closely resembles the action spectrum for amoebal phototaxis, leading to the conclusion that this protein might play an important role in the photoreception of *Dictyostelium* amoebae. For further characterization we employed phase partition in an aqueous polymer two-phase system, which was developed by Widell and Larsson for the separation of plasma membrane proteins of higher plants. This method clearly shows that the 45.5 kDa protein is a plasma membrane protein and not an intracellular protein. Furthermore, by using phase systems with increasing polymer concentrations, this simple and rapid purification of plasma membrane proteins allowed us to isolate the putative photoreceptor in one single step. Compared to standard biochemical methods phase partition provides an enormous facilitation of the isolation of *D. discoideum* membrane proteins.

Key words: Dictyostelium discoideum amoeba; Phototaxis; Phase partition; Plasma membrane protein

1. Introduction

Dictyostelium discoideum is a haploid eukaryotic organism which offers a number of features for biochemical investigations. Because of its easy handling this organism has developed into a eukaryotic model system which serves as a useful object to study basic cellular processes at a molecular level [1–3].

The organism which was first described by Raper in 1935 [4] shows an interesting alteration in its vegative cycle between unicellular amoebae and multicellular pseudoplasmodia, also called slugs because of their morphology. The free-living amoebae feed upon bacteria which are sensed chemotactically [5], or in the case of axenically grown cells, take up organic media by fluid phase endocytosis. Upon starvation the cells undergo a developmental cycle, leading to the formation of slugs and, in the end, to the formation of fruiting bodies [2].

Both slugs and amoebae use a number of external stimuli to orientate themselves in their habitat [6–8] and are both able to detect light and react to it: the slugs move towards a given light source, thus showing positive phototaxis. The action spectrum for slug phototaxis shows two peaks at about 420 and 440 nm, a broad maximum at 560 nm and a minor peak at 610 nm [9]. The mechanism for light perception is supposed to be based on a lens effect where the light is focussed at the distal side of the slug [8,10], inducing the production of a yet unknown, low molecular weight metabolite, called slug turning factor (STF) [11].

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The photoreceptor for slug phototaxis has not yet been isolated and characterized. Poff and co-workers connected the primary photochemical reaction with a light-induced oxidation of a high spin heme [12]. This hypothesis was modified by assuming the involvement of a flavin and a cytochrome b_2 as chromophoric groups located on the same protein [13]. Amoebae show positive phototaxis at low irradiances (<0.1 $W \cdot m^{-2}$) [14] which changes to negative phototaxis at higher irradiances [15]. The assumption that slug phototaxis differs from amoebal phototaxis is based on three additional facts:

(i) The action spectrum for amoebal phototaxis differs significantly from that of slug phototaxis [9,14].

(ii) Amoebae cannot use the above discussed lens effect because of their small size and highly variable form.

(iii) A mutant was isolated lacking slug phototaxis, but showing clear amoebal phototaxis [16].

Experiments with light spots directed at the cell surface of the amoebae suggested that the photoreceptor is located in the cytoplasmic membrane or in the ectoplasm [17]. Based on these experiments, we focussed our interest on membrane proteins of *D. discoideum* amoebae and were able to isolate a 45.5 kDa protein. The absorption spectrum of this protein closely resembles the action spectrum for photoaccumulation of amoebae in light traps [18,19]. The pattern of difference spectra of light minus dark grown cultures are similar to the absorption spectrum of protoporphyrin IX, indicating that this may be the chromophoric group localized on the 45.5 kDa protein [20].

The aim of the present study was to obtain information on the exact localization of the 45.5 kDa protein within the cell, by using a two phase aqueous polymer system. Similar extractions are described for the isolation and characterization of membrane proteins of higher plants, such as maize [27], pea [28,29] and cauliflower [30].

2. Materials and methods

2.1. Strains and culture conditions

The axenic strain AX2 of *Dictyostelium discoideum* was used for all experiments. The cells were grown in 5 l Erlenmeyer flasks filled with 2 l HL5-medium [21]. Streptomycin (250 μg/ml) was added to prevent bacterial growth. Fresh flasks were inoculated with 1 ml of cell suspension in the late exponential phase ($3–5 \times 10^6$ cells/ml) and kept on a rotary shaker (125 rpm) for 6 days at 21°C in the dark.

2.2. Membrane isolation

Unless otherwise indicated all of the following steps were carried out at 4°C in the dark. Cells in the late exponential phase were harvested by centrifugation (refrigerated centrifuge J2–21M/E, 600 × *g*, 30 min, rotor JA10, Beckman, Palo Alto, USA) and washed twice with 5 mM potassium phosphate buffer, pH 8.0. The washed cells were resuspended in 80 ml of the same buffer and sonicated for 90 s at 40% duty cycle (output 5, Branson sonifier 450, Danburry, USA). Unbroken cells and cell debris were removed by a low speed centrifugation (600 × *g*, 10 min, rotor JA10, Beckman). The membrane particles were pelleted

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by ultracentrifugation (100,000 × g, 45 min, rotor 75TI, L8-M centrifuge, Beckman). To remove all of the supernatant (containing the cytosol fraction) the membrane pellet was washed twice and ultracentrifuged as above. The resulting pellet was resupended in 6.4 ml of 0.25 M sucrose, 4 mM KCl, 5 mM potassium phosphate, pH 8.0, to give a final protein concentration of 1.45 mg/ml. Protein concentration was quantified using the Bradford assay [22].

2.3. Partition in an aqeous polymer two-phase system

Two different polymers were used for the phase partition: polyethylene glycol (PEG) 3350 from a 40% (w/w) stock solution (SIGMA) and dextran T 500 (Pharmacia Fine Chemicals, Uppsala, Sweden). The exact concentration of the dextran solution was calculated according to Widell and Larsson (1987) [23] and adjusted to 20% (w/w) with water. The compositions of the various phase systems are given in Table 1; 0.8 g of the sample (corresponding to 1.16 mg protein) was added to 7.2 g phase mixture to yield 8.0 g phase system. The solution was thoroughly mixed and centrifuged at 1000 × g for 5 min in a swinging bucket rotor (rotor SW 40, Beckman) to facilitate phase separation. The upper phase, containing the plasma membrane fraction was removed and added to a tube containing fresh lower phase; this mixture was then repartitioned as described above to increase the purity of the plasma membrane preparation. The original lower phase, containing the bulk of intracellular membranes was treated in a similar way.

To lower the density of the solutions and to facilitate membrane pelleting, both the final upper and lower phase were diluted 10-fold with 5 mM potassium phosphate buffer, pH 8.0, and the membranes were pelleted at 100,000 × g for 45 min (rotor 75TI). The membrane pellets were resuspended in 1 ml 0.2% (w/v) Triton X-100, 5 mM potassium phosphate, pH 8.0 and stirred for 2.5 h. Undissolved material was removed by ultracentrifugation (100,000 × g, 1 h, rotor 75TI) and the supernatant was subjected to further experiments.

2.4. Gel electrophoresis

SDS polyacrylamide gradient gel electrophoresis was carried out in a vertical system (2001, Pharmacia LKB) with gels of 140 mm × 110 mm, 1.5 mm thick using the method described by Lämmli [24] with a gradient (8 to 20% T) in the resolving gel. The samples contained 0.1 mg protein per ml and were diluted with an equal amount of sample buffer. The gels were silver stained according to [25]. To determine the molecular weight, protein test mixtures 4 and 5 (SERVA) were co-separated.

3. Results and discussion

In previous investigations [18–20,26], a 45.5 kDa membrane-bound protein was isolated showing an absorption spectrum which closely resembles the action spectrum for amoebal phototaxis [14]. Based on the assumption that the action spectrum of a given organism and the absorption spectrum of a photoreceptor must match, it was concluded that this specific protein might be the photoreceptor and/or an antenna pigment. Furthermore, we could show that the binding of polyclonal antibodies, directed against the 45.5 kDa protein differed with

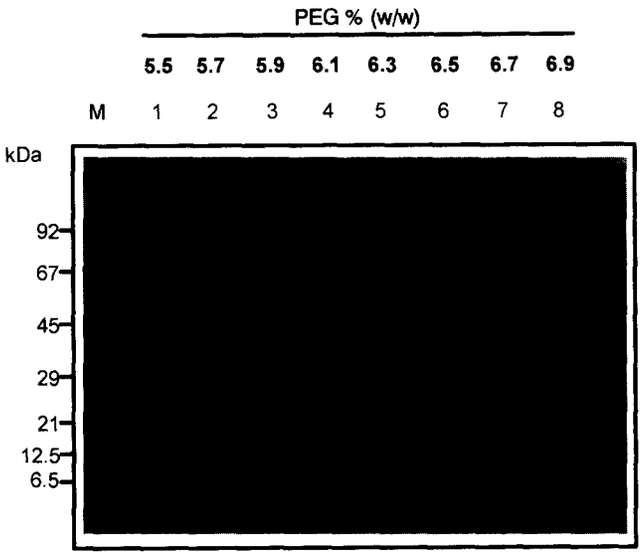

Fig. 1. SDS-PAGE with aliquots taken from different concentrations of the upper phase (PEG-phase). Each lane contained 100 μl with a protein concentration of 0.1 mg/ml. The gel was stained with silver nitrate according to [25]. M: marker proteins with molecular weights indicated at the left; lanes 1 to 8: aliquots of the upper phases. The different polymer concentrations are given at the top.

differing light conditions showing that irradiation triggered modifications of a yet unknown nature.

In this study we used a partition in an aqeous polymer two-phase system [23] to further characterize the membrane-bound proteins of *Dictyostelium* amoebae. Right-side-out membrane vesicles [30] were prepared using a phase partition with a phase system composed of two different polymers: PEG 3350 and dextran T500. Optimal polymer concentrations were determined by partitioning the material in a series of phase systems with increasing polymer concentrations from 5.5% to 6.9% (w/w). For the separation we used 8.0 g phase systems (Table 1).

After the phase settling the upper and lower phases were separated by a sharp interface. The bulk of the proteins (up to 60% of the total protein, depending on the polymer concentration) was found in this interface (data not shown). This material could not be characterized in terms of belonging to either the plasma or the intercellular membrane fraction.

During optimization a polymer concentration of 5.7% was found, where the 45.5 kDa protein is only detectable in the PEG

Table 1
Composition of 8.0 g phase systems in a polymer series

	Conc. of dextran and PEG % (w/w), respectively							
	5.5	5.7	5.9	6.1	6.3	6.5	6.7	6.9
Dextran, 20% (w/w) (g)	2.20	2.28	2.36	2.44	2.52	2.60	2.68	2.76
PEG 40% (w/w) (g)	1.10	1.14	1.18	1.22	1.26	1.30	1.34	1.38
Buffer medium[a] (g)	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
H_2O (g)	2.10	1.98	1.86	1.74	1.62	1.50	1.38	1.26
Sample (ml)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80

[a] 1 M sucrose, 16 mM KCl, 5 mM potassium phosphate, pH 8.0.
The solutions were weighed into transparent centrifuge tubes and then centrifuged to facilitate phase settling. After separating the two phases and pelleting the membrane fraction of each phase, aliquots were subjected to SDS-PAGE.

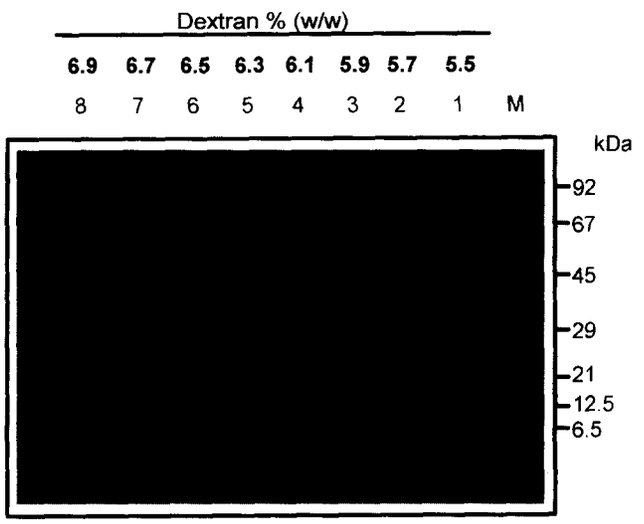

Fig. 2. SDS-PAGE with aliquots taken from different concentrations of the lower phase (dextran-phase). Details as in Fig. 1.

phase (lane 2, Fig. 1). In the corresponding 5.7% dextran concentration (lane 2, Fig. 2) the 45.5 kDa was not found, thus giving clear evidence that this specific protein is localized in the plasma membrane.

What was originally planned to be a method of characterization turned out to be a very effective way of isolating the 45.5 kDa protein. Using higher polymer concentrations (6.5 and 6.7%, lanes 6 and 7, Fig. 1), we were able to extract the putative photoreceptor into the PEG phase without any detectable contamination of other proteins.

In conclusion, two phase partitioning is a very powerful tool for the isolation and characterization of membrane proteins of *D. discoideum* amoebae. We are currently using this method on a larger scale (500 g phase system) to harvest larger amounts of pure material for spectroscopic measurements.

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