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SPECTRAL ANALYSIS OF FLUORESCENCE OF 8-ANILINO-1-NAPHTHALENESULFONATE IN CHEMORECEPTION WITH A WHITE PLASMODIUM OF *PHYSARUM POLYCEPHALUM*

EVIDENCE FOR CONFORMATIONAL CHANGE IN CHEMORECEPTIVE MEMBRANE

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Summary

A plasmodial mutant (strain Lu 647 × Lu 861) of *Physarum polycephalum* contains much less yellow pigment compared with the wild type. Thus, extrinsic fluorescence measurements should be possible with the use of this white plasmodium. We adopted 8-anilinonaphthalenesulfonate (ANS) as a fluorescent anion, and measured fluorescence spectra and polarization in conjunction with the membrane potential when the cell was stimulated with various salts. Results are:

(1) The membrane potential, $\Delta\varphi$, did not change until the concentration of stimulus chemicals exceeded respective thresholds, C_{th}. Above C_{th}, $\Delta\varphi$ depolarized linearly with log C. The Schulze-Hardy rule was applicable to C_{th}; i.e.

$$\log C_{th} = -6 \log z + \log K_H$$

where z is the valency of the cations. The Hamaker constant, K_H, displayed two distinct values depending on the cation species: K_H = 3 mM for Ca, La and Th (Group I) and K_H = 0.2 mM for Na, K, NH_4, Mg, Ba and Al (Group II). The value of K_H in the wild type was 3 mM for all polyvalent cations examined.

(2) When excited at 360 nm, ANS gave two emission maxima of 500—510 nm and 460 nm through interaction with the plasmodial membrane. Application of an Mg or Na salt of ANS below the respective C_{th} gave a single peak at 500 nm, while that above C_{th} caused an increase in fluorescence intensity at 460 nm.

(3) Fluorescence polarization, p, at 460 nm decreased appreciably when an

Mg or Na salt of ANS above the respective C_{th} was applied to the plasmodium. Addition of $CaCl_2$, $LaCl_3$ or $Th(NO_3)_4$ caused an increase in the p value, while that of KCl, NaCl, $MgCl_2$ or $AlCl_3$ did not.

Results described above indicate that the plasmodial membrane undergoes a conformational change upon salt reception. Based on the physicochemical properties of ANS, we may conclude that chemoreceptive membane of the plasmodium becomes more hydrophobic above C_{th}.

Introduction

Sensing and its transducing systems in response to various chemicals are widely found in lower organisms [1]. Plasmodia of *Physarum polycephalum* are well suited for studying the mechanisms of chemoreception and transduction because the large aggregate of protoplasm without boundary cell walls allows us to measure various physicochemical properties of the motile system as well as of the membrane [2,3].

In previous papers we reported results demonstrating that the surface membrane of the plasmodium changes its structure in responding to chemical stimuli: e.g., threshold phenomena in chemoreception and taxis [4], changes in charge density at the membrane surface [5], interference between chemical stimuli [6,7], changes in adhesive properties [8], etc. However, the evidence was largely phenomenological and indirect.

Here, fluorescence technique is applied to the study of molecular processes of chemoreception in the plasmodium of *Physarum polycephalum*. The fluorescence method has been found fruitful in the study of membrane systems such as mitochondria, lymphocytes, nerves, sarcolemma, etc. [9—14]. However, application of the method to the plasmodium of the wild type encounters severe difficulty due to the large amount of yellow pigment which emits strong fluorescence at 500—520 nm and interferes with the extrinsic staining of fluorescent dyes. Therefore, we used a white plasmodium of *Physarum polycephalum*, a mutant containing less yellow pigment.

8-Anilino-1-naphthalenesulfonates (ANS) can be considered as chemical stimuli having dormant fluorescence rather than as a dye used for membrane labeling. This possibility lies in the fact that ANS emits virtually no fluorescence in water and fluoresces strongly only in hydrophobic environments such as are found in the surface membrane. Spectral analysis and measurements of fluorescence polarization will elucidate the molecular nature of the conformational changes of the membrane on chemoreception.

Materials and Methods

Materials

The white plasmodium used was a strain (Lu 647 × Lu 861) supplied kindly by Dr. M. Carlile at Imperial College of London, U.K. The plasmodia were cultured on moist oats according to the method of Camp [15]. Before use, plasmodia were allowed to crawl on 0.8% agar gel overnight without feeding.

Magnesium and sodium salts of ANS, both of analytical grade, were pur-

chased from Tokyo Kasei Co. and Eastman Kodak Co., respectively. Other salts were analytical grade and used without further purification.

Measurement of membrane potential

Changes in the membrane potential in response to chemical stimuli were measured by applying a double-chamber method as described before [2,4]. Salts were dissolved in distilled water and used as chemical stimuli without buffering to avoid complicated interference between receptions of different chemical stimuli [7]. Solutions of monovalent and divalent salts and $LaCl_3$ gave a pH value of 5.3—5.5, while those of $AlCl_3$ and $Th(NO_3)_4$ gave a slightly acidic pH, of about 4.5.

Fluorescence measurement of ANS

Fig. 1 shows a schematic illustration of the experimental procedure and apparatus used for measuring fluorescence of ANS on chemoreception in the plasmodia. The advancing front region of the plasmodia (approx. 60 mg/cm^2) was cut with agar gel (1.2 × 2.0 cm), and placed in a chamber made of black Lucite (5 mm thick) (Fig. 1a, b). The use of a dense population of the plasmodia was favourable for obtaining sufficient fluorescence intensity and also for avoiding regional differences in the plasmodia. The chamber was closed with a slide glass after filling with an aqueous solution containing ANS. The chamber

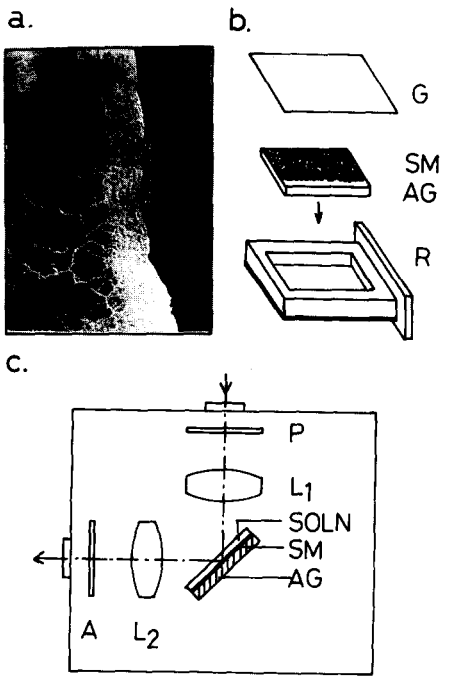

Fig. 1. Schematic illustration of experimental procedure and arrangement for measuring fluorescence of ANS on chemoreception in the plasmodia. (a) Advancing plasmodia of *Physarum polycephalum*: a part shown by dotted lines was cut with agar gel. (b) Details of experimental procedure and arrangement: G, slide glass; SM, slime mold; AG, agar gel; R, receptacle made of black Lucite. (c) top-side view of an arrangement for measuring fluorescence: P, polarizer; A, analyzer; L_1 and L_2, lenses.

was then set in a spectrophotofluorometer (Hitachi, model MPF-2A) as shown in Fig. 1c. Excitation light wavelength 360 nm, illuminating 10×1 mm plasmodium. Hereupon, the incident light was linearly polarized and the electric vector was vertical. Parallel and vertical components of fluorescence intensity with respect to the incident light, $I_{\parallel}$ and $I_{\perp}$, respectively, were measured as a function of wavelength.

The fluorescence polarization, p, was calculated according to the following definition [9,10]:

$$p = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

At a given concentration of chemicals, experiments were repeated three to four times and gave reproducible results.

All experiments were performed at a room temperature, 17—20° C.

Results

Changes in membrane potential in response to salts

Fig. 2a shows the dependence of the membrane potential, $\Delta\varphi$, on concentration of monovalent salts, NaCl, NaSCN, KCl and NH_4Cl. Here $\Delta\varphi$ is plotted against log C. For the sake of comparison, the corresponding data for the plasmodia of wild type in response to NaCl, KCl and NH_4Cl are shown by dotted lines in the same figure.

The membrane potential did not change until the concentration of salts exceeded respective thresholds, C_{th}. Above C_{th}, $\Delta\varphi$ depolarized linearly with log C, and changed about 20 mV for a ten-fold increase of concentration. These characteristics in the membrane potential are the same both for the mutant and the wild type. However, thresholds for monovalent salts in the white plasmodia decreased by 1/10 to 1/100 times in comparison with those in the wild type.

Changes in anion species, Cl to SCN in sodium salts, for example, as denoted by open and closed circles in the figure, respectively, produced no appreciable

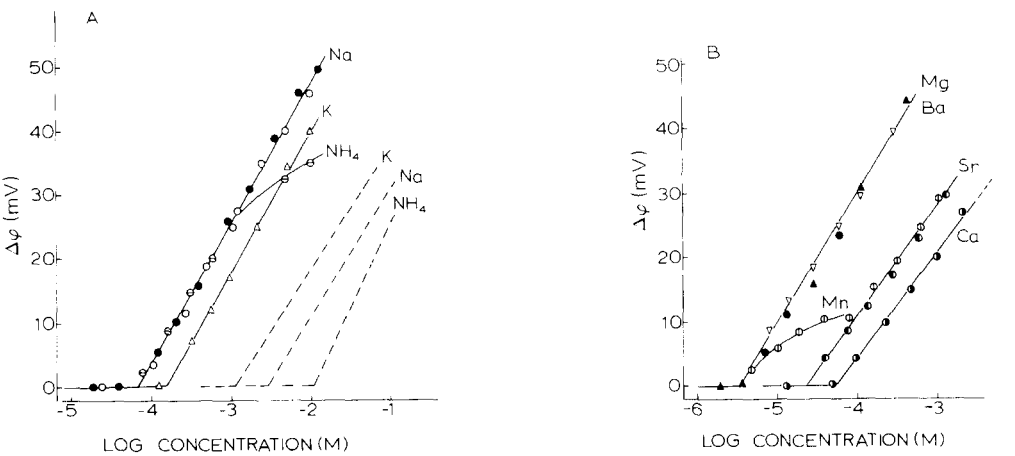

Fig. 2. Dependence of membrane potential on concentration salts. (a) ○, NaCl; ●, NaSCN; △, KCl; ⊖, NH_4Cl. (b) ▲, $MgCl_2$; △, $BaCl_2$; ⊕, $MnCl_2$; ◐, $SrCl_2$; ◑, $CaCl_2$.

difference in $\Delta\varphi$. This result contrasts to that for the wild type, where NaCl and NaSCN presented a distinct difference in respective thresholds [4].

Fig. 2b shows the same plots for divalent salts as in Fig. 2a. As in the case of monovalent salts, $\Delta\varphi$ did not change until the concentration of stimulus chemicals exceeded respective thresholds, C_{th}, and depolarized linearly with $\log C$ above C_{th}. $MnCl_2$ is an exception and produced complicated changes in $\Delta\varphi$. Plasmodia of the wild type gave an identical variation in responding to all the divalent salts examined (Ca, Sr, Mg, Ba, Mn) [4]. On the other hand, this degeneracy in the specificity of divalent cations splits into each cation species in the white plasmodia. Mg and Ba salts became 10 times more sensitive in the white plasmodia than in the wild ones. Sr and Mn caused intermediate responses.

$LaCl_3$, $AlCl_3$ and $Th(NO_3)_4$ induced similar changes in the membrane potential, giving a different C_{th} and 20 mV depolarization with a decade increase of salt concentration above the respective C_{th}.

Cation specificity in the Schulze-Hardy rule

The thresholds, C_{th}, for various salts decreased as the valency of cation, z, increased. Fig. 3 shows the plot of $\log C_{th}$ against $\log z$. Results indicate that the Schulze-Hardy rule is applied to the C_{th} [16]:

$$\log C_{th} = -6 \log z + \log K_H$$

Here, the constant K_H has two distinct values depending on cation species: 3 mM for Ca, La and Th (Group I) and 0.2 mM for Na, K, NH_4, Mg, Ba, Mn and Al (Group II). In short cations belonging to Group II are about ten times more sensitive than the corresponding ones with the same valencies in the white plasmodia. Note that the value of K_H is 3 mM for all polyvalent cations examined in the wild type [4].

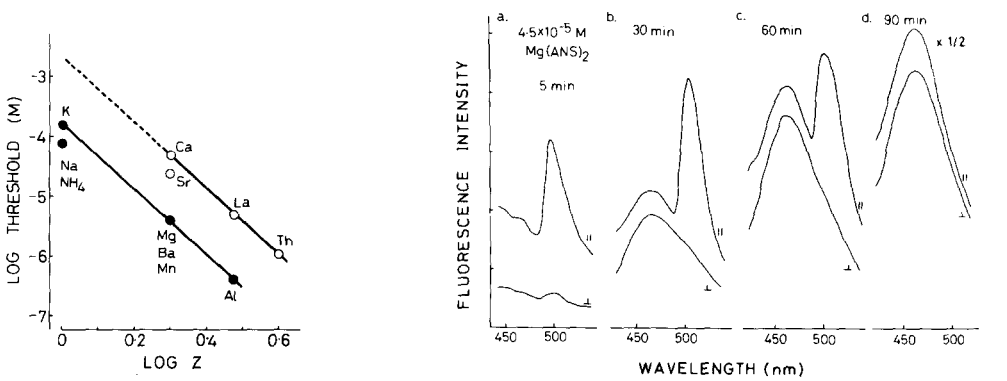

Fig. 3. Dependence of the threshold concentration on valences, Z, of cations.

Fig. 4. Time course of spectral pattern of ANS fluorescence after immersion of the plasmodia in $4.5 \cdot 10^{-5}$ M $Mg(ANS)_2$. ANS was excited by a vertically polarized light at 360 nm. $\parallel$ and $\perp$ indicate parallel and vertical components of fluorescence intensity with respect to incident light. Fluorescence intensity at the final stage (d) became a few times stronger than that of the initial level (a).

Spectral analysis of ANS on chemoreception of ANS salts

Fig. 4 shows changes in spectral pattern of ANS fluorescence with time when 4.5×10^{-5} M $Mg(ANS)_2$, well above C_{th} for Mg, was applied to the white plasmodia. Just after the application, ANS spectra exhibited a maximum at 500 nm. As time went on, fluorescence at 460 nm grew larger and larger and became the major emission at the final stage. An application of $Mg(ANS)_2$ below the C_{th} ($1 \cdot 10^{-5}$ M) did not induce any change in spectral patter, but fluoresced steadily at 500 nm, as shown in Fig. 4a.

Similar variation in spectral pattern with time was also observed when NaANS was applied to the white plasmodia. In this case, the threshold appeared at $1 \cdot 10^{-4}$ M of NaANS, which agreed with the threshold of NaANS determined from the membrane potential measurements as shown in Fig. 2a.

Fluorescence polarization of ANS on chemoreception

Data such as shown in Fig. 4 can also be analysed in terms of fluorescence polarization, p, a parameter of membrane fluidity as regards to the binding sites of ANS. Fig. 5 shows the time course of the fluorescence polarization, p, at 460 nm when various concentrations of $Mg(ANS)_2$ were applied to the white plasmodia. When the concentration was below C_{th} of $Mg(ANS)_2$ ($1 \cdot 10^{-5}$ M), p remained at a constant level, while it decreased when the concentration was above C_{th}, where changes in p followed a virtually identical time course irrespective of the concentration of ANS.

Similar changes were also observed for NaANS. In this case, the threshold was $1 \cdot 10^{-4}$ M, identical with chemoreceptive threshold for NaANS.

In Fig. 6, comparison is made between changes in membrane potential and in fluorescence polarization at 460 nm when magnesium and sodium salts of ANS were applied to the white plasmodia. The membrane potential changed in a manner similar to that of the corresponding chlorides (see Fig. 2). Repeated stimulation by ANS or long immersion in a concentrated ANS solution induced

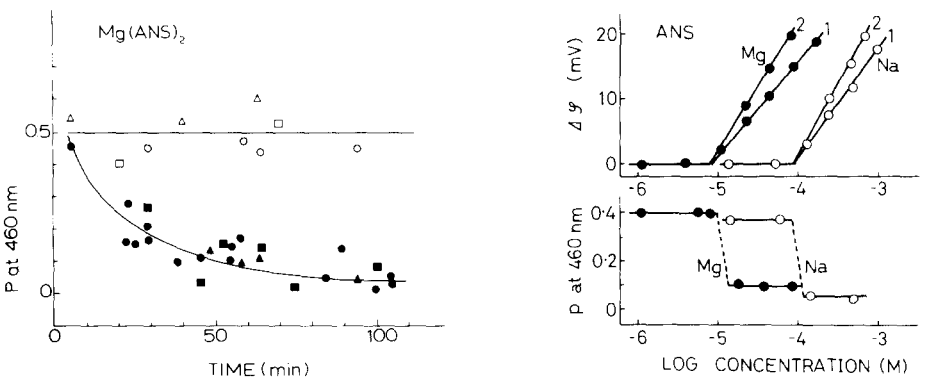

Fig. 5. Time course of fluorescence polarization, p, of ANS at 460 nm after immersion of the plasmodia in various concentrations of $Mg(ANS)_2$. ○, $1 \cdot 10^{-6}$ M; △, $6 \cdot 10^{-6}$ M; ○, $9 \cdot 10^{-6}$ M; ■, $2 \cdot 10^{-5}$ M; ●, $4.5 \cdot 10^{-5}$ M; ▲, $9 \cdot 10^{-5}$ M.

Fig. 6. Dependence of the membrane potential and fluorescence polarization, p, at 460 nm on the concentration of ANS salts. ○, NaANS; ●, $Mg(ANS)_2$. The number 2 in the upper trace indicates cases after repeated stimulation or by lengthy immersion in concentrated ANS solutions.

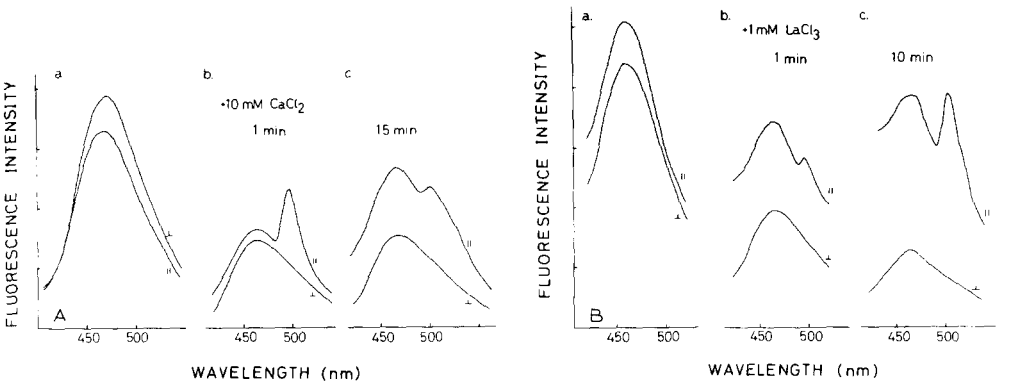

Fig. 7. Spectral pattern of ANS fluorescence after an addition of $CaCl_2$ (A) or $LaCl_3$ (B). Beforehand, the plasmodia were immersed in $4.5 \cdot 10^{-5}$ M $Mg(ANS)_2$ for 60—100 min. Addition of Ca or La increased the fluorescence polarization at 460 nm, an inverse of the effect observed for Na or Mg.

larger potential responses without changing the respective thresholds, as shown by the number 2 in the figure. The lower trace of Fig. 6 shows the concentration dependence of fluorescence polarization at 460 nm 30 min after the application of chemicals. Contrary to the membrane potential, p changed in an all-or-nothing manner at the thresholds. The values of p decreased from 0.4 to 0.1 and to 0.05 for Mg and Na salts in these trials.

Different action of cations on membrane fluidity

An application of Mg or Na salts of ANS caused a decrease in the rigidity of the membrane as regards the binding sites of ANS, as described above. Fig. 7 shows changes in the spectral pattern of ANS when 10 mM $CaCl_2$ or 1 mM $LaCl_3$ was added to a solution of $4.5 \cdot 10^{-5}$ M of $Mg(ANS)_2$ 60—100 min after immersion of the plasmodia in ANS solution. Fluorescence of ANS at 500—520 nm increased transiently after an addition of Ca or La. However, fluorescence at 460 nm remained as a major emission even after the addition of salts.

Fluorescence polarization at 460 nm increased after adding Ca or La. Thorium had a similar effect, but the extent of this was relatively small. Contrary to these, Al, Mg, Na and K did not increase p at 460 nm at all; they rather enhanced a decrease in p.

The different actions by cation species on membrane fluidity classify cations into two groups: group I includes Ca, La and Th, and group II, Na, K, Mg and Al. This grouping coincides with that based on the Schulze-Hardy rule as shown in Fig. 3.

Discussion

Nature of the structural change in the plasmoidal membrane caused by chemoreception

In previous papers we showed that both physical and chemical properties of the membrane changed on reception of chemical stimuli, suggesting an involvement of a conformational change in membrane structure in the process of chemoreception [4—8]. Our results described above afford a molecular picture

of the conformational change in the membrane caused by chemoreception in the plasmodia of *Physarum polycephalum*.

Physicochemical studies on ANS fluorescence showed that the emission maximum of ANS is located at 500–520 nm in polar environments, while it shifted to 460 nm in hydrophobic environments [9]. Based on these properties of ANS, our data shown in Fig. 3 are interpreted as follows. Below C_{th}, the surface of the plasmodial membrane is maintained predominantly in a hydrophilic state, protecting a penetration of ANS into the hydrophobic regions. On the other hand, the hydrophobic region on the membrane having access to ANS increases discontinuously above C_{th}. This interpretation is consistent with the effects of adhesive properties in the plasmodia [8].

Tasaki et al. reported that the nerve membrane of squid axon became hydrophilic during excitation [11]. An inverse result is obtained in chemical excitation in the plasmodia of *Physarum* as described above.

Fluidity of the membrane

Fluorescence polarization, p, is related to the rotational motion of the chromophore by the well-known Perrin equation [17,9,10]:

$$1/p - 1/3 = (1/p_0 - 1/3)\,(1 + 3\tau/\rho)$$

where p_0 is a constant, τ the fluorescence lifetime, and ρ the rotational relaxation time of the solute. ρ may be considered proportional to the microviscosity. Thus, a change in p is attributed to that in the microviscosity under the assumption that τ stays constant. Our interpretation of p in terms of fluidity of the membrane is based on this assumption.

Results shown in Fig. 4 indicate that ANS binds at least two distinct sites on the membrane. It would be possible to determine the microviscosity around respective binding sites, were we able to know the respective τ values.

The plasmodia can also be vitally stained with various cationic dyes such as rhodamine G, acridine orange, rose bengal, etc. Preliminary results on the fluorescence polarization of these dyes showed that the values of p were very small (nearly zero). This fact indicates that the surface membrane of the plasmodia has very high fluidity.

As shown in Figs. 4 and 7, fluidity of the membrane is influenced by external chemical environments and differs for the various binding sites of the membrane. It is of interest to determine the values of p for various dyes in various environments.

In the discussion above we have assumed that the observed fluorescence was not attributable to that ANS which had penetrated into the cell, but rather to that bound to the surface membrane. Some plausible reasons are as follows: The time course of changes in the fluorescence was independent of the concentration of ANS, as seen in Fig. 5. C_{th} determined from the fluorescence agreed with that from the membrane potential. The latter is known to be ascribed to the membrane surface. Too lengthy an immersion of the plasmodia (more than 2 h) in ANS solution induced 460 emission even at a concentration lower than C_{th}. This may result from the penetration of ANS into the cell interior.

Chemoreceptive mutant in the plasmodia

Results shown in Fig. 2 show that the white plasmodium used in this study is also a chemoreceptive mutant, at least in discriminating various cations. How many receptors are influenced in this mutant? How is this mutation determined genetically? The answers must await further studies.

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