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The surface and membrane potentials of chromatophores of photosynthetic bacteria as studied by carotenoid absorbance changes

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The change in surface potential induced by addition of mono- or divalent cations to a chromatophore suspension was monitored by carotenoid absorbance changes (a probe which is intrinsic to the membrane). The change in carotenoid absorbance elicited by an alteration of the surface potential is strongly dependent on the presence of ionophores; the absorbance changes (due to addition of $MgCl_2$) in the presence of valinomycin or gramicidin are larger than those in the presence of carbonyl cyanide *m*-chlorophenylhydrazone or cabonyl cyanide *p*-trifluoromethoxyphenylhydrazone. These differences in carotenoid absorbance change reflect the degree in which the membrane resistance has been shunted. Gramicidin or high concentrations of valinomycin (10^{-6} M) appear to be sufficiently effective as shunt in order that the totality of the change in external surface potential is seen as an intramembrane potential difference as sensed by the carotenoids. It is also shown that the decay of the carotenoid changes induced by the addition of salt to the medium is a measure of the intrinsic permeability of the chromatophore membrane for the added cation.

Introduction

It is now well recognized that electrochemical potential differences of protons across biological membranes play a central role in a variety of processes such as energy transduction, transport and control of biological function [1,2]. The measurement of the transmembrane potential difference ($\Delta\Psi^m$), therefore, is very important [3,4]. However, large discrepancies still exist between the values of $\Delta\Psi^m$ obtained with different techniques [5–7]. Also, it has been recognised that the bulk to bulk electrical potential difference, $\Delta\Psi^m$, is composed of two contributions: the intramembrane (surface-to-surface) potential difference, $\Delta\Psi_{int}$, and the difference between the two surface-to-bulk potentials, $\Psi^{so}-\Psi^{si}$ (corresponding to the outside and inside of the membrane vesicle, respectively) [8]. The surface potentials are created by the negatively charged groups located on both sides of the photosynthetic membrane [9–11]. Estimation of these surface charge densities is thus a prerequisite for the evaluation of the electrical potential profile across the energy-transducing membrane.

In photosynthetic bacteria, the absorption changes of the intrinsic carotenoid molecules [12,13] have been since long a favourite probe for the measurement of $\Delta\Psi^m$ [6,14]. More recently, Matsuura et al. [10] and Swysen et al. [15] demonstrated that the carotenoid absorption spectra are

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinepropanesulphonic acid.

also sensitive to changes in surface potential induced by addition of salts. Hitherto, it has been tacitly assumed that the resistance of the photosynthetic membrane is so low that, upon alteration of the ionic strength of the external bathing medium the inner and outer bulk potentials remain equilibrated (see Fig. 1 in Ref. 16), implying that the change in the outer surface potential is seen as an intramembrane potential difference. For the chromatophore membrane prepared from photosynthetic bacteria it was suspected that this assumption was not valid but it was thought that the protonophore CCCP would provide a sufficiently effective shunt across the membrane [10]. In this paper we show that this in fact is not the case and that the valinomycin-K^+ complex or gramicidin appear to yield a better shunt.

Negatively charged merocyanine dyes, which also have been used as a probe for transmembrane potentials [17,18], were shown both in liposomes [19] and in photosynthetic membranes [20,21] to respond also to surface potentials, the dye distributing itself according to the potential difference between its adsorption site in the membrane and the bulk aqueous phase.

Materials and Methods

Rhodopseudomonas capsulata wild type was grown anaerobically in a medium described by Cohen-Bazire et al. [22] and harvested after two days. Growth temperature was 30°C. Chromatophores were prepared by sonication (two periods of 30 s separated by 5 min) in the presence of 20 μM $MgCl_2$, 2 mM (KCl + KOH), and 2 mM Mops at pH 7. Concentrated suspensions of chromatophores were stored on ice or on liquid nitrogen. The former were used for the experiments within three days of preparation, the latter were used the day following the thawing. Measurements were carried out on a home-made Chance-type double-beam spectrophotometer provided with a continuous stirring mechanism (mixing time, less then 0.5 s). The wavelengths were set at 508 and 530 nm for carotenoids, and 570 and 600 nm for merocyanine. A split-beam spectrophotometer (Cary 17) was used to check that for all conditions the kinetics do not depend on the measuring wavelength; artefacts due to scattering changes are negligible for all kinetic measurements. This was not the case in the presence of high concentrations (10^{-6} M) of gramicidin, where increases in scattering was observed upon addition of $MgCl_2$ to the sample. The measurement medium was the same as the preparation medium. The chromatophores were allowed to equilibrate for about 2–5 min after dilution in medium, before the measurement of the absorbance change (the time of equilibration depending on the ionophore used: valinomycin gave a stable base line more rapidly than did CCCP). For the salt additions, 10–100 μl of salts were added to 2.5 ml of chromatophore suspension. The dilution artefact was corrected by a previous injection of the same volume of medium in a test cuvette. All the experiments were carried out at room temperature. The bacteriochlorophyll concentration was about 6 μg/ml. Merocyanine-540 was purchased from Eastman Kodak and used without further purification. Valinomycin, FCCP, CCCP and Mops were purchased from Sigma.

Results

The kinetics of the carotenoid absorbance change (commonly called 'carotenoid shift') induced by addition of $MgCl_2$ to a suspension of chromatophores of *Rhodopseudomonas capsulata* (wild type) in the presence of an optimal amount of various antibiotics are shown in Fig. 1. From the figure, it can be seen that the extent of the carotenoid shift is proportional to the membrane conductance: it is larger in the presence of gramicidin than it is in the presence of CCCP, for example. It should be noted that in the concentration range used, ionophores are not expected to alter the surface charge to any significant degree: if there would be influence of the ionophore itself on the surface charge, one would expect that the negatively charged FCCP or CCCP, upon binding, would increase the surface charge, which should cause a large carotenoid shift. This obviously is not the case: the absorbance change induced in the presence of optimal amounts of FCCP or CCCP was smaller than it was in the presence of valinomycin or gramicidin. The assumption that the ionophores themselves do not alter the surface charge is substantiated by the fact that no significant carotenoid absorbance changes were observed

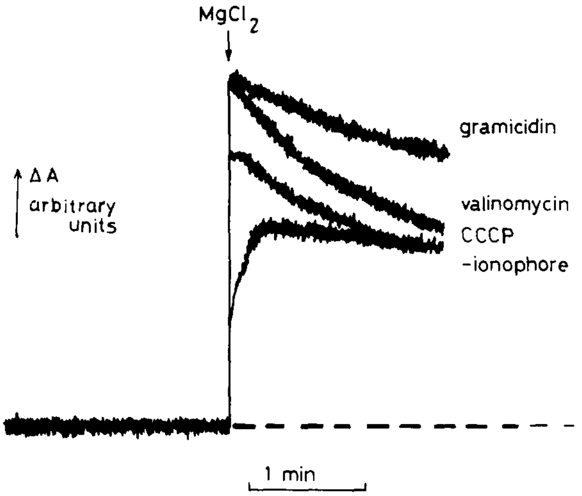

Fig. 1. Carotenoid absorbance changes induced by addition of 4 mM of $MgCl_2$ in the presence of various ionophores. The absorbance changes were due to a blue shift of the carotenoid absorption spectrum, and were determined by measuring the absorbance change (508–530 nm) in arbitrary units. The concentration of the ionophores was 1 μM. For conditions, see text.

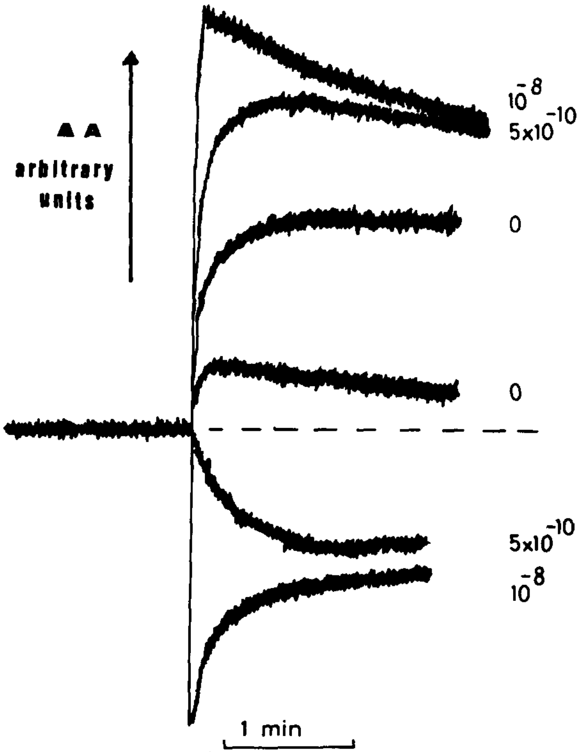

Fig. 2. Comparison of the kinetics of Mg^{2+} (4 mM)-induced carotenoid changes (three top curves) and the Rb^+ (20 mM)-induced absorbance changes (three bottom curves), for different valinomycin concentrations (M).

upon addition of ionophores to the sample (not shown).

The involvement of the membrane permeability in the surface potential induced carotenoid band shift is further substantiated by a comparison of the valinomycin concentration dependencies of the surface- and diffusion-potential-induced carotenoid absorbance changes. Kinetics of the absorbance changes induced by diffusion potentials (red shift of the carotenoid band) and by Mg^{2+} induced changes in surface potential (blue shift of the carotenoid band) are shown in Fig. 2. As can be expected from the size of the absorbance change induced by a change in surface potential, in the absence of a sufficient quantity of (surface charge) screening ions, the carotenoid absorbance changes induced by a K^+ diffusion potential are contaminated by the response of the carotenoid absorption bands to the change of the surface potential caused by the addition of the K^+. This is most obvious in the absence of valinomycin in which case the change of the surface potential is larger than the diffusion potential induced by K^+ * (see Fig. 2). Therefore, in order to obtain the values for the diffusion potential in Fig. 3 (filled circles), the absolute value of the absorbance change corresponding to the surface potential change induced by an amount of Li^+ (20 mM), equal to the amount of K^+ previously added, was added to the absorbance change of the diffusion potential of K^+, for each valinomycin concentration. Also, in order to be able to compare the sole effect of valinomycin on the surface and diffusion potential-induced changes, the carotenoid response in the absence of valinomycin was subtracted for each time-course. It is clear, from Fig. 3, that up to a valinomycin concentration of about 10^{-8} M, surface and diffusion potential induced carotenoid changes are affected in a similar manner by valinomycin.

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* For most preparations, in the absence of valinomycin, only small K^+ diffusion potentials were observed. This is at variance with an earlier report from our laboratory (see Table I of Ref. 28). In those experiments, however, the large diffusion potentials in absence of valinomycin appeared to be an artefact due to improper rinsing the measuring cuvette, rather than by leakiness of the membranes (cf. Ref. 6). This does not alter to any degree the other results presented in that paper.

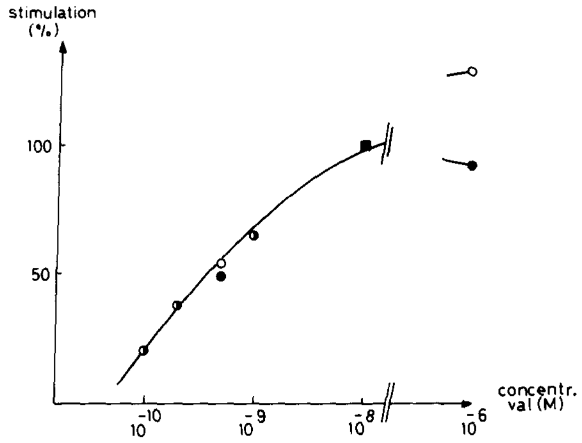

Fig. 3. Comparison of valinomycin concentration dependencies of surface- and diffusion potential-induced carotenoid absorbance changes. Conditions as in Fig. 1. The absorbance changes were normalised to the common maximum response (10^{-8} M valinomycin) for both screening and diffusion effect. ○, surface potential induced by addition of Li^+; ●, diffusion potential induced by addition of Rb^+; ■, reference point.

For larger valinomycin concentrations, the surface potential-induced carotenoid shift continues to increase, while the diffusion potential changes become smaller. The valinomycin concentration dependence of the surface potential effect reaches a plateau at about 10^{-6} M. We considered 10^{-6} M valinomycin as an optimal concentration regarding the surface potential-induced absorbance changes. Virtually the same saturation phenomenon was observed for CCCP, FCCP and gramicidin (not shown).

It is well documented that for valinomycin concentrations of 10^{-7} M and above its functioning is no longer optimal [23–28]. The fact that the surface potential-induced carotenoid shift is larger at these high concentrations of valinomycin can also be explained by the side effects the carrier has on the membrane permeability (see Discussion).

Circumstantial evidence for the effectiveness of these high concentrations of valinomycin in shunting the membrane comes from computer fittings of the carotenoid absorbance induced by addition of mono- and divalent cations using the Gouy-Chapman theory [29]. In spite of the numerous assumptions intrinsic to this theory (for a discussion, see Refs. 9 and 30), we observed a very close fitting for the experiments in the presence of 10^{-6} M valinomycin. There were considerable deviations, however, in the presence of CCCP. It is important to remark in this respect that no further difference was observed between the surface potential-induced carotenoid changes in the presence of this high concentration of valinomycin and in the presence of gramicidin.

Fig. 4 shows the absorbance changes of the surface potential-indicating dye merocyanine 540 in absence and presence of valinomycin (10^{-6} M). Clearly, the difference between the results obtained from the two experimental conditions is very small, showing that, indeed, the change of the surface potential as indicated by the merocyanine dye [19–21] is not altered by valinomycin. The small residual difference can be explained by the fact that merocyanine also senses part of the in-

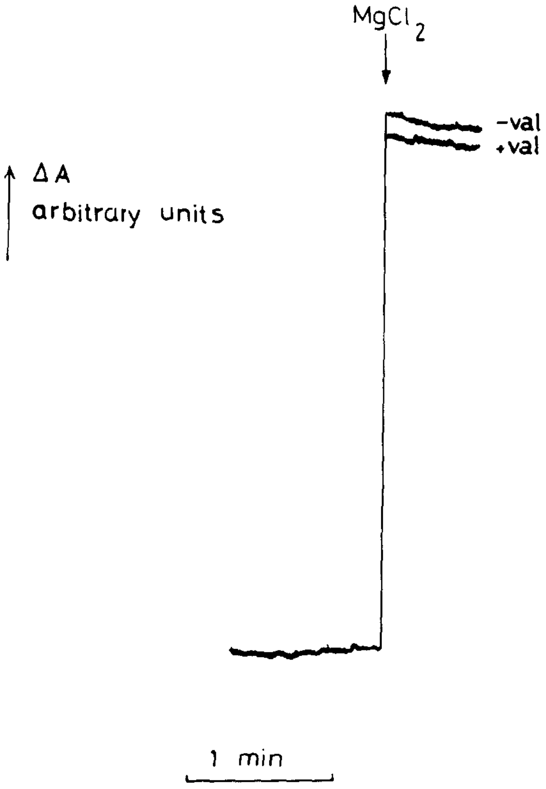

Fig. 4. Merocyanine absorbance changes (570–600 nm) induced by addition of $MgCl_2$ (4 mM) in the presence and absence of valinomycin (10^{-6} M). Merocyanine concentration was 10^{-6} M.

tramembrane potential difference, as pointed out by Masamoto et al. [21].

In the absence of ionophore, an interesting situation is obtained. The slowly rising carotenoid band shift observed upon addition of $MgCl_2$ (Fig. 1) reflects in fact the intrinsic membrane permeability. The question that arises then is: which ions contribute to this intrinsic permeability? We tried to answer this question in a qualitative manner by evaluating the relative permeabilities of all the present ions. A significant Mg^{2+} permeability can be excluded, since this could give rise to a diffusion potential which is positive inside, which would cause a red shift of the carotenoids, opposite to what is observed. In fact, we will argue below that it is the small Mg^{2+} permeability which is responsible for the slow decay of the surface potential-induced carotenoid change. In order to investigate whether the slow rise of the carotenoid change reflects an inward diffusion of the added anion we compared the absorbance change induced by addition of $MgSO_4$ and $MgCl_2$.

Both in the presence and the absence of valinomycin the extent of the change induced by $MgCl_2$ was less than 4% larger than the one induced by $MgSO_4$, which is about as large as the experimental error. It has been well documented on the contrary that both in *Rps. capsulata* [31] and in *Rps. sphaeroides* [32] the permeability for Cl^- is substantially higher than for SO_4^{2-}. This indicates that, in our conditions, the contribution of the anions to the total intrinsic membrane permeability must be small. the K^+ permeability is also relatively small. This is substantiated by the observation that the diffusion potential generated by addition of K^+ in the absence of valinomycin is only 10% of the diffusion potential created in the presence of an optimal amount of valinomycin (see Fig. 2, bottom). Thus it might be that the protons are the predominant ions determining the membrane permeability. Leakage through the coupling factor seems to be nihil in this case, since the $MgCl_2$ induced carotenoid shift is the same in presence or absence of oligomycin which blocks the ATPase channel [33]. However, the solution of the proper equations taking the permeabilities of all the present ions simultaneously into account, should establish whether the above considerations do not represent oversimplified view.

Discussion

In this paper, we show that the carotenoids are an indirect probe of the external surface potential. Moreover, only if the membrane is efficiently short-circuited, these carotenoid absorbance changes yield at least a qualitative measure of the change in surface potential (see fig. 2 of Ref. 29). The data obtained with carotenoid absorbance changes and with merocyanine absorbance changes show that the presence of protonophores such as CCCP or FCCP is not as effective to shunt the membrane perfectly. The gramicidin channel appears to be the most effective shunt.

In Fig. 5, we depict the membrane potential profile thought to be responsible for the observed surface potential-induced carotenoid absorbance changes. Fig. 5A represents the situation in which the membrane is ideally shunted: the bulk-to-bulk potential does not change upon addition of the poorly permeable $MgCl_2$. Gramicidin or high concentrations of valinomycin in the presence of Rb^+ or K^+ probably approach this situation. In this case, the totality of the change in surface potential is seen as an intramembrane potential difference. Fig. 5B depicts a situation in which the membrane is not sufficiently shunted: upon addition of $MgCl_2$ only part of the change in surface potential will be translated in an intramembrane potential difference. This effect occurs in the presence of CCCP or FCCP (probably because the concentration of

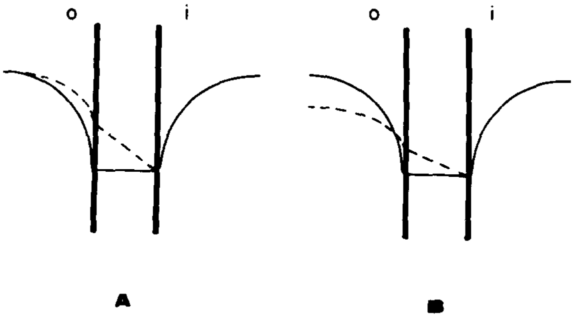

Fig. 5. Hypothetical electrical potential profiles across the chromatophore membrane (o = outside, i = inside) to show the shunting effectivity of various ionophores. Continuous line: before addition of a screening cation, 2 mM K^+/20 μM Mg^{2+} background present at both sides. Dashed line: after addition of Mg^{2+} to suspension medium. (A) Chromatophore plus 10^{-6} M valinomycin; (B) without ionophore.

protons is limiting) or in the absence of ionophore; if the membrane is not correctly shunted, an addition of salt leads to a decrease of the bulk phase potential [34].

The discrepancy between the valinomycin concentration dependence of the diffusion- and surface potential-induced carotenoid changes at high valinomycin concentrations (Fig. 3) obviously is caused by the non-ideal functioning of the carrier molecule, a feature which has been extensively discussed in the literature [23–28]. It can be observed from Figs. 1 and 2 that the salt-induced carotenoid changes have a slow decay. It is obvious that this decay must be due to permeation of Mg^{2+}, since in the conditions of our experiment it is the only ion which is capable of eliciting a positive diffusion potential (but see Ref. 10). This conclusion is substantiated by the fact that the carotenoid shift induced by impermeable cations (such as tetraethylammonium, tetramethylammonium and methyl viologen ions) does not exhibit any decay (not shown). As can be noticed from Fig. 2, increasing concentrations of valinomycin have an accelerating effect on this decay. Since we found no report in the literature that valinomycin also transports Mg^{2+}, the most likely explanation for this effect is that the presence of increasing valinomycin concentrations progressively perturbs the membrane (see also Ref. 24), thus increasing its intrinsic permeability for cations.

In this respect, it has been shown that valinomycin can alter the microviscosity in lipid vesicles as monitored by diphenylhexatriene fluorescence polarization (Shinitzky, M., personal communication). This is altogether not suprising; a very simple calculation shows that at a valinomycin concentration of 10^{-6} M, with a bacteriochlorophyll concentration of 6 μM, an average surface area per bacteriochlorophyll of 4.1 nm^2 [35], a surface area of one valinomycin molecule of 1.8 nm^2 [36] and assuming that all the valinomycin goes into the membranes, 7% of the total surface is occupied by valinomycin! It is interesting to note that at high concentrations of CCCP and FCCP, a similar acceleration of the decay of the salt-induced carotenoid changes can be observed.

An additional feature of the decay of the induced carotenoid change is that it allows us to determine the relative intrinsic cation permeability of the chromatophore membrane. For the ions which were tested, the sequence (as evaluated in the presence of 10^{-6} M FCCP) reads as follows Rb^+, K^+, $Cs^+ > Na^+ > Li^+ > Ca^{2+} > Mg^{2+} > Sr^{2+}$. It is interesting to note that aged chromatophore preparations become leaky, as is obvious from the more pronounced decay of the surface potential-induced carotenoid shift. These aged chromatophores upon addition of K^+ or Rb^+ to the medium also show substantial diffusion potentials in the absence of valinomycin. As can be observed from Fig. 1, the decay phenomenon is significantly slower in the presence of gramicidin. This can be explained by the fact that in this case, due to the very short transfer time of ions in the gramicidin channel, the rise of the Mg^{2+} diffusion potential is considerably slowed down by cation counter transport.

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