Photobiochemistry and Photobiophysics, 2 (1981) 105—110

POLAROGRAPHIC ASSAY OF THE BINDING OF CERTAIN "PROBE" MOLECULES TO ILLUMINATED BACTERIORHODOPSIN SHEETS

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Received September 10, 1980
Accepted September 18, 1980

SUMMARY

It is pointed out that many commonly used probes for one or other of the components of the proton-motive force are electroactive at the mercury electrode, and that a novel polarographic assay for changes in their binding upon membrane energisation is thus possible.

The assay is applied to the probes safranine, 9-aminoacridine and 1-anilinonaphthalene-8-sulphonate in the presence of illuminated bacteriorhodopsin sheets, where the generation of a bulk-phase proton-motive force is impossible. Illumination-dependent binding changes are readily observed in each case.

It is concluded that these probes are unreliable as quantitative indicators of ΔpH and $\Delta\psi$ as defined in the chemiosmotic theory.

INTRODUCTION

Of the many possible approaches to gaining an understanding of the mechanisms of free energy transduction catalysed by biological membrane systems which pump protons, the use of so-called "probe" molecules with spectral features which report on their environment has assumed popularity [1—4]. In many cases it has been assumed that proton-motive activity causes a redistribution of the probe molecule between the outer aqueous phase and the lumen of the vesicular preparation under study, with subsequent change in spectral properties, for instance for the probe 9-aminoacridine [5], which allegedly reports upon the pH gradient component of the proton-motive force [6]. Other probes, which may be assumed to leave the outer bulk aqueous phase of a vesicular suspension when they change their spectral properties in response to proton-motive activity, include safranine [7—11]

and 1-anilinonaphthalene-8-sulphonate [12]. The former, in particular, has gained currency as a reliable probe for the mitochondrial membrane potential (e.g. refs. 11,13), probably because it gives a linear response to artificially induced diffusion potentials of magnitude up to 170 mV, regardless of the nature of the cation creating the diffusion potential [9]. Yet both Åkerman and Wikström [9] and Zanotti and Azzone [11] agree that the probe response is at least partially dependent upon binding to the membrane. It is thus questionable, to say the least, as to whether such probes, even when calibrated using artificially induced ionic diffusion potentials, give signals which correspond exactly to the bulk-phase transmembrane potential when proton-motive activity is initiated by electron transport or by ATP hydrolysis. Reports using spin-labelled probes [14] or resonance Raman spectroscopic probes [15] add weight to the idea that many of these probes are bound to biological membranes in the steady state. Unravelling the extent of probe binding is an extremely arduous (and infrequently performed) task, since most energy-transducing systems, for thermodynamic reasons [16], form vesicles upon isolation, and it is exceptionally difficult to ascertain the extent to which probe molecules are bound or free within the lumen of the vesicle. However, the proton-pumping protein bacteriorhodopsin from the halophile *Halobacterium halobium* (reviews in refs. 17,18) forms sheets upon isolation which are not topologically closed. The generation of a bulk-phase proton-motive force [6,19,20] is thus impossible in this system since both sides of the sheets have access to the same bulk aqueous phase. The purpose of the present article is thus two-fold: a) to point out that a very useful control of whether a probe is sufficiently hydrophilic as not to bind to energised membranes is to study its binding to illuminated bacteriorhodopsin sheets, and b) to describe a novel assay, based upon polarography at the hanging mercury drop electrode, for the extent of the binding of such electroactive probes to biological membranes and macromolecules. A preliminary account of this work has been presented [21].

EXPERIMENTAL

Preparation of bacteriorhodopsin sheets

Halobacterium halobium strain R_1 was grown as described by Clarke and Morris [22], following the method described by Oesterhelt and Stoeckenius [23]. Cells were harvested and stored as a concentrated suspension (2 ml/l culture) in growth medium lacking peptone [23] at −20°C. To prepare bacteriorhodopsin cells equivalent to 20 l of culture were homogenised in the presence of DNase and RNase (20 μg/ml each) and dialysed versus 10 l of water for 17 h at 4°C. All succeeding centrifugations were performed on a Sorvall RC5B centrifuge, using an SS34 rotor, at 20,000 r.p.m. (48,000 × *g*). Cell debris was removed in a 10 min centrifugation and membranes sedimented by centrifugation for 1 h. Membranes were washed once in water and repeatedly in 0.5 mM EDTA until the supernatant no longer displayed a

visible red colour, a procedure usually requiring 3 EDTA washes. The relatively pure bacteriorhodopsin was now centrifuged for 2 h in the presence of 25% (v/v) Percoll and the resulting purple layer removed and washed 3 or 4 times in water until all Percoll (which pellets below the bacteriorhodospin layer under these circumstances) had been removed. The bacteriorhodopsin sheets were then finally sedimented and stored as a stock suspension (approx. 0.5 mM—1 mM) in distilled water in 0.5 ml aliquots under liquid nitrogen. The bacteriorhodopsin so prepared was judged to be greater than 99% pure, using the usual spectroscopic [23] and gel electrophoretic [22] criteria. The main advantage over previous procedures for purifying bacteriorhodopsin [23,24] is that the lower centrifugation speeds possible using Percoll as a centrifugation medium allow much larger volumes of protein to be processed in one preparation, avoiding the bottleneck caused by the messy and tedious sucrose step gradient methods [23,24]. Similar results to those described were obtained when bacteriorhodopsin was prepared according to the methods of Oesterhelt and Stoeckenius [23]. A more rigorous comparison of these methods will be presented elsewhere.

Polarographic methodology. Linear sweep voltammograms were performed using a hanging mercury drop electrode (Princeton Applied Research Model 303), and a PAR 174A Polarographic Analyser, as described previously [25]. Illumination was from a 250 W projector lamp, filtered as described [26]. The Ag/AgCl reference electrode contained one drop of Indian ink to exclude possible illumination-dependent artefacts.

Chemicals. Safranine O (greater than 99% pure dimethylphenosafranine (cf. ref. 27)) was obtained from the Aldrich Chemical Company, and Percoll was from Pharmacia Ltd. Other chemicals were from BDH Chemicals, Poole, Dorset, or the Sigma Chemical Company, Poole, Dorset, and were of the highest grade available. Water was doubly distilled in an all-glass apparatus.

RESULTS

In a typical experiment, linear sweep voltammograms (e.g. ref. 28) were performed on a deaerated solution of the probe of interest, both in the dark and under illumination. Then an appropriate concentration of bacteriorhodopsin sheets was added, and linear sweep voltammograms were performed again, both in the dark and under illuminated conditions. Such experiments are displayed for the probes safranine (Fig. 1) and 9-aminoacridine (Fig. 2). In the former case, illumination of the bacteriorhodopsin sheets caused an increase in the extent of probe binding compared with that seen in the dark (Fig. 1), whilst in the latter case membrane deenergisation was accompanied by a desorption of some of the previously bound probe (Fig. 2). No observable photoreduction of probe molecules, as may take place for safranine in the presence of electron donors [29] such as EDTA [30], was observed in the present system. However, a time dependence of these energy-dependent binding changes could be observed, possibly due to aggregation of the bacteriorhodopsin sheets.

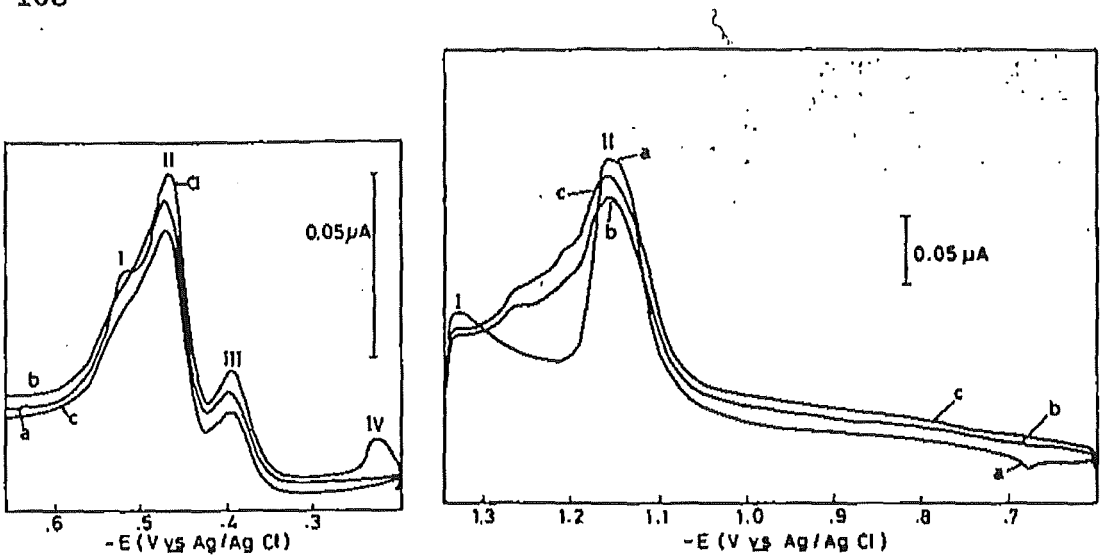

Fig. 1. Binding of safranine by illuminated bacteriorhodopsin sheets. Linear sweep voltammograms were run at a scan rate of 20 mV/s of a safranine solution (33 μM) in 0.1 M Tris—HCl pH 7.0 in the dark or the light (trace a). In traces b (dark) and c (illuminated as described in Methods), bacteriorhodopsin sheets (3.2 μM) were also present. All scans were taken in a cathodic direction on a fresh Hg drop, with a starting potential of —0.2 V (vs. Ag/AgCl).

Fig. 2. Illumination-dependent desorption of 9-aminoacridine from bacteriorhodopsin sheets. Linear sweep voltammograms were performed as described in the previous figure. Trace a) 13 μM 9-aminoacridine (dark or illuminated); trace b) plus 0.7 μM bacteriorhodopsin sheets (dark); trace c) dito, illuminated as described in Methods. Starting potential = —0.6 V vs. Ag/AgCl.

Similar observations to those made with 9-aminoacridine, namely an illumination-dependent desorption of previously bound probe molecules, were made with the probe 1-anilinonaphthalene-8-sulphonate (data not shown), at a peak potential of —1.1 V vs. Ag/AgCl, even under conditions of a probe : protein molar ratio of 150 : 1. Finally, we may point out that any possible reduction of bacteriorhodopsin itself [31] would not have been observed using the present concentrations of bacteriorhodopsin. A more detailed discussion of the nature of the voltammetric peaks observed is not presented here, since it is of little relevance to the present considerations.

DISCUSSION

The observation (Figs. 1, 2) of changes in the binding of commonly used probe molecules upon illumination in the presence of bacteriorhodopsin sheets, where the generation of a bulk-phase proton-motive force is impossible, clearly indicates that a simple analysis of the behaviour of these probes, based solely upon the notion that they are probes for one or other component of the proton-motive force, is inapplicable. Whilst it is of course inappropriate (but tempting) to extend this observation to the general case, we would reiterate the comment [32] that "only in the case of ion-distribu-

tion methods using hydrophilic ions which do not bind to biological membranes to any significant degree is it credible that the Nernst potential and bulk-phase pH gradients are being measured". Whether the present observations indicate a lack of equilibration of the proton electrochemical potential between the interfaces and the bulk aqueous phases [32,33] under energised conditions, or constitute merely a more trivial criticism of the use of these probes, cannot be definitely established until the nature of the probe response to energisation is more fully understood. However, it is germane to point out that the stoichiometry, *per photon absorbed*, of protein translocation to the bulk aqueous phase by bacteriorhodopsin sheets is a function of the ionic conditions in the suspension [34—37], indicating that, under some conditions at least, part of the free energy change associated with photon absorption is not converted completely into a bulk-phase proton gradient on the time scale under consideration. We would certainly point out that the present observations add further weight to criticisms (reviewed most recently in refs. 32,38) of the use of 9-aminoacridine as a monitor of the pH gradient component of the proton-motive force.

Whilst the present method would be confined to the study of probe molecules which are electroactive in the region of applicability of the Hg electrode (circa 0 V to −2 V vs. Ag/AgCl at neutral pH) [39], it is noteworthy that this restriction still allows the use of this method for most of the lipophilic spectroscopic probes in common use as supposed monitors of ΔpH or $\Delta\psi$ [1—4]. The rapidity of response of the present method and the applicability of indicator electrodes other than the hanging Hg drop, together with more sophisticated electrochemical techniques, will allow a much more general extension of the present method to other probes and other energy-transducing systems.

ACKNOWLEDGEMENTS

D.B.K. is indebted to the Science Research Council, London, for generous financial assistance, and to the Royal Society, London, for an equipment grant.

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