COMPARISON OF THE CAROTENOID BANDSHIFT AND OXANOL DYES TO MEASURE MEMBRANE POTENTIAL CHANGES DURING CHEMOTACTIC STIMULATION OF *RHODOPSEUDOMONAS SPHAEROIDES* AND *ESCHERICHIA COLI*

Judith P. ARMITAGE and Michael C. W. EVANS

Department of Botany and Microbiology, University College London, Gower Street, London WC1E 6B, England

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1. Introduction

Flagellate bacteria are able to respond to changes in their environment by biasing their overall direction of travel towards attractants and away from repellents. This is achieved by altering the time intervals between the brief periods of tumbling which result in a change of direction, achieved by reversing the rotation of the flagella from normal anticlockwise to clockwise. To bring about this change in tumbling frequency the chemoattractant or repellent must initially be bound to a receptor protein which interacts with specific methyl-accepting chemotaxis proteins (MCPs) probably causing a perturbation in the membrane potential and resulting in changes in internal cyclic GMP levels and thus in the methylation level of the MCP (reviews [1,2]).

The early stages of the signalling process remain unresolved. Work on heterotrophic bacteria has used the movement of permeant ions to suggest that although perturbation of the membrane potential can change the swimming behaviour of bacteria, the chemotactic response itself may not involve the membrane potential [3]. Using the electrochromic absorbance shift of the natural, membrane-bound carotenoids of photosynthetic bacteria as an indicator of membrane potential changes we have shown that chemotactically stimulated *Rhodopseudomonas sphaeroides* undergoes a fast transient increase in membrane potential [4].

Chemotaxis in photosynthetic bacteria has not, as yet, been genetically characterised. To establish therefore where in the sequence of sensory events the increase in membrane potential occurred, it would be helpful if the work could be extended to heterotrophic bacteria.

Oxanol VI (OX VI) is a well-characterised, potential-sensitive dye undergoing a very fast absorbance change in a region not masked by the carotenoids [5]. Although OX VI is a permeant anion, work here shows that it will respond in right-side out membrane systems as well as inside-out chromatophore, and suggests that the membrane potential change seen during chemotaxis as indicated by the carotenoid bandshift is probably not the result of ion transport, but may be due to a charge separation across the membrane.

2. Materials and methods

2.1. *Organisms and growth conditions*

Rhodopseudomonas sphaeroides wild-type 2.4.1. and *Escherichia coli* C600 were used throughout this study. Both organisms have been shown to exhibit both positive and negative chemotaxis.

Rhodopseudomonas sphaeroides was grown anaerobically in the light in batch culture to late exponential phase as in [4]. After centrifuging, the cells were resuspended in 20 mM K-Hepes (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) pH 6.8 to $\sim 10^9$ cells/ml.

Escherichia coli was grown aerobically in Oxoid Nutrient Broth no. 2 and was resuspended in the same K-Hepes buffer.

2.2. *Spheroplast preparation*

Spheroplasts of both *Rps. sphaeroides* and *E. coli* were prepared as in [6]. For experiments using OX VI to measure potential changes across spheroplast membranes the final gentle lysis was completed by resuspending the spheroplasts in 20 mM Hepes (pH 6.8) containing 10 mM ascorbate, 150 μM OX VI, 10 μg

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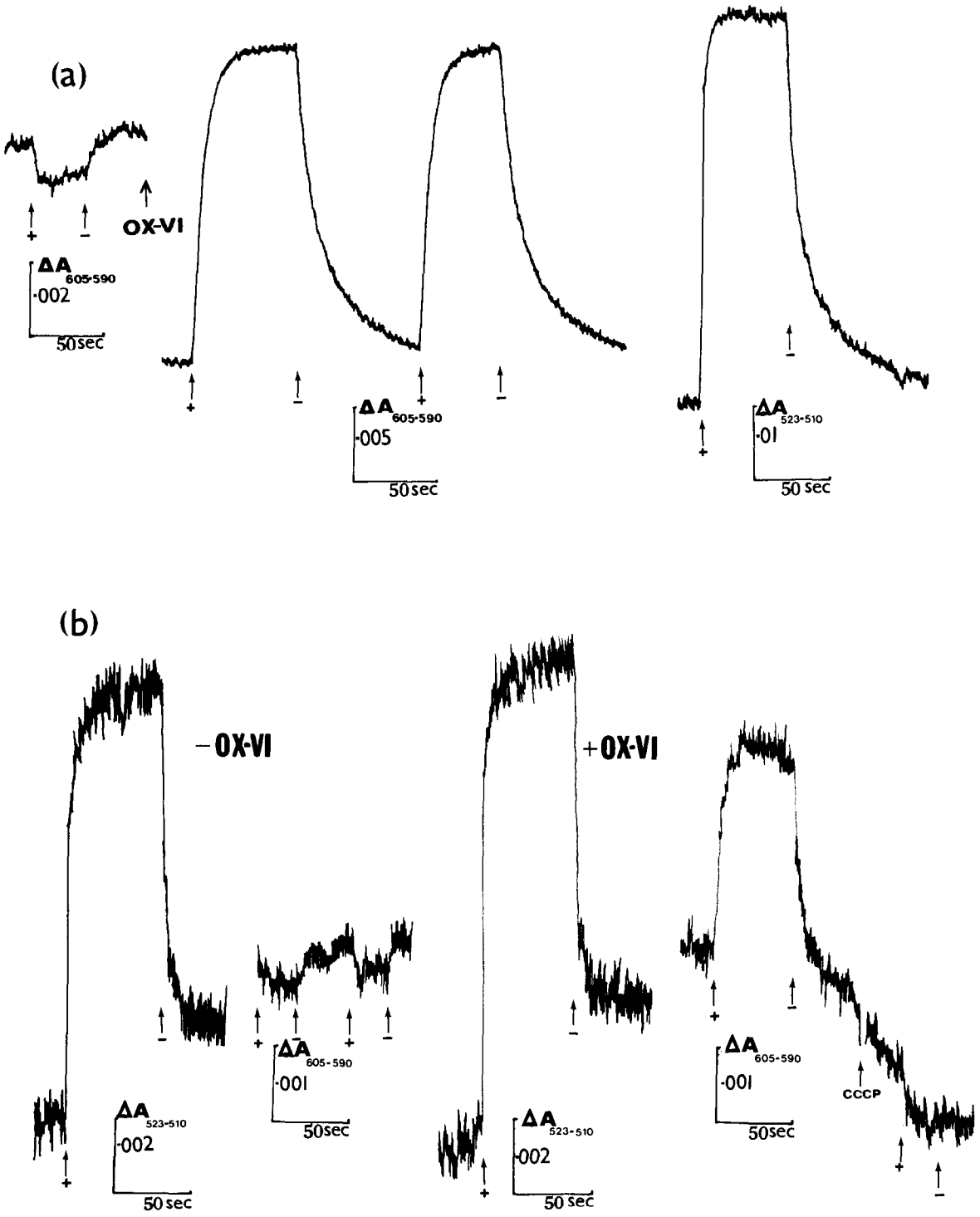

Fig.1. (a) Light-induced increase in membrane potential in chromatophores of *Rps. sphaeroides* measured after the addition of OX VI and by the carotenoid bandshift. (b) Light-induced increase in membrane potential in spheroplasts of *Rps. sphaeroides* measured by the carotenoid bandshift and OX VI. (↑+) Light on; (↑−) light off.

RNase/ml, 5 μg DNase/ml. After stirring at 0°C for 30 min the spheroplasts were centrifuged and washed 3 times in 20 mM Hepes containing 10 mM ascorbate (pH 6.8). After 3 washes the absorbance of OX VI in the spheroplasts stabilised and little was measurable in the suspending buffer. The final absorbance of oxanols in the spheroplast suspension was consistent with a level of ~10 μM. When *E. coli* spheroplasts were examined 20 mM sodium nitrate was added to the Hepes buffer.

2.3. *Preparation of chromatophores and membrane vesicles*

Inside-out membrane vesicles were prepared by sonication as in [6], but purified chromatophores were resuspended in 20 mM Hepes (pH 6.8).

2.4. *Measurement of carotenoid and oxanol absorbance changes*

Spectrophotometric measurements of the carotenoid and oxanol bandshift was carried out using an Aminco DW2 spectrophotometer in dual wavelength mode, using the difference $A_{510}-A_{523}$ to measure the carotenoid shift and $A_{590}-A_{625}$ to measure the oxanol shift. The light-induced response was achieved by illuminating with actinic light at 861 nm at right angles to the sample. Interference from scattered light was minimised by a blue/green filter on the photomultiplier.

To study the effect of chemotactic agents 20 μl of 0.1 M solutions of chemotactic chemicals were added to 3 ml stirred cuvettes as previously described.

When the response of OX VI in membrane vesicles or chromatophores was examined, OX VI was added to 5 μM to the membrane suspension.

3. Results and discussion

Fig.1 compares the light-induced carotenoid bandshift in spheroplasts and chromatophores of *Rps. sphaeroides* with the response of OX VI. The carotenoid bandshift of OX VI in chromatophores showed similar response, but OX VI respond more slowly

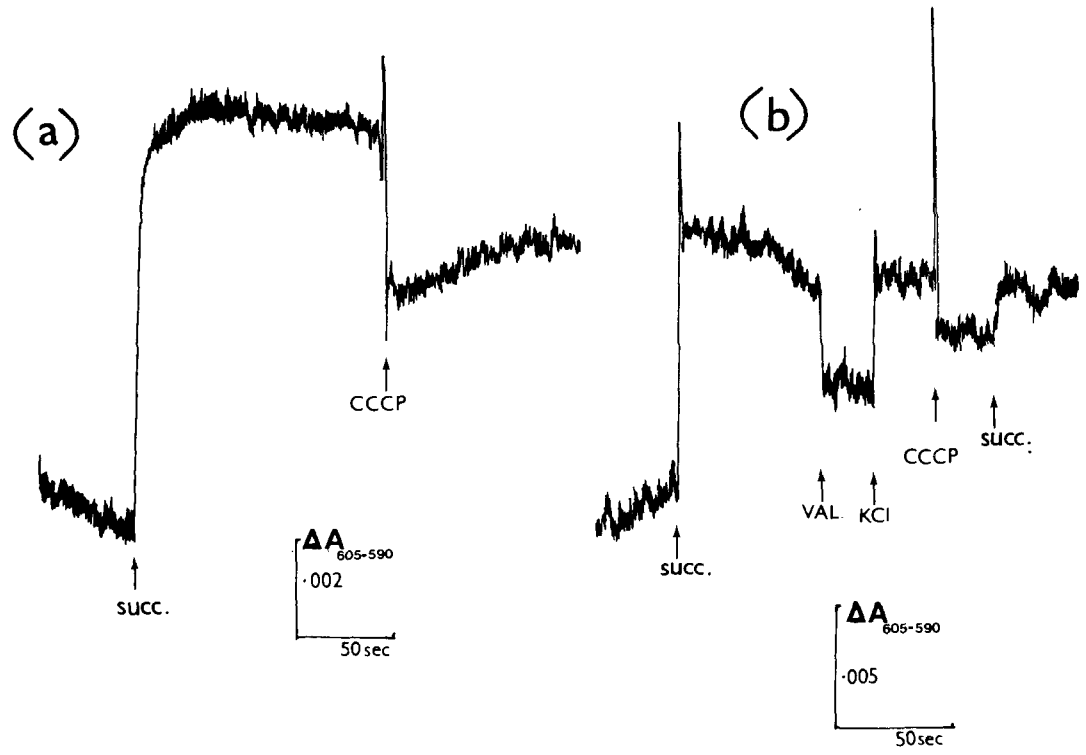

Fig.2. (a) Energisation of spheroplasts of *E. coli* with succinate measured by OX VI. (b) Energisation of membrane vesicles of *E. coli* with succinate, measured by OX VI. (↑succ) 10 μl 0.1 M succinate, (↑CCCP) 10^{-5} M *m*-chlorocarbonyl cyanide phenyl hydrazone; (↑VAL) 10 μg valinomycin/ml.

and only had ~20% of the absorbance shift of the carotenoids. The addition of OX VI to the chromatophores had no effect on the size of the carotenoid bandshift and without OX VI the wavelengths used showed no interference from the carotenoid bandshift. The response of OX VI in chromatophores to energisation with actinic light has been reported [5], however, fig.1(b) shows that right-side out spheroplasts containing OX VI will also respond to actinic light. The half rise time of the OX VI shift in spheroplasts was ≃100 ms, and sensitive to uncouplers, which is comparable to the half rise time in chromatophores. The slower response of oxanols to energisation compared to the response of the carotenoids in natural membrane system was similar to the times noted in [5], oxanols losing their most rapid changes when added to natural rather than artificial membrane systems.

Having shown that oxanols are able to respond, if somewhat more slowly than the carotenoids, to potential changes in spheroplasts of photosynthetic bacteria, without affecting the nature of the potential as measured by the carotenoid bandshift, we have used the oxanol with *E. coli* spheroplasts and membrane vesicles. Fig.2(b) shows the energisation of membrane vesicles with succinate and fig.2(a) shows the response of spheroplasts. Clearly OX VI was able to respond to the increase in membrane potential when *E. coli* membranes were energised with succinate; however, subsequent addition of chemotactic

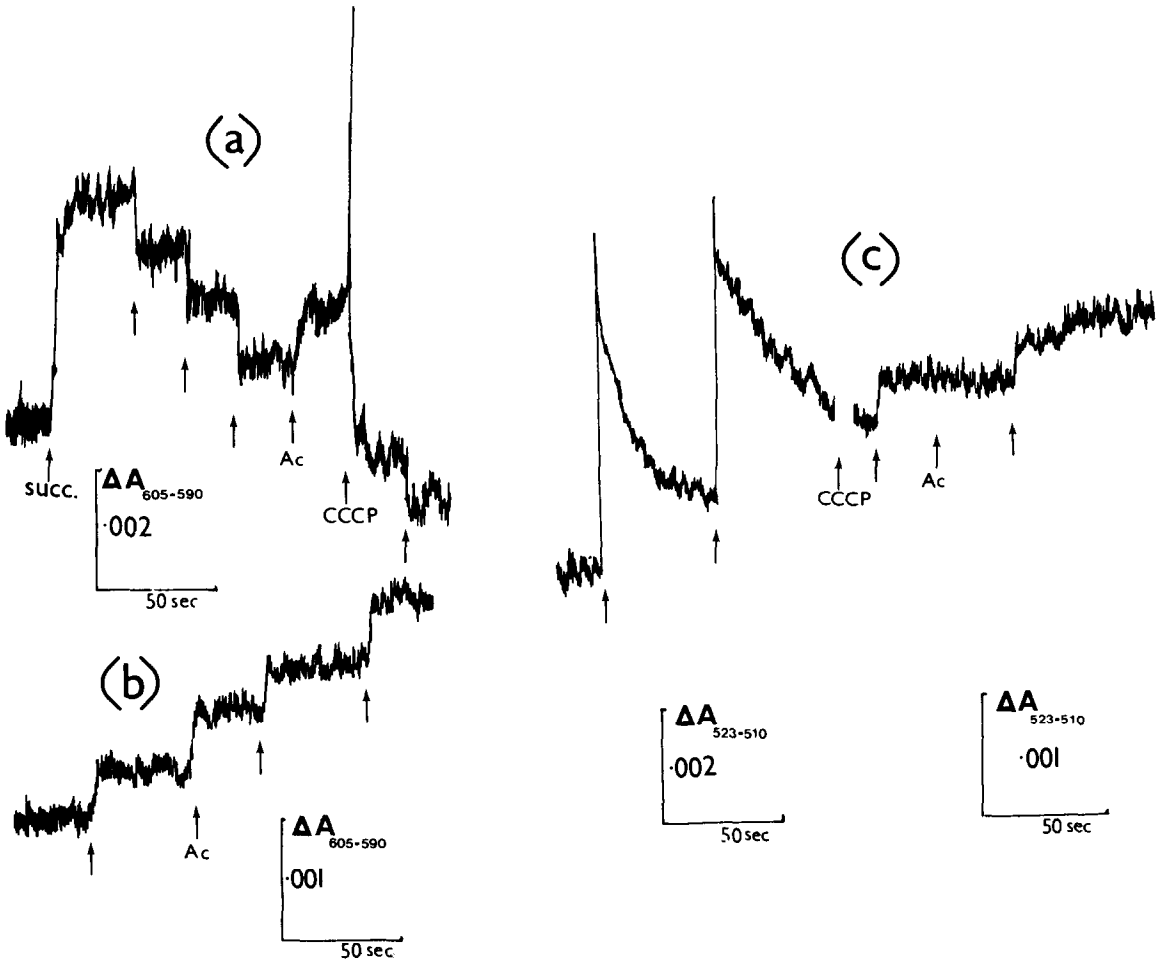

Fig.3. (a) Effect of addition of chemoeffectors to spheroplasts of *E. coli* containing OX VI. (b) Effect of chemoeffectors on membrane potential of spheroplasts of *Rps. sphaeroides* measured by OX VI; (c) the carotenoids on the same sample. (↑) 20 μl 0.1 M L-aspartate; (↑Ac) 20 μl 0.1 M sodium acetate.

metabolites showed no significant alteration in the membrane potential (fig.3(a)). The only exception appeared to be the addition of acetate, however, it is possible that acetate has a direct effect on the ionic distribution, being able, as a weak acid, to cross the membrane directly. Anaerobic *E. coli* spheroplasts in succinate showed a similar potential increase when energised by the addition of oxygenated buffer. Fig.3(b),(c) shows that in the same sample of *Rps. sphaeroides* spheroplasts it was possible to see a chemotactically-induced carotenoid bandshift but not an oxanol shift.

These results suggest 3 possibilities:

(i) The response time of the oxanols is too slow to reveal the potential change seen by the carotenoids; or

(ii) The carotenoid shift seen during chemotaxis has a different basis to that seen during energisation; or

(iii) The technique is not sensitive enough.

The response time of OX VI, although slower than the carotenoids, should reveal at least the latter part of the slow decay of the response seen by the carotenoids. Fig.1 shows that the oxanols show only ~20% of the absorbance change of the carotenoids in the same sample, and the chemotactically-induced response has been shown to be only ~10% of the light-induced carotenoid bandshift. The oxanol shift would therefore be very small but should be detectable. Membrane-bound carotenoids may be able to respond to all charge changes across the cell membrane [7,8], whether gross ion movements or a change in charge separation localised at the membrane level. Oxanol dyes, however, as most other potential-sensitive dyes, respond to changes in ion concentrations in the bulk aqueous phase only. The failure to detect changes with oxanols in response to chemotactic agents suggest that the chemotactic membrane-potential change is localised on the membrane. Binding of a chemotactic agent to the outer surface of the specific receptor may cause a localised change in surface charge, revealed by a carotenoid bandshift. This alteration in surface charge, probably at the MCP, may result directly or indirectly in the release of an intracellular messenger, possibly cyclic GMP, which controls flagellar rotation. Such a mechanism would explain why it is only binding, not transport of the chemotactic agent to the receptor and its interaction with the MCP, that is required for chemotactic signalling. In [9] conformational changes occurred in membrane receptors on binding of the chemoeffector and it is possible that the membrane potential measured here is part of the same phenomenon.

Acknowledgement

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