Flash-Induced Electrochromic Band Shifts, is It a Simple Mechanism?

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The flash-induced carotenoid bandshifts have been studied for various strains of both *Rhodopseudomonas sphaeroides* and *capsulata*. A technique for calculating shifts of isobestic points down to 0.05 nm is described. To this end, special attention has been paid to the appropriate correction for reaction center absorbance changes occuring concommitantly with the carotenoid bandshifts. Plots of the wavelength of the isobestic point versus the corresponding absorption changes at the maximum of the difference spectrum have been made, suggesting the existence of different pools of carotenoids. The various pools of carotenoids seem to have different sizes, inducing non-linearities in the plots. In some cases spectral differences of the pools have to be assumed. A possible interpretation of the results would be that each electrogenic span of the electron transport chain is to be associated with its own pool of carotenoids, all the pools behaving in somewhat independant way. We discuss possible difficulties in making those measurements.

Introduction

Carotenoid absorption changes upon energization of chromatophores and thylakoids are generally accepted to reflect the creation of transmembrane electrical fields [1–5]. Recently, the light-induced carotenoid absorption difference spectrum in *Rhodopseudomonas sphaeroides* has been attributed to only a fraction of the total carotenoid content, with absorption bands shifted to the red by about 5 nm in respect to the bulk carotenoids [6, 7]. However, the question of whether this field-indicating carotenoid pool is homogeneous or not, remained unanswered. It has been shown for instance that reaction centers contain specific carotenoids with absorption bands shifted to the blue by about 5 nm in respect to the field-sensitive pool showing a red shift upon oxidation of the reaction center [8]. The flash-induced carotenoid absorption changes showing three distinct phases which are correlated with different electron transport sequences [9] provides an excellent system to investigate the homogenity of the field sensitive carotenoid pool. Using a method which allows the determination of carotenoid bandshifts within an error smaller than 0.05 nm, we were able to resolve the shifts attributed to the different phases.

Materials and Methods

Chromatophore preparation

Rhodopseudomonas sphaeroides wild type NCIB 8253 (obtained by courtesy of Dr. R. Niederman, Rutgers University, USA) and 2.4.1., mutant G 1 C (kindly provided by Dr. V. A. Saunders, University of Bristol, U. K.) and *Rhodopseudomonas capsulata* mutants BY 761 and N 22 (isolated and kindly provided respectively by Dr. B. Marrs, Washington University, St-Louis, USA and Dr. N. G. Holmes, University of Bristol, U. K.) were grown anaerobically in a medium described by Sistrom [10]. The cells were washed in a medium containing 50 mM MOPS and 100 mM KCl, pH 7.0, resuspended in the same medium and broken in a French Pressure Cell at 985 kg cm^{-2}. The fraction sedimenting between 20000×g (20 min) and 110000×g (90 min) was resuspended in a minimal volume of the same medium and kept at 3 °C or at −30 °C in a deep freeze (with 40% glycerol).

Spectrophotometric methods

Changes in carotenoid absorbance at a single wavelength were measured in a rapidly responding

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Abbreviations: MOPS, 3-(N-morpholino) propane sulphonic acid; DAD, diaminodurene; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyldiazone.

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single beam spectrophotometer [11]. Signals were averaged and processed by a home-made microcomputer based on an LSI-11 system (Digital Equipment Co. Ltd., Reading, U. K.) and using a transient recorder (Datalab DL 901, Mitcham, U. K.) as a fast data acquisition peripheral. Actinic illumination was provided by a saturating Xenon flash (special snaked flash tube, Wingent, Cambridge, U. K.) of about 20 μs duration at half-width.

Calculating techniques

In order to calculate the wavelength of the isosbestic point corresponding to an absorbance change at a given wavelength around the isosbestic point for a given time after a flash, normalized difference spectra were calculated for the red-most band, which shows the largest bandshift [7]. The contribution of the second band to absorbance changes around the isosbestic point of the red-most one are negligible, as well as the electrochromic extinction changes of the bands [7]. The simulated difference spectrum for the long wavelength band is given by the following function (where the subscript i stands for the different species studied):

$$\Delta_i(\lambda) = \exp - \frac{(\lambda_0 + \Delta\lambda - \lambda)^2}{\omega_i} - \exp - \frac{(\lambda_0 - \lambda)^2}{\omega_i}. \quad (1)$$

In this formula, $\Delta_i(\lambda)$ is the absorbance change at a wavelength λ, $\Delta\lambda$ is the bandshift, λ_0 is the center of the non-shifted band and $\lambda_0 + \Delta\lambda$ is the center of the shifted band, ω_i is the width of the band at 1/e of its maximum value. When two conditions are met, *viz.* (1) the bandwidth does not change during the bandshift and (2) $\Delta\lambda$ remains smaller than about 7 nm, then the difference spectrum resulting from the bandshift is equal to the first derivative of the non-shifted band, but shifted by $\Delta\lambda/2$ and multiplied by a constant c, such that c is proportional to $\Delta\lambda$.

Both conditions are met in the light-induced carotenoid shift, see ref. [7]. Moreover it has been verified that the bandwidths of the field indicating carotenoids do not change during the different phases of the flash induced absorbance change (not shown), so that the same simulation curve could be used for all the phases of the flash induced carotenoid bandshifts. Therefore, assuming that the model is correct, and ω_i is known, we can in principle calculate the position of the isosbestic point of the light minus dark difference spectrum by measurement of the absorbance change at only two wavelengths, *viz.* one at the wavelength λ_{max}, where the absorbance is maximal and one at an arbitrary wavelength λ_x near the isosbestic point. When the absorbance change $\Delta\lambda_{max}$ at λ_{max} is normalized to some fixed value, then from the normalized absorbance change at λ_x we can graphically determine on the simulation curve the distance of λ_x to the isosbestic point and obtain in this way a calculated value for the isosbestic point. Before carrying out this normalization, certain corrections of the measured absorbance changes have to be carried out.

In a typical experiment, about 40 groups of flashes were fired with a fixed delay between them. At certain time intervals the flash induced absorbance change at λ_{max} was measured so that possible instabilities of the light induced absorbance changes could be monitored; usually a decay of the changes not larger than 10% was observed. Taking a normalized value for ΔA_{max}, each flash induced absorbance change was corrected for the instabilities of ΔA_{max} observed.

The dark spectrum of the field sensitive populations, which appeared to change significantly after continuous illumination [12], remained quite constant during one series of flash illumination (changes were less than 0.05 nm for 40 flashes) so that correction was not necessary. A (very important) correction was made for the light induced reaction center absorbance changes (see results section).

In most cases in order to obtain the flash induced absorbance changes ΔA_x at a wavelength near the isosbestic point λ_x, averages of 4 traces were made. Taking all the necessary corrections into account for each ΔA_x measured, the isosbestic point was calculated for about 15 time points. The flash induced absorbane changes were recorded for at least 5 different wavelengths λ_x, so that for each time point the calculated isosbestic point could be averaged. The standard deviation on the isosbestic point varied from 0.02 to 0.08 nm.

Results and Interpretation

A difference spectrum of the flash induced absorbance changes due to photochemistry of the reaction center was obtained by collapsing the "usual" carotenoid bandshift upon addition of valinomycin,

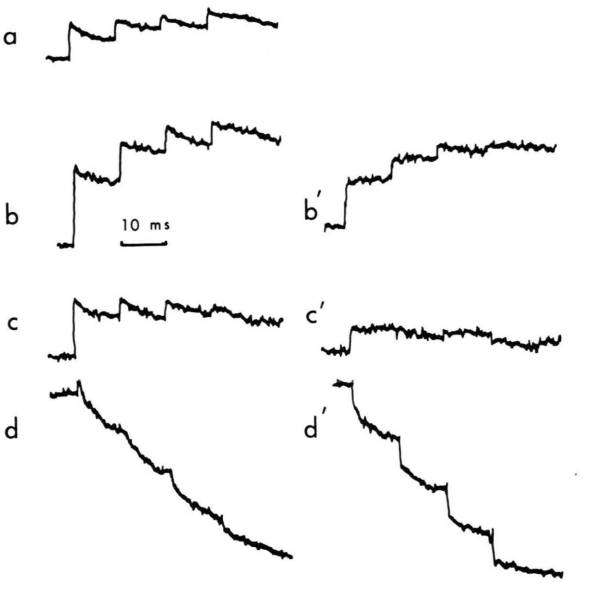
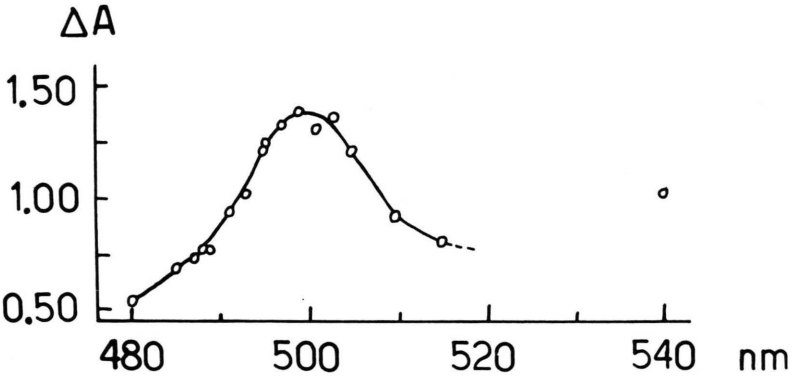


Fig. 1. Kinetics of carotenoid absorption change induced by multiflash illumination with and without correction for reaction center changes. Chromatophores of *Rps. capsulata* mutant N 22 where suspended (to an O. D. of 1.3 at the maximum of the red carotenoid band) in 100 mM MOPS, pH 7.0, 100 mM KCl, 2 mM KCN, 10 mM ascorbate, 100 μM DAD and 0.1 μg/ml nigericin. Each trace was an average of 2. Absorbance changes are in arbitrary units. (a) calibrated reaction center change at 540 nm, with an average calibration factor of 1.26; (b) change 494 nm; (c) change at 495.3 nm (isosbestic for the total change); (d) change at 497 nm; (b'), (c') and (d') changes are the same as (b), (c) and (d) minus (a).

Insert: Normalized spectrum of the reaction center changes ($A_{540} = 1$) obtained 40 ms after a flash in the presence of 10 μM valinomycin.



for example, the N 22-mutant of *Rhodopseudomonas capsulata*, see Fig. 1 insert. The flash induced difference spectrum originates from two changes: first a bandshift to the red of carotenoids closely linked to the reaction center [8] and with bands absorbing at wavelengths which are shifted to the blue by about 5 nm in respect to the transmembrane field sensitive carotenoids; and secondly, a change with a very flat spectrum due to bacteriochlorophyll oxidation. Since the reaction center carotenoid difference spectra vary slightly with the type of mutant, for each species a set of correction factors has to be calculated. The decay kinetics of these flash induced reaction center changes correlate very well with those of P 870 oxidation monitored at 540 or 605 nm (not shown); this indicates that the reaction center carotenoid bandshifts correspond to charge separation in the reaction center itself as suggested by Cogdell *et al.* [8]. This fact allows us to multiply the 540 nm trace by the calculated normalization factor in order to obtain the appropriate correction for the reaction center changes. The reason why the absorbance change at 540 nm was used instead of the absorbance change at 605 nm is that the latter are contaminated by absorption changes due an electrochromic bandshift of the 590 nm band of bacteriochlorophyll (J. Bowyer, private communication).

For all species studied, except for the *Rhodopseudomonas sphaeroides* wild types, the reaction center changes at 540 nm are almost completely free from contributions due to carotenoid band shifts. Fortunately, for these wild types the reaction center

changes are negligibly small (at the time scales measured). Cytochrome changes being much smaller in the wavelength regions of interest than the reaction center changes, were not taken into account. Flash induced absorbance changes in the N22-mutant of *Rps. capsulata* at a few wavelengths around the isosbestic point are shown in Fig. 1 with and without reaction center contribution, which clearly have a great influence on both the extent and the kinetics of the traces.

Plots of the isosbestic point versus the corresponding absorption changes at the maximum of the difference spectrum show directly whether the carotenoids which undergo a bandshift form a homogeneous population or not. Indeed, if there is only one carotenoid shifting one would expect a linear plot, as is deduced from the following formula taken from reference [7], where α, the amount of field-sensitive carotenoid, is a constant:

$$\Delta A_{max} = \epsilon \times \alpha \times \Delta\lambda \qquad (2)$$

where ΔA_{max} is the absorption change at the maximum of the difference spectrum, $\Delta\lambda$ the wavelength shift of the isosbestic point and ϵ a constant. Such a plot is shown in Fig. 2 for the 2.4.1. wild type of *Rhodopseudomonas sphaeroides.* Clearly from the non-linearity of the plot one can see that there is more than one population involved.

The apparent slow phase seems to be biphasic, the first phase being completed in about 1 ms and showing a larger bandshift than the second which takes about 10 ms (depending on the redox conditions) to be completed. After the additon of Antimycin A to the chromatophore suspension, which abolishes the slow phase of ΔA_{max} almost completely, there still remains about 70% of the apparent slow change wavelength shift of the isosbestic point. The fact that the Antimycin-sensitive phase, which is responsible for the larger part of the absorbance change ΔA_{max} of the total slow phase, contributed only for about 30% to the isosbestic point shift substantiates the conclusion that the slow phase contains contributions from two different pools of carotenoids, one correlated to the electron transport from cytochrome b to cytochrome c, and the other, being insensitive to Antimycin A, probably reflecting the slow phase of cytochrome c oxidation.

A particularly interesting feature of plots of the kind shown in Fig. 2 is observed for the change in isosbestic point after the last flash of a series of 4 flashes with 10 ms interval. It appears that the maximum wavelength shift is reached at about 50 ms after the last flash, at which time the absorbance change ΔA_{max} has already decay by 5%. This indicates that the various populations of field-sensitive carotenoids can behave independantly to some extent.

From all the previous arguments, we have to assume that there exist various pools of field-sensitive carotenoids which have different sizes and can show different bandshifts. The same result was obtained with the N. C. I. B. 8253 wild type of *Rhodopseudomonas sphaeroides.*

Fig. 3 shows plots obtained from two different preparations of the G 1 C-mutant of *Rhodopseudomonas sphaeroides.* Apparently, the situation here is even more complicated. In any case one has to assume that there are about three carotenoid pools each absorbing at slightly different wavelengths. The carotenoid pool reacting in the phase which is too fast to be resolved by the apparatus, absorbs apparently more to the red than the other pools. The possibility that each pool has a slightly different

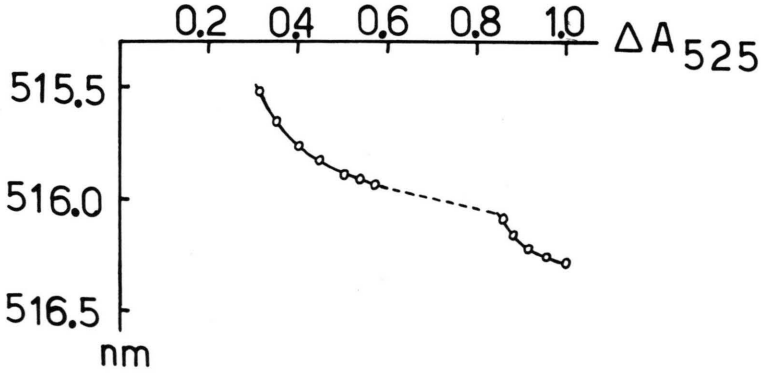


Fig. 2. Plot of the isosbestic point as a function of the relative absorbance change measured at the maximum of the difference spectrum (525 nm) for *Rps. sphaeroides* strain 2.4.1. The maximal absorbance change at 525 nm was arbitrarily set to 1.0. Measuring medium was the same as in Fig. 1.

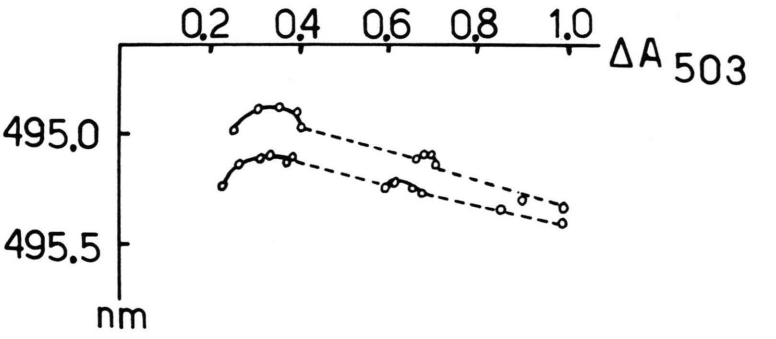


Fig. 3. Similar plots as in Fig. 2 for two different preparations of *Rps. sphaeroides* mutant G1C. The maximal absorbance change at 503 nm, maximum of the red band, after 4 flashes was arbitrarily set to 1.0. Measuring medium was the same as in Fig. 1. Lower trace, bacteria grown at high light intensity; upper trace, bacteria grown at low light intensity.

absorption spectrum makes it difficult to quantify the results, since the shifts of the isosbestic point may be masked by the spectral differences of the various pools. This, in fact, remains true for the results obtained with the *Rhodopseudomonas sphaeroides* wild types. Results obtained with the *Rhodopseudomonas capsulata* mutants lead to the same conclusions. Experiments with the N22 mutant, containing predominantly neurosporene (60%) and chloroxanthin (30%) (N. G. Holmes, private communication) lead to plots similar to those obtained with the *Rhodopseudomonas sphaeroides* wild type strains although they have a completely different carotenoid composition. The BY761 mutant, containing only neurosporene (B. Marrs, private communication) gives plots resembling those of the G1C mutant of *Rhodopseudomonas sphaeroides*, which has the same carotenoid composition.

Discussion

The results presented in this paper emphasize the importance of the correction for reaction center changes if one wants to calculate the carotenoid bandshift, with an error less than 0.2 nm. The reaction center changes have to be analyzed very carefully for this reason. It is of importance to notice here that the reaction center difference spectrum is insensitive to the ionophores used to collapse the membrane potential (valinomycin, which is neutral or FCCP, bearing a positive charge), justifying the way of correction we used in this work.

It has to remembered that the analysis of the carotenoid bandshift measured under continuous illumination reported in a previous paper [7] was only a first order approach. The analysis in fact ammounted to some kind of averaging of all the possible populations giving rise to the band shifts. Jackson and Dutton [5] have related the different phases of the carotenoid absorbance changes to electron transport events after a flash both by kinetic and redox potentiometric resolution. The present results strongly suggest the existence of several pools of carotenoids, each being associated with a particular segment of the electron transport chain and acting independently from each other, at least to some measurable extent.

At least three pools can be resolved. The first one, responding to the formation of P^+X^- (where P is the reaction center bacteriochlorophyll and X the primary acceptor) has to include also the fast component of P^+ reduction by cytochrome c. The second pool, probably corresponding to the slow component of cytochrome c oxidation seems to be very small. This pool, however, would give rise to large bandshifts (0.5 to 0.6 nm for the first flash). The third pool, which is Antimycin A sensitive, corresponds probably to the electron transport sequence between cytochrome b and cytochrome c.

The spectral differences of the various pools could be caused by different carotenoid-environment interaction. Sewe and Reich [13] pointed out that carotenoids can undergo asymmetrical complex formation with other molecules (in the case of chloroplasts chlorophyll b) creating a permanent field. This gives rise to a much larger electrochromic bandshift (with a pseudolinear field dependence) than for the unpolarized carotenoids (showing a quadratic field dependence).

Our results clearly show that the absorbance change at the maximum of the difference spectrum cannot be used to measure the electrical fields accompanying the different phases of electron transport. In the case of electrochromic carotenoid ab-

sorbance changes the only valid measure of electrical field strength is the wavelength shift of the isosbestic point since the value ΔA_{max} as indicated by equation [2], is dependant on the size of the different pools involved. However, it seems to be very difficult to get quantitative values for the different field strengths developped in each electrogenic site out of the plots as shown above.

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