Biochimica et Biophysica Acta, 462 (1977) 247–258

BBA 47375

ELECTROCHROMIC ABSORBANCE CHANGES OF PHOTOSYNTHETIC PIGMENTS IN *RHODOPSEUDOMONAS SPHAEROIDES*

II. ANALYSIS OF THE BAND SHIFTS OF CAROTENOID AND BACTERIOCHLOROPHYLL

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(Received January 4th, 1977)

SUMMARY

An analysis was made of the changes of pigment absorption upon illumination of chromatophores of *Rhodopseudomonas sphaeroides* at −35 °C, described in the preceding paper (de Grooth, B. G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 237–246). Comparison of the light-induced difference spectra in the carotenoid region obtained without additions, and in the presence of *N*-methylphenazonium methosulphate and ascorbate as donor-acceptor system showed that the latter spectrum was not only about 10 times larger in amplitude, but also red-shifted with respect to the first one. Together with the shape of the difference spectrum, this indicated that the spectrum obtained in the presence of a donor-acceptor system is due to an electrochromic shift of the absorption spectrum of a carotenoid by a few nm towards longer wavelength, caused by a delocalized potential across the chromatophore membrane. The results of an analysis of the kinetics of the absorbance changes near the zero points of the spectrum were in quantitative agreement with the extent of the red shift and indicated a shift of 0.25 nm for a single electron transfer per reaction center, and shifts of up to 4 nm when the electron transport is stimulated by a donor-acceptor system. For bacteriochlorophyll *B*-850 the shift is three times smaller.

Analysis of the overall absorption spectrum showed that there are at least two pools of carotenoid. The carotenoid that shows electrochromism has absorption bands at 452, 481 and 515 nm, and comprises about one-third of the total carotenoid present; the remaining pool absorbs at about 7 nm shorter wavelength and does not show an electrochromic response to illumination. Both pools presumably consist of spheroidene; the differences in band location may be explained by the assumption that only the first pool is subjected to a local electric field which induces an electric dipole even at zero membrane potential. Similar results were obtained at room temperature and with a mutant of *Rps. sphaeroides* (G1C)-containing neurosporene.

Abbreviation: PMS, *N*-methylphenazonium methosulphate.

INTRODUCTION

In the preceding paper [1] it was shown that chromatophores of *Rhodopseudomonas sphaeroides*, when illuminated in the presence of an electron donor-acceptor system at sub-zero temperature, show large absorbance changes in the region of carotenoid and bacteriochlorophyll absorption. These changes were abolished by gramicidin D and presumably reflect the generation of large membrane potentials across the chromatophore membrane. The difference spectrum was similar to difference spectra observed earlier at room temperature [2, 3, 4] both with chromatophores and intact bacteria, and indicated the red shift of the absorption bands of a carotenoid and of bacteriochlorophyll (*B*-850).

This paper gives an analysis of the kinetics and the spectrum of the low-temperature absorbance changes. It will be shown that, contrary to earlier conclusions [2, 5–7] the absorbance changes can be quantitatively ascribed to electrochromic band shifts, the extents of which increase with increasing potential across the chromatophores membrane. Measurements at room temperature, though less precise, were in agreement with this conclusion.

MATERIALS AND METHODS

The methods for preparing chromatophores and for measuring absorbance changes induced by continuous illumination were described in the preceding paper [1]. All experiments were done in a phosphate buffer (50 mM) containing 10 mM $MgCl_2$ and unless otherwise indicated in the presence of 50 % (v/v) glycol. The pH before mixing was 7.5, corresponding to an effective pH (pH*) of 8.0 at −35 °C [8]. Flash-induced changes were measured with a single beam apparatus attached to a signal averager (Data Lab. DL 102.S) to enhance the signal to noise ratio. The optical pathlength was 1.0 mm in these experiments. Absorption spectra at −35 °C were obtained using a Cary model 14R spectrophotometer connected to a computer (PDP 9) used for analysis of the data. The optical pathlength was 1.0 mm. The absorption spectra were analyzed into Gaussian components using the method of Fraser and Suzuki [9].

RESULTS AND INTERPRETATION

Difference spectra and kinetics of the carotenoid shift

The dashed line of Fig. 1 shows the spectrum of the gramicidin-sensitive part of the light-induced absorbance changes measured at −35 °C with chromatophores of *Rps. sphaeroides*. As was discussed in the previous paper [1], these changes most likely reflect a red shift of the absorption spectrum of a carotenoid, spheroidene, which is caused by a change in the membrane potential due to a single electron transfer in each reaction center. After addition of an artificial electron donor and acceptor, much larger potentials are created due to secondary electron transport, as was concluded from the 10–15 times stimulation of the carotenoid absorbance changes. The solid line of Fig. 1 shows the difference spectrum of the enhanced changes plotted on a reduced scale. The spectra of Fig. 1 were measured with the same batch of chromatophores. The spectra are very similar in shape, but the spectrum of the stimulated changes is shifted by about 1 nm towards longer wavelength.

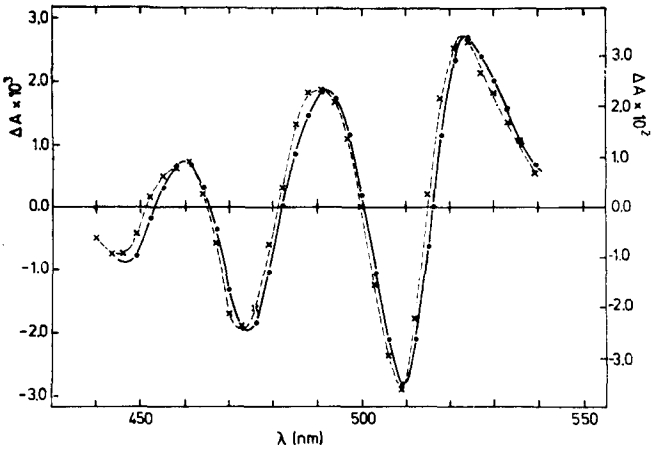

Fig. 1. Light minus dark absorbance difference spectrum of chromatophores of *Rps. sphaeroides* (wild strain) measured at −35 °C. X---X, absorbance changes obtained upon the second and subsequent illumination (see ref. 1) after subtraction of the signals obtained with 50 μM gramicidin D. Illumination 880 nm, 60 nE/cm^2 per s (left hand scale). ● — ●, absorbance changes induced by 5 s of illumination (6 nE/cm^2 per s) in the presence of 160 μM PMS and 3.3 mM ascorbate (right hand scale). Bacteriochlorophyll concentration: 80 μM. The absorbance at 477 nm was 0.56.

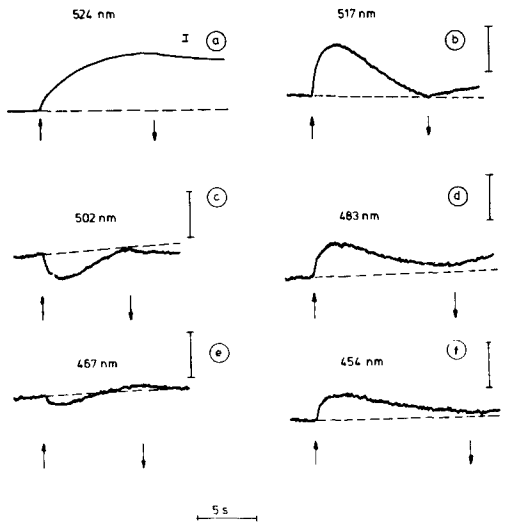

Fig. 2. Kinetics of the light-induced absorbance changes in the presence of PMS and ascorbate. Continuous illumination was switched on and off as indicated by the up- and downward pointing arrows. Conditions as for Fig. 1 (solid line). The vertical bars denote a change in transmittance of 1.25 %. Recordings a and b were measured simultaneously.

The above observations are easily explained by the hypothesis [10, 11] that the carotenoid shifts are an increasing function of the membrane potential, caused by an electrochromic mechanism. If one assumes that a single electron transfer per reaction center produces a red shift by 0.25 nm of the absorption spectrum, then the difference spectrum of the stimulated changes (solid line) should reflect a 10 times larger shift (2.5 nm). In first approximation, the shape of both spectra will be identical, and proportional to the inverted first derivative of the absorption spectrum. However, the first difference spectrum will be shifted by 0.5×0.25 nm, the second one by 0.5×2.5 nm with respect to the first derivative. Thus the second spectrum will be located at 1.13 nm longer wavelength than the first one. It is clear that the results are not in agreement with the assumption [2, 5–7] that the difference spectra reflect a discrete shift of the spectrum of an increasing number of pigment molecules, since this would imply that the two difference spectra should differ only in amplitude.

The kinetics of the absorbance change were also in agreement with the assumption that they are caused by a spectral shift that increases with increasing membrane potential. This is illustrated by the recordings of Fig. 2 which were measured under conditions where the stimulation was even larger than in Fig. 1 and the distance between the spectra of the stimulated and the "normal" changes was about 2 nm. Recording a shows the kinetics at 524 nm where the absorbance changes show an approximately exponential increase. However, the kinetics at 517, 483 and 454 nm showed an absorbance increase followed by a decrease, whereas at 502 and 467 nm the opposite behaviour was observed (Fig. 2, recordings b–f). At these wavelengths the spectrum of the stimulated changes intersects the zero line; they are located at slightly longer wavelengths than the maxima and the minima, respectively, of the absorption spectrum of the carotenoid that undergoes an electrochromic absorbance change. From extrapolation towards a membrane potential of zero it followed that the absorption maxima of this carotenoid are located at 452, 481 and 515 nm. The absorbance changes at 517, like those at 524 nm [1] were abolished by gramicidin D.

For a quantitative analysis of the relation between the kinetics at 517 and 524 nm we assume that they are caused by the shift of a single Gaussian band. For mathematical convenience we shift the wavelength axis such that $\lambda = 0$ at the maximum. The absorption spectrum then is given by:

$$A(\lambda) = A \cdot \exp[-\tfrac{1}{2}(\lambda/\sigma)^2]$$

where $A(\lambda)$ is the absorbance as a function of wavelength λ, 2σ is the distance between the inflection points and A is the absorbance at the maximum. This absorption band then shifts over a distance $p\Delta\lambda$ where p goes from 0 to 1 in time and $\Delta\lambda$ is the ultimate wavelength shift for a particular experiment. For the absorbance change we obtain:

$$\Delta\varepsilon(p, \lambda) = A \cdot \exp[-\tfrac{1}{2}(\lambda/\sigma - p\Delta\lambda/\sigma)^2] - A \cdot \exp[-\tfrac{1}{2}(\lambda/\sigma)^2] \quad (1)$$

For a number of values of $\Delta\lambda$ we computed the relation between the absorbance changes in the inflection point ($\Delta\varepsilon(p, \sigma)$), measured at 524 nm, and p. Measurements of the kinetics of $\Delta\varepsilon(\sigma)$ then gave p as a function of time and also the constant A. Subsequently, by substituting these parameters into the right hand side of Eqn. 1, and taking $\lambda = \frac{1}{2}\Delta\lambda$, at which wavelength the absorbance changes are zero for $p = 1$, we computed both the amplitude and the time dependence of the absorbance changes at this wavelength (517 nm). The result is shown in Fig. 3; the solid line is a hand-

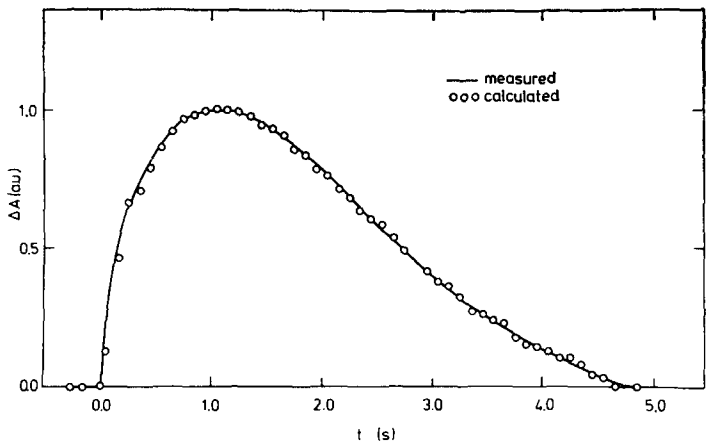

Fig. 3. Kinetics of the light-induced absorbance changes at 517 nm. Conditions as for Fig. 1 (solid line). Solid line, measured kinetics, obtained from a hand-smoothed recording. Open circles, calculated from the kinetics at 524 nm as discussed in the text.

smoothed recording of the absorbance changes measured at 517 nm whereas the open circles were calculated from the kinetics at 524 nm which were measured simultaneously. The best fit was obtained with $\Delta\lambda = 4.4$ nm. Since this value is in good agreement with that calculated from the relative position of the difference spectra (4.0 ± 0.5 nm), we may conclude that the model is in close agreement with the observations.

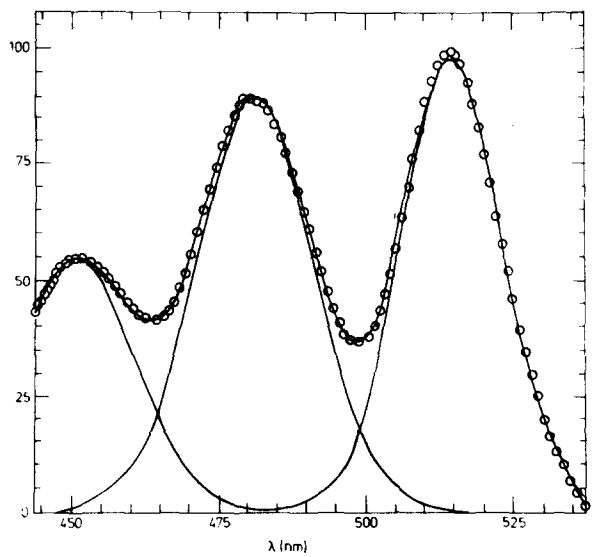

Fig. 4. Circles, absorption spectrum of the carotenoid which shows an electrochromic shift, calculated from Fig. 1 (solid line). The spectrum is analyzed into three Gaussian components, the sum of which gives the solid line through the circles.

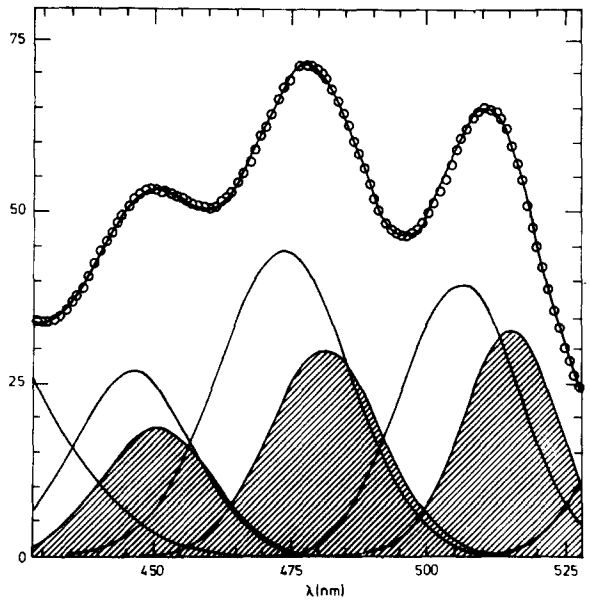

Fig. 5. Open circles: absorption spectrum of chromatophores at −35 °C. The solid line through the circles is the sum of the Gaussian bands into which the spectrum is resolved. The shaded areas denote the bands that comprise the absorption spectrum of the carotenoid that shows a wavelength shift (see Fig. 4). See text for further explanation.

Carotenoid absorption spectra

In the previous section we have shown that the light-induced absorbance changes at −35 °C can be described as an electrochromic shift of the absorption spectrum of a carotenoid with maxima at 452, 481 and 515 nm. The absorption spectrum of this carotenoid, apart from an "integration" constant, can be obtained in a simple way from the difference spectra; Fig. 4 shows this absorption spectrum as calculated from the spectrum (solid line) of Fig. 1. The constant was chosen such that the spectrum could be fitted by three Gaussian curves. The spectrum was clearly different from the overall absorption spectrum of the chromatophores in the carotenoid region; not only was it lower, but the bands were also narrower and located at about 4 nm longer wavelength. The question thus arises whether the spectrum of the carotenoid that undergoes an electrochromic shift can be accommodated in the overall spectrum. In order to answer this question, we measured the overall absorption spectrum at −35 °C (Fig. 5, open circles) and analyzed it into Gaussian components (Fig. 5, solid line through the circles). Among these components were the ones mentioned above, corresponding to the absorption spectrum of carotenoid which undergoes the electrochromic shift. Apart from these components, three additional bands were necessary to fit the absorption spectrum. These bands are due to carotenoids which do not shift. At the edges of the spectrum, two extra bands were needed. These bands, however, were also found in a more trivial analysis in which the carotenoid part of the spectrum was analyzed into only three bands and are presumably due to other pigments.

From the above analysis it can be concluded that the simplest model to describe our results assumes the existence of two different "pools" of carotenoid in

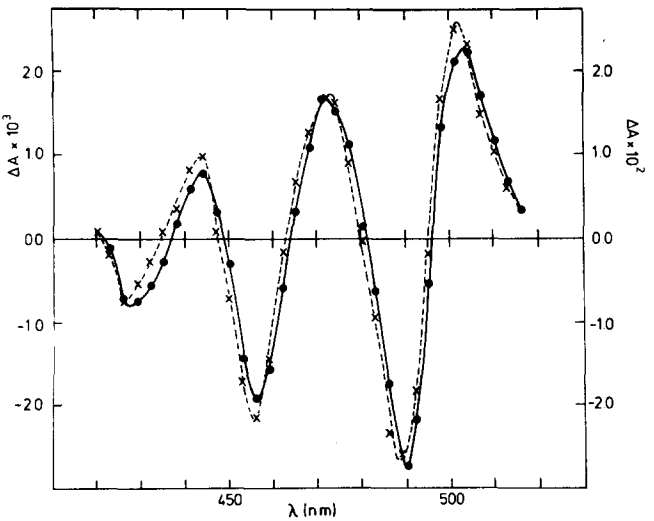

Fig. 6. Light minus dark absorbance difference spectra of chromatophores of the G1C mutant of *Rps. sphaeroides*. Conditions and symbols as for Fig. 1, except a bacteriochlorophyll concentration of 70 μM.

Rps. sphaeroides. The first pool, comprising about 35 % of the total carotenoid, has absorption maxima at 515, 481 and 452 nm and its absorption spectrum shifts upon illumination; the remaining carotenoid has absorption maxima at 5–9 nm shorter wavelengths and does not show absorbance changes.

Experiments with the G1C mutant

Essentially the same results as described in the previous sections, were obtained with chromatophores of the G1C mutant of *Rps. sphaeroides*. This mutant has only

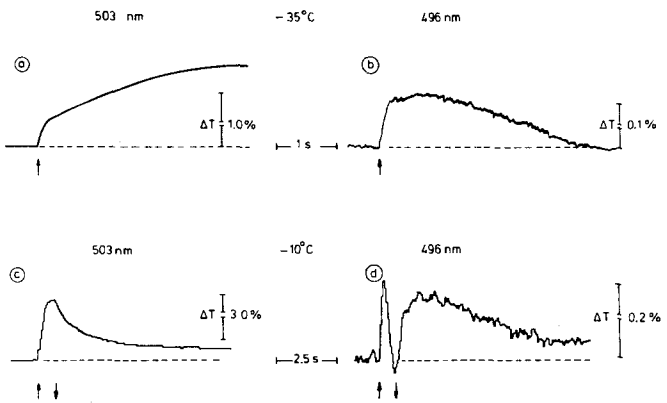

Fig. 7. Light-induced absorbance changes of chromatophores of the G1C mutant in the presence of 160 μM PMS and 3.3 mM ascorbate. Recordings a and b: temperature, −35 °C; bacteriochlorophyll concentration, 23 μM; illumination, 6 nE/cm² per s. Recordings c and d: temperature, −10 C°; bacteriochlorophyll concentration, 70 μM; illumination, 6 nE/cm² per s. Changes which were not sensitive to 50 μM gramicidin were subtracted.

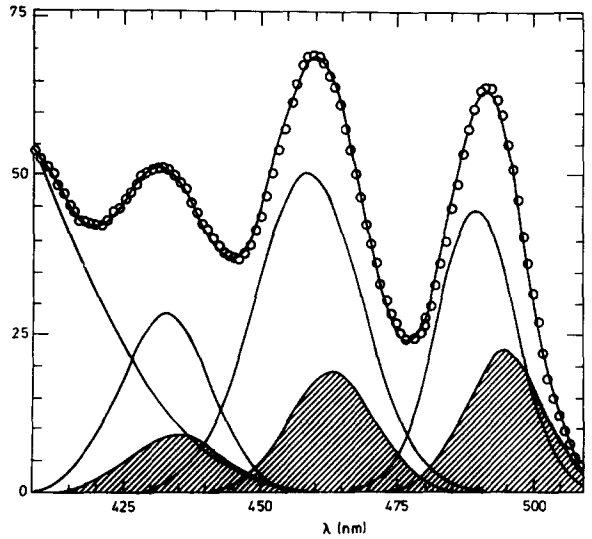

Fig. 8. Absorption spectrum of chromatophores of the G1C mutant of *Rps. sphaeroides* at −35 °C, analyzed into Gaussian bands. See Fig. 5 for further explanation.

one major carotenoid, neurosporene [6]. The carotenoid absorption maxima of this mutant are located at about 20 nm shorter wavelengths than those of the wild type. Fig. 6 shows the difference spectra at −35 °C in the absence and in the presence of a donor-acceptor system. The spectra, like those of the wild strain were shifted with respect to each other. Kinetics of the absorbance changes are shown in Fig. 7, recordings a and b. The kinetics at 496 nm (near the zero point) could be fitted by the same method as described above, and the analysis indicated a red shift of 0.3 nm of the absorption spectrum upon a single electron transfer per reaction center. The reconstituted absorption spectrum of the carotenoid that showed a red shift upon illumination could be accommodated in the overall absorption spectrum as shown in Fig. 8. As with the wild strain, our results indicate the existence of at least two pools of carotenoid, of which only one, with absorption maxima at 436, 463 and 495 nm shows absorbance shifts.

Fig. 7 shows in addition absorbance changes measured at −10 °C (recordings c and d). At this temperature the decay upon darkening was fast enough to be measured with reasonable precision. It can be seen that at 495 nm this decay showed the expected behavior: an absorbance increase followed by a decrease, whereas at 503 nm it showed a decrease of absorbance only.

Measurements at room temperature

The model discussed above is based on observations done at low temperature and in the presence of 50 % glycol. In order to investigate whether the model is also valid under more "normal" conditions, we did some experiments without glycol at room temperature. Before discussing these results it is necessary to take a closer look at Eqn. 1. For small $\Delta\lambda$ compared to σ, developing the right hand side into a Taylor

series around $p\Delta\lambda/\sigma = 0$, we obtain for the absorption changes in the inflection point ($\lambda = \sigma$):

$$\Delta\varepsilon(p, \sigma) = A(\sigma)p\,\frac{\Delta\lambda}{\sigma} \tag{2}$$

where terms of order higher than $(\Delta\lambda/\sigma)^2$ are neglected. (The coefficient of $(\Delta\lambda/\sigma)^2$ is zero). For the absorption changes at the "zero" point, $\lambda = \frac{1}{2}\Delta\lambda$ we obtain:

$$\Delta\varepsilon(p, \tfrac{1}{2}\Delta\lambda) = \tfrac{1}{2}A(\tfrac{1}{2}\Delta\lambda)\,(p-p^2)\left(\frac{\Delta\lambda}{\sigma}\right)^2 \tag{3}$$

neglecting terms of higher order than $(\Delta\lambda/\sigma)^3$. (The coefficient of $(\Delta\lambda/\sigma)^3$ is zero). Thus we see that the absorbance increase at the inflection point (524 nm) is linear with the shift $\Delta\lambda$, whereas at $\lambda = \frac{1}{2}\Delta\lambda$ it is proportional to $(\Delta\lambda)^2$.

Furthermore, combining Eqns. 2 and 3 gives a simple formula for calculating $\Delta\lambda$. Let $\Delta\varepsilon(\sigma)_{\max}$ and $\Delta\varepsilon(\frac{1}{2}\Delta\lambda)_{\max}$ be the maximal absorbance changes for $\lambda = \sigma$ and $\lambda = \frac{1}{2}\Delta\lambda$ (which are obtained for $p = 1$ and $p = \frac{1}{2}$, respectively). Since for small $\Delta\lambda$, $A(\frac{1}{2}\Delta\lambda) \approx A = 1.65\,A(\sigma)$, we find:

$$\Delta\lambda = 4.85\,\sigma\,\frac{\Delta\varepsilon(\frac{1}{2}\Delta\lambda)_{\max}}{\Delta\varepsilon\,(\sigma)_{\max}} \tag{4}$$

For a Lorentzian band we obtain in a similar way:

$$\Delta\lambda = 4.50\,\sigma\,\frac{\Delta\varepsilon(\frac{1}{2}\Delta\lambda)_{\max}}{\Delta\varepsilon\,(\sigma)_{\max}} \tag{5}$$

The symbols are defined in the same way as for Eqn. 4.

The maximum absorbance change at 524 nm was about four times lower at room temperature than at −35 °C. This implies that the changes to be expected at $\lambda = \frac{1}{2}\Delta\lambda$ (515 nm) were 16 times reduced in amplitude. To observe these small absorbance changes we had to correct for other spectral changes. Fig. 9 shows the result of such an experiment. Absorbance changes were induced by a train of nine short, saturating, xenon flashes given at intervals of 40 ms in the presence of PMS and ascorbate. With an averager 16 experiments were added. After injecting 50 μM gramicidin in the sample, 16 experiments were subtracted. The kinetics obtained in this way at 515 and 524 nm are in agreement with the observations at −35 °C. Substituting the observed ratio between the maximal absorbance changes at 524 and 515 nm into Eqn. 4 we obtain $\Delta\lambda = 1.0\pm0.3$ nm. The stimulation in this experiment was about three times, as can be seen by comparing the absorbance change induced by the first flash with the maximal changes at 524 nm. Consequently, for a single electron transfer this gives a band shift of 0.3 ± 0.1 nm which is in agreement with the value of 0.25 nm calculated at −35 °C.

Bacteriochlorophyll shifts

Eqns. 4 and 5 are general equations that can be used to calculate the magnitude of $\Delta\lambda$ for a shift of an absorbance band, provided the changes at $\lambda = \frac{1}{2}\Delta\lambda$ are large enough to be measured. We did this for the bacteriochlorophyll shift around 850 nm.

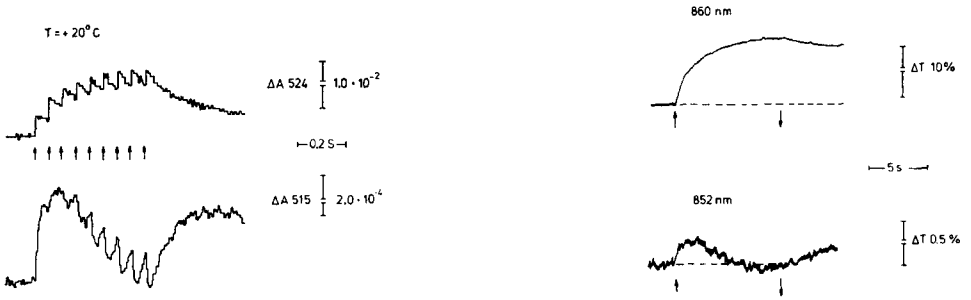

Fig. 9. Absorbance changes in chromatophores of wild type *Rps. sphaeroides* measured at room temperature in the absence of glycol, induced by a train of xenon flashes (duration a few μs) given at the times indicated, obtained as discussed in the text. Additions: 160 μM PMS, 3.3 mM ascorbate. Bacteriochlorophyll concentration, 120 μM.

Fig. 10. Light-induced absorbance changes measured at −35 °C in the presence of PMS and ascorbate. Illumination, 600 nm; 1 nE/cm² per s. The absorbance at 850 nm was 2.0, corresponding to a bacteriochlorophyll concentration of 160 μM. In the same sample, the corresponding changes at 524 nm showed a stimulation of about nine times under these conditions.

As discussed previously, this shift is most likely caused by the same mechanism as the carotenoid shift. The absorption spectrum of the bacteriochlorophyll that participates in the shift, reconstructed from the difference spectrum of Fig. 5 of the preceding paper [1], showed an approximately Lorentzian band shape. Fig. 10 shows the kinetics measured at 860 and 852 nm, corresponding to $\Delta\varepsilon(p, \sigma)$ and $\Delta\varepsilon\ (p, \frac{1}{2}\Delta\lambda)$, respectively, at −35 °C in the presence of PMS and ascorbate. Although the signal to noise ratio at 852 nm was low, the general shape of the kinetics supports the hypothesis that the absorbance changes are caused by a gradual band shift. From Eqn. 5 we calculated a shift of 0.75 nm under these conditions; this gives a value of 0.08 nm for a single electron transfer, if it is assumed that the stimulation was the same as at 524 nm, measured with the same sample. From the magnitude of the absorbance increase at 860 nm we conclude that about 45 % of the pigments absorbing at 850 nm participate in this shift.

DISCUSSION

The results presented in this paper strongly indicate that the pigment shifts observed in chromatophores of *Rps. sphaeroides* are due to electrochromic band shifts of photosynthetic pigments. The analysis indicates that a carotenoid which comprises about 25–35 % of the total carotenoid present shows a red shift of 0.25–0.3 nm if a single electron is transferred across the membrane by each reaction center. The remaining carotenoid absorbs at shorter wavelengths and does not show electrochromism. For the mutant G1C both pools of carotenoid presumably consist of neurospherene, which comprises 96 % of the total carotenoid present in this bacterium [6]. For wild type *Rps. sphaeroides* the situation is complicated by the presence of a variable, but significant amount of spheroidenone in addition to the major carotenoid, spheroidene [12, 13]. However, the shape and the location of the absorption spectrum of spheroidenone in organic solvent [12], suggests that the carotenoid that shows electrochromism is probably spheroidene, and that the remaining spheroidene belongs to the pool that does not show an absorbance change upon illumination.

As pointed out by Schmidt [11] and Reich and Schmidt [14], the size and linearity with membrane potential [3] of the electrochromic reponse of carotenoids which do not possess a permanent electric dipole moment, requires the additional assumption of a permanent local electric field to which the pigment is subjected at zero membrane potential. This field was estimated to be about $2 \cdot 10^{-6}$ V/cm, on basis of in vitro experiments with lutein. The extent of the electrochromic shift observed by us, 0.25–0.3 nm for an estimated membrane potential of about 100 mV [15] (see also ref. 1), is in agreement with this hypothesis, if it is assumed that the polarizabilities of spheroidene or neurosporene and lutein are about the same. It should be noted that the local field is of the right order of magnitude to explain the difference of about 7 nm in the location of the absorption spectra of the two "pools" of carotenoid, since only the long-wavelength form is supposed to be subjected to the field. In addition, spheroidene may have a permanent dipole moment on its own, since the molecule is not symmetrical [13].

Two additional remarks should be made regarding the analyses given by Figs. 5 and 8. Firstly, the pool of carotenoid that shows electrochromism may not be completely homogeneous, but consist of carotenoid molecules with slightly different peak wavelengths and band shifts, depending on the strength of the local field and orientation of the molecule. Secondly, it is assumed that the wavelength shift is the same for all three bands of the carotenoid, whereas the relatively low amplitude of the middle and short-wave bands may suggest that these bands actually may shift by a somewhat shorter distance than the long-wave band. However, this would not materially change the conclusion, except that it would give a somewhat larger estimate for the size of the pool of carotenoid that shows electrochromism. The difference spectra of Fig. 1 do not allow a sufficient accurate determination of the extents of the shifts of the short-wave bands, whereas the analysis given in Fig. 3 becomes more complicated because of overlap of the bands involved.

The absorption difference spectrum in the near-infrared region [1] is mainly due to a red shift of *B*-850, as was earlier observed at room temperature [2, 4]. As with the carotenoid, electrochromic broadening of bacteriochlorophyll absorption bands does not seem to occur to any significant extent. This indicates that the bacteriochlorophyll dipoles, permanent as well as induced, are oriented. Neither the bacteriochlorophyll nor the carotenoid difference spectra suggest a significant increase in oscillator strength as proposed by Conjeaud and Michel-Villaz [16], since the positive and negative areas are approximately equal to each other.

The older model of Amesz and co-workers [2, 5] to explain the band shifts in the carotenoid region was based on the observation that the absorption difference spectrum could be roughly fitted by shifting the overall absorption spectrum by about 10 nm (see also refs. 6 and 7). This model thus assumed a discrete shift of an increasing number of spheroidene molecules with time or light intensity and does not explain the kinetics of the absorbance changes near zero points of the difference spectrum (Fig. 2). The same applies to a model based upon a combination of a discrete and a gradual shift: such a model would predict smaller absorbance changes at e.g. 517 nm in relation to those at 524 nm than were actually observed.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. N. G. Holmes for giving a culture of the G1C mutant of *Rps. sphaeroides* and to Mr. D. Los for aid with the computer calculations. The investigation was supported by the Netherlands Foundation for Chemical Research (S.O.N.), financed by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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