Biochimica et Biophysica Acta, 548 (1979) 153—160

BBA 47733

THE RESONANCE RAMAN SPECTRUM OF CAROTENOIDS AS AN INTRINSIC PROBE FOR MEMBRANE POTENTIAL

OSCILLATORY CHANGES IN THE SPECTRUM OF NEUROSPORENE IN THE CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHAEROIDES* *

Y. KOYAMA ***, R.A. LONG, W.G. MARTIN and P.R. CAREY **

Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A OR6 (Canada)

(Received February 26th, 1979)

Key words: Membrane potential; Neurosporene; Carotenoid; Chromatophore; Resonance Raman spectroscopy; (Rhodopseudomonas sphaeroides)

Summary

The resonance Raman spectrum of the carotenoid neurosporene is shown to be a sensitive monitor of absorption shifts, and thus changes in membrane potential, in chromatophores of the GlC mutant of *Rhodopseudomonas sphaeroides*. For a Raman excitation wavelength at 472.7 nm, the intensities of the two most prominent resonance Raman features (ν_1 and ν_2) respond very differently to small shifts in the absorption maxima. Thus, the ratio intensity ν_1/intensity ν_2 is a sensitive probe for absorption shifts. Changes in this ratio of ~20% were observed during a valinomycin induced diffusion potential. At 5°C changes in the average intensity ratio of +6, -4 and —14% were brought about by oligomycin, FCCP and sodium deoxycholate, respectively. The changes in intensity ratio were temperature dependent and, in addition, effects due to the laser beam acting as an actinic light could be detected. Oscillatory changes were observed in absolute Raman and Rayleigh scattering intensities for chromatophores at 5°C and for intact cells under growing conditions.

Introduction

In both plant and bacterial cells the electronic absorption spectra of membrane bound carotenoids have shown considerable promise as intrinsic probes

* Issued as NRCC No. 17628.

** Author to whom correspondence should be addressed.

*** Present address: Kwansei Gakuin University, Faculty of Science, Nishinomiya, Japan.

of membrane potential. In chromatophores from *Rhodopseudomonas sphaeroides* an artificially generated diffusion potential was shown to cause small shifts in the position of the carotenoids' $\pi \rightarrow \pi^*$ absorption transition [1]. Furthermore, electrochromism has been reported from dried chromatophores [2] and from model carotenoid-multilayers [3]. Recently, the absorption spectrum of chromatophores from the GlC mutant of *Rps. sphaeroides*, chosen for the predominance of a single carotenoid species viz. neurosporene, has been analyzed by several workers and interpreted in terms of two separate carotenoid pools [4—6].

In the present paper we report that the resonance Raman spectra of carotenoids can provide an additional sensitive probe of membrane potential in chromatophores and that the technique may be applied to intact photosynthetic cells. The resonance Raman method enables one to study directly carotenoid absorption spectra at higher resolution than that obtained by absorption spectroscopy. Importantly, the technique offers an especially sensitive measure of shifts in carotenoid absorption maxima. The resonance Raman spectra of carotenoids in various biological environments have provided new insights into the nature of these environments [7] and the resonance Raman spectra of several membrane-bound carotenoids have been reported [8—10]. However, only the study of Szalontai et al. [10] has touched upon the effect of an electric field upon the resonance Raman spectrum of carotenoids, in this case in a frog sciatic nerve. Crucially, no attempt has been made to follow the effect of membrane potential on the resonance Raman spectrum of carotenoids in systems, such as the chromatophores from photosynthetic bacteria, where absorption studies exist for comparison. By chosing the GlC mutant of *Rps. sphaeroides* only the resonance Raman spectrum of the dominant (96%) carotenoid neurosporene [4] is detected. With this simplification, the idea behind the present work was that by the proper choice of resonance Raman excitation wavelength the intensities of the two most prominent resonance Raman features of neurosporene, ν_1 and ν_2, will respond very differently to small changes in absorption maxima. Therefore, the intensity ratio of ν_1/ν_2 should be a sensitive probe for shifts in absorption maxima and thus membrane potential.

Materials and methods

Chromatophores. *Rhodopseudomonas sphaeroides* mutant GlC was a gift from Dr. A.R. crofts. The cells were grown under anaerobic conditions in the light, by the method of Cohen-Bazire et al. [11]. Each culture was started by innoculating from the original slant. Chromatophores were prepared by the procedure of Symons et al. [6] using an ultrasonic cell-disruptor. Usually the chromatophores were suspended in a buffer, 10 mM glycylglycine and 10 mM choline chloride, pH 7.8. The chromatophore suspensions used for resonance Raman measurements had absorbances of ~0.2 and ~0.35 at 493 and 853 nm, respectively, in 1-cm path length absorption cells. The results described here are from approximately 15 separate preparations.

Chemicals. Oligomycin (lot 115B-1580), valinomycin (lot 37C-0274) and gramicidin (lot 77—0195) were purchased from Sigma and sodium deoxy-

cholate (lot 791521) from Fisher Scientific. FCCP (carbonylcyanide-*p*-trifluoromethylphenylhydrazone) was a gift from Dr. H. Schneider.

Raman measurements. A Jarrell-Ash 25-400 Raman spectrometer equipped with an Ar laser, Spectra-Physics 164, was used. The laser beam (40—60 mW) was focussed as close as possible to the wall of a rotating Raman cell, and scattered light was collected at 90°. Typically a rotatable Raman cell was totally filled with a chromatophore suspension and then fully immersed in a temperature-controlled, transparent water bath. Thus, essentially anaerobic conditions were achieved. We hereafter refer to such measurements as 'rotating'. 'Stationary measurements were achieved simply by stopping the rotation of the Raman cell. 'Flow' measurements used horizontal 2-mm square quartz tubing as the Raman cell and a peristaltic pump to move the chromatophore suspension through the cell. The same type of rotating Raman cell was used for intact microbial cell suspensions after filtering the suspensions through a Millipore filter with a pore size of 8.0 μm. For simultaneous measurement of the pH value of the medium a temperature-controlled Raman cell with a magnetic stirrer and a pair of Calomel and glass electrodes connected to a pH meter, Radiometer Copenhagen Model 26, was used.

Results and discussion

Fig. 1 shows the excitation profile (the dependence of Raman intensity on the excitation wavelength) of the ν_1 (C=C stretching) and ν_2 (C—C stretching) bands [12] of neurosporene in the chromatophores of the GlC mutant of *Rps. sphaeroides*. The frequencies of the ν_1 and ν_2 band for different excitation lines agreed with each other to within the limit of experimental error; $\nu_1 = 1526 \pm 3$ cm^{-1} and $\nu_2 = 1157 \pm 3$ cm^{-1}. Because of oscillatory change in Raman intensity (see below), the peak height of each band was measured 6 times at each excitation and then averaged. The water band at ~3300 cm^{-1} was used as an internal standard. Two water band measurements were averaged after each series of recordings of ν_1 or ν_2. Corrections for spectrometer sensitivity and the ν^4 factor were made for each measurement. The excitation profile of neurosporene in the chromatophores is essentially the same as that of β-carotene in isopentane [13]. The analysis of the excitation profile of β-carotene by Inagaki et al. [13] provides us with unequivocal assignments of the peaks in Fig. 1; the structure in the spectrum is vibronic in origin and is due to electronic transitions from the ground vibrational level of the ground electronic state (π) to the vibrational levels of the excited electronic state (π^*). Thus, the peak around 490 nm is due to the (0—0) transitions of the ν_1 and ν_2 modes and the peak around 460 nm is the (1—0) transition of the ν_1 mode. Absence of the (1—0) peak of ν_2 is characteristic and was ascribed to a different molecular structure in the first electronic excited state [13].

Fig. 1 reveals the potential utility of resonance Raman spectroscopy for measuring small shifts in the $\pi \rightarrow \pi^*$ transition. By choosing the 472.7 nm line for excitation, the resonance Raman intensity ratio of ν_1 and ν_2 is very sensitive to small changes in the overall position of the electronic transitions since the slope of the excitation profiles for ν_1 and ν_2 are of opposite sign in this region. With the reasonable assumption that small changes in the absorption peak posi-

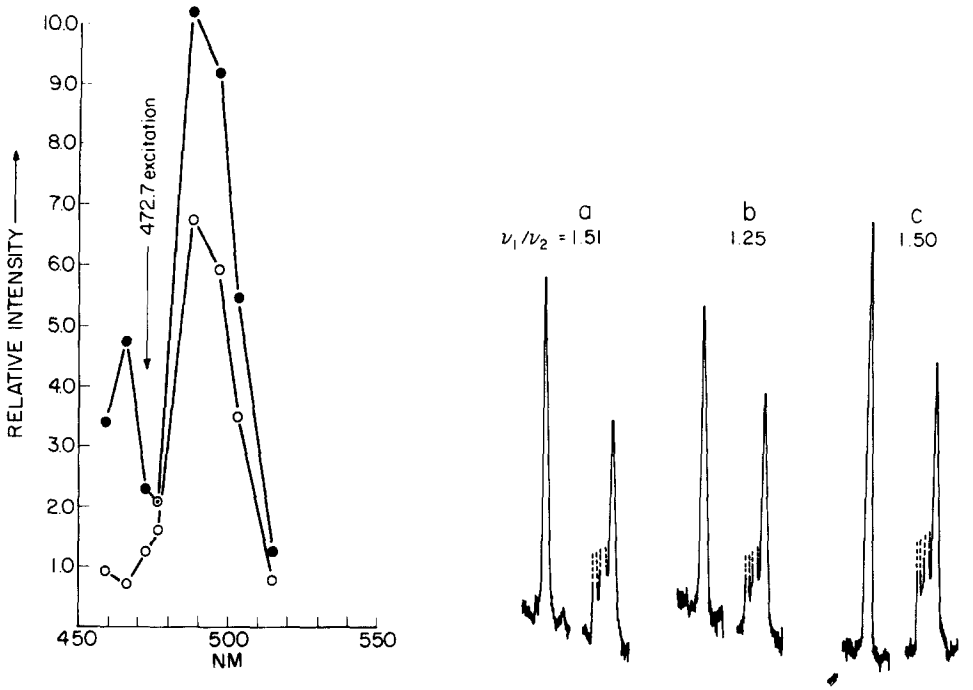

Fig. 1. Excitation profiles for • ν_1 (C=C stretching) and ○ ν_2 (C—C stretching) bands of neurosporene in chromatophores of the mutant G1C of *Rhodopseudomonas sphaeroides*. Lines of an Ar-laser at 457.9, 465.8, 472.7, 476.5, 488.0, 496.5, 501.7 and 514.5 nm were used for excitation.

Fig. 2. Effect of valinomycin-induced efflux of potassium on the relative intensity of ν_1/ν_2. The resonance Raman spectrum of K^+-loaded chromatophores is shown after rapidly mixing with an equal volume of buffer. Experiment 'a' is a control in which the buffer is potassium and valinomycin free, in 'b' a diffusion potential is set up by including valinomycin in the buffer and in 'c' the buffer contains both valinomycin and 50 mM KCl. 50 mW 472.7 nm excitation, at room temperature. Dashed lines are due to laser plasma.

tions will not change the vibrational structure for each normal mode, it can be seen that a red-shift of 3 nm in 0—0 and 0—1 transitions will theoretically increase the ν_1/ν_2 intensity ratio by nearly 100%. Similarly, a blue-shift of 3 nm reduces this ratio by ~25%.

The above ideas were tested by following the effect of a diffusion potential on the resonance Raman spectrum. Jackson and Crofts [1] showed that a valinomycin-induced K^+-efflux from chromatophores, in the dark, produces a blue-shift of the $\pi \rightarrow \pi^*$ band due to an inside-negative diffusion potential. In our system, the laser beam used for exciting Raman scattering may act as an actinic light [14,15] and cause an influx of H^+. This will result in an inside-positive membrane potential with a concomitant red-shift of the carotenoid absorption band [16]. However, the results shown in Fig. 2 indicate that the diffusion potential dominates any possible potential resulting from the actinic light. The K^+-loaded chromatophores were suspended in a potassium-free buffer containing 10 mM choline chloride and 10 mM glycylglycine, pH 7.8, (stock solution A). Solution A was mixed rapidly with an equal volume of (a) exactly the same buffer solution, (b) buffer +0.2 μg/ml valinomycin, and (c) buffer

TABLE I

THE ν_1/ν_2 INTENSITY RATIO AT 472.7 nm EXCITATION FOR CHROMATOPHORES OF *RHODOPSEUDOMONAS SPHAEROIDES* MUTANT GlC

Values are averages ± S.D. Conditions in (A) were approx. 5°C and rotating Raman cell.

(A) Effects of oligomycin, FCCP and sodium deoxycholate

	Oligomycin (40 μg/ml)	FCCP (2 μM)	Sodium deoxycholate (0.25%)
Before addition	1.71 ± 0.03	1.70 ± 0.05	1.71 ± 0.05
After addition	1.81 ± 0.03	1.64 ± 0.03	1.47 ± 0.04
		(overnight	(+ heat
		1.66 ± 0.12)	1.41 ± 0.04)

(B) Dependence on conditions of Raman measurement

	Stationary	Rotating	Flow
Room temperature	1.50 ± 0.03	1.50 ± 0.03	1.50 ± 0.03
~5°C	1.74 ± 0.11	1.64 ± 0.03	1.58 ± 0.02

+0.2 μg/ml valinomycin +50 mM KCl. Experiment (a) was a control and (b) and (c) were designed to monitor the effect of fast and slow K^+-efflux from the chromatophores. Solution A was mixed with the second solution through a T-junction, fitted with a stirring bar, by pushing with a syringe pump. Spectra were recorded 2 s after mixing while the resulting suspension was flowing at 5 cm/s through a horizontal 2-mm square tube. Room temperatures were used in these experiments to promote K^+-diffusion. Decrease in the ν_1/ν_2 intensity ratio from 1.51 to 1.25 in experiment (b) indicates a blue-shift and thus reflects the perturbation due to an inside-negative membrane potential. In the presence of 50 mM KCl the ratio remains at 1.50 (Fig. 2c).

The resonance Raman method was tested also by the effects of oligomycin, FCCP and sodium deoxycholate Table Ia). Control experiments for the chromatophores, in the absence of these factors, in a rotating Raman cell at ~5°C showed that the ν_1/ν_2 intensity ratio did not vary for at least a day except for the oscillatory change discussed below. To allow for errors due to the oscillatory behaviour, the values in Table I are the mean of six measurements on the ν_1/ν_2 ratio. The function of the first factor reported in Table I, oligomycin, is to inhibit the membrane-bound ATPase [17]. This enzyme pumps out H^+ and degrades the inside-high H^+-gradient produced by the photoreaction; thus, oligomycin has been used to prevent the decay of red-shift of chromatophores under continuous illumination of infrared light [4]. Therefore an additional red-shift of the $\pi \rightarrow \pi^*$ band and an increase in the ν_1/ν_2 ratio are expected upon addition of oligomycin. Experimentally this is borne out since an addition of oligomycin resulting in a concentration of 40 μg/ml increased the ratio from 1.71 to 1.81. In contrast to oligomycin, FCCP makes the membrane leaky to H^+ and apparently decreases the inside-positive potential; a blue-shift of the $\pi \rightarrow \pi^*$ band was observed under continuous illumination of actinic light [18]. In keeping with these expectations resonance Raman measurements showed a

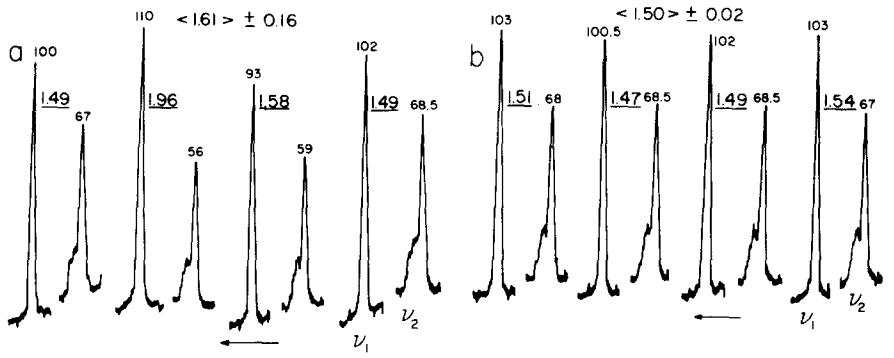

Fig. 3. Oscillatory changes in resonance Raman intensity and their damping by gramicidin for the chromatophores of the mutant G1C. Peak height in an arbitrary scale is marked at the top of each peak and the ν_1/ν_2 intensity ratio between two peaks is underlined (a) a preparation showing a large oscillation (see text), (b) after addition of 70 μM gramicidin and warming up to room temperature (2 h). Before measurement, the Raman cell was set up in exactly the same way as (a) (5°C and rotating).

decrease in the ν_1/ν_2 ratio from 1.70 to 1.64 on addition of 2 μM FCCP. The last perturbant listed in Table Ia, sodium deoxycholate, destroys the H^+ gradient and thus inhibits the red-shift of the $\pi \rightarrow \pi^*$ band completely even under the effect of actinic light [19]. Consonant with this, upon treatment with deoxycholate the ν_1/ν_2 ratio decreased from 1.71 to 1.47, indicating a large blue-shift.

The average value and the standard deviation of the ν_1/ν_2 intensity ratio (calculated from six recordings) depends on temperature and measuring conditions (Table Ib). The measurements in Table Ib were made for a preparation having the ratio of 1.64 under rotating conditions at 5°C, which was lower than the ratios 1.70—1.71 shown in Table Ia. For chromatophore suspensions at room temperature, no difference was observed among stationary, rotating and flow measurements (see Materials and Methods). However, at 5°C the effects of the laser beam acting as an actinic light bean became apparent. The average value as well as the standard deviation were largest for stationary measurements, intermediate for rotating measurements, and smallest for flow measurements. For both stationary and rotating measurements, decrease of the ratio was observed when the temperature was raised. For rotating measurements, no dependence on the rotating speed was observed in the range 300—30 rev./min, which corresponds to a linear speed through the laser beam 100—10 cm/s. In the case of flow measurements, dependence on flow speed was detected around 1 cm/s. Assuming the diameter of the laser beam to be 100 μM, the sample is then in the beam for 10^{-2} s. Typically the value of the ν_1/ν_2 intensity ratio changed from 1.58 to 1.64 and the range of oscillation (see below) changed from 0.05 to 0.11, as the flow rate dropped below 1 cm/s. The higher ν_1/ν_2 ratio at 5°C compared to room temperature and its dependence on measuring conditions may indicate a 'slow' build-up (on the basis of the dependence on flow rate, on the time scale of 10 ms) of an additional component of membrane potential at lower temperatures. The present observation possibly corresponds to the larger carotenoid shift at lower temperature reported by Baltscheffsky [20] and by De Grooth and Amesz [5].

The large standard deviation, observed at 5°C for stationary measurements, was due to an oscillatory change in absolute resonance Raman intensity. No evidence for irreversible photodecomposition, using 50 mW of 472.6 nm excitation, was obtained under any set of observation conditions. However, all samples showed oscillatory behaviour although it varied quantitatively from preparation to preparation. In some preparations, very large oscillatory changes were observed even using a rotating Raman cell. Fig. 3a shows a series of alternate measurements of the ν_2 and ν_1 bands. Because of the limitations imposed by a scanning spectrometer the interval between each measurement (ν_1, ν_2, ν_1, etc) was 4—4.5 min. For seven measurements, the average value and the standard deviation of ν_2, ν_1 and ν_1/ν_2 were 62 ± 5, 99 ± 3 (in an arbitrary unit) and 1.60 ± 0.16, respectively. After the addition of gramicidin resulting in a final concentration of 70 μM and warming up to room temperature for 2 h no intensity fluctuation were observed under the standard conditions of 5°C and rotating sample cell. The average value and the standard deviation of ν_2, ν_1 and ν_1/ν_2 became 68 ± 0, 102 ± 1 and 1.50 ± 0.02, respectively (Fig. 3b).

Returning to fresh chromatophores, repeated measurement of ν_1 or ν_2 alone revealed that the period of oscillation is in the range of 11—16 min and that the oscillatory change of the ν_1/ν_2 ratio is mainly due to the time lag in recording ν_1 and ν_2. The background at both sides of the Raman peak, the foot of the Rayleigh scattering at 20 cm^{-1} from the laser excitation line and the resonance Raman lines excited by the 501.7 nm line all oscillated with the same period. Simultaneous measurements of the pH value of the medium was attempted, but no oscillatory change related to the resonance Raman and Rayleigh oscillations was detected. The pH value was constant within the range of 0.03 when the buffer solution was used; the pH value increased continuously when the chromatophores were suspended in 0.1 M KCl. The average ν_1/ν_2 intensity-ratio as well as the range of oscillatory intensity-change varied from one preparation to another. We are presently unable to identify the factors controlling those values. However, the chromatophores with larger oscillation tend to have a lower ν_1/ν_2 ratio. It is to be noted that addition of FCCP and leaving the sample at 5°C in the dark, overnight, caused large oscillations (Table Ia). The fact that oscillations were observed in the scattered Rayleigh light, in addition to the Raman light, suggests that the size of particles in solution is varying. Oscillatory changes in Raman intensity were observed also for an aliquot of intact cells in suspension. The average value and the standard deviation of ν_1/ν_2 (15 measurements) were 1.73 ± 0.17. Chromatophores prepared from a second aliquot of the same cells gave a value of 1.72 ± 0.03. Callis et al. [21] have reported size changes, on the millisecond time scale, for *Chromatium* chromatophores following flash excitation. However, the differences in time scale between the *Chromatium* studies and most of the present work prevents correlation of the two sets of results. Finally, we note that oscillatory behaviour in particle size has been reported for mitochondria [22].

The present work has not provided any evidence for or against the existence of more than one carotenoid pool. Carotenoids existing in more than one conformation or in clearly different environments would be expected to have different resonance Raman spectra [23], especially in their double bond stretching frequencies [24]. However, if the second carotenoid pool is only a

minor constituent its resonance Raman spectrum may be lost in the spectral background, or in the tails of the peaks of the spectrum of the major pool.

Ackowledgement

We are indebted to Dr. A.R. Crofts for the gift of the GlC mutant of *Rhodopseudomonas sphaeroides.*

References

1 Jackson, J.B. and Crofts, A.R. (1969) FEBS Letters 4, 185—189
2 Borisevich, G.P., Kononenko, A.A., Venediktov, P.S., Verkhoturov, V.N. and Rubin, A.B. (1975) Biofizika 20, 250—253
3 Reich, R., Scheerer, R., Sewe, K.-U. and Witt, H.T. (1976) Biochim. Biophys. Acta 449, 285—294
4 Holmes, N.G. and Crofts, A.R. (1977) Biochim. Biophys. Acta 459, 492—505
5 De Grooth, B.G. and Amesz, J. (1977) Biochim. Biophys. Acta 462, 247—258
6 Symons, M., Swysen, C. and Sybesma, C. (1977) Biochim. Biophys. Acta 462, 706—717
7 Carey, P.R. (1978) Q. Rev. Biophys., 11, 309—370
8 Verma, S.P. and Wallach, D.F.H. (1975) Biochim. Biophys. Acta 401, 168—176
9 Lutz, M., Kleo, J. and Reiss-Husson, F., (1976) Biochem. Biophys. Res. Commun. 69, 711—717
10 Szalontai, B., Bagyinka, Cs. and Horvath, L.I. (1977) Biochem. Biophys. Res. Commun. 76, 660—665
11 Cohen-Bazire, G., Sistrom, W.R. and Stanier, R.Y. (1957) J. Cell. Comp. Physiol. 49, 25—68
12 Inagaki, F., Tasumi, M. and Miyazawa, T. (1975) J. Raman Spectroscopy 3, 335—343
13 Inagaki, F., Tasumi, M. and Miyazawa, T. (1974) J. Mol. Spectrosc. 50, 286—303
14 Leigh, J.S. Jr., Netzel, T.L., Dutton, P.L. and Rentzepis, P.M., (1974) FEBS Lett. 48, 136—140
15 Govindjee and Rajni Govindjee (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 1—50, Academic Press, New York
16 Crofts, A.R. (1974) Perspectives in Membrane Biology (Strada, O.S. and Gitler, C., eds.), pp. 373—412, Academic Press, New York
17 Schneider, E., Schwulêra, U., Müller, H.W. and Dose, K. (1978) FEBS Lett. 87, 257—260
18 Jackson, J.B. and Crofts A.R. (1971) Eur. J. Biochem. 18, 120—130
19 Clayton, R.K., (1962) Photochem. Photobiol. 1, 313—323
20 Baltscheffsky, M. (1976) Structure of Biological Membranes (Abrahamsson, S. and Pascher, I., eds.), pp. 41—62, Plenum Press. London
21 Callis, J.B., Parson, W.W. and Gouterman, M. (1972) Biochim. Biophys. Acta 267, 348—362
22 Packer, L., Utsumi, K. and Mustafa, M.G. (1966) Arch. Biochem. Biophys. 117, 381—393
23 Lutz, M., Agalidis, I., Hervo, G., Cogdell, R.J. and Reiss-Husson, F. (1978) Biochim. Biophys. Acta 503, 287—303
24 Salares, V.R., Young, N.M., Bernstein, H.J. and Carey, P.R. (1979) Biochim. Biophys. Acta 576, 176—191