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ON THE LOCATION OF THE CAROTENOIDS IN THE LIGHT-HARVESTING PIGMENT-PROTEIN COMPLEXES OF THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS CAPSULATA*

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Measurements of pronase-induced shifts of the absorption spectrum and of the isobestic point of the light-induced difference spectrum of the carotenoids show that the pool responsible for the light-induced absorption changes in *Rhodopseudomonas capsulata* wild type is more sensitive to pronase treatment than the bulk carotenoids. The most likely explanation for this, in the context of the work of Kakitani et al. (Kakitani, T., Honig, B. and Crofts, A.R. (1982) Biophys. J. 39, 57–63), is that the field indicating carotenoids, or at least that part of the molecules which determines their spectral characteristics, are imbedded in the LHC II pigment-protein complexes, close to the membrane surface. The importance of the location of the carotenoids for the measurement of the electrical potential differences is briefly discussed.

Light-induced carotenoid absorption changes as observed in various photosynthetic organisms are now widely used as an indicator of transmembrane potential. It is generally believed that the mechanism of the band shifts has an electrochromic character.

The literature has been reviewed in Refs. 1 and 2. In photosynthetic bacteria, it has been shown that the carotenoids consist of two spectrally different pools, of which only the smallest one, containing 20–35% of the total carotenoid content, shows a substantial shift as a result of energization of the membrane [3,4]. Recently, it has been shown that the field-sensitive pool is associated with the light-harvesting complex LHC II characterized by bacteriochlorophyll bands at 800 and 850 nm. In support of this, we can mention the correlation of the extent of the carotenoid absorbance changes with the amount of B800 and B850 present in *Rhodopseudomonas sphaeroides*, the different spectral characteristics of carotenoids belonging to the two isolated light-harvesting complexes of the same bacterium [5–7], mutant studies with *R. capsulata* [8] and the studies with pronase digestion of the LHC in the same bacterium [9]. However, it is clear that the field-sensitive carotenoid pool constitutes only part (less than half) of the carotenoids contained in LHC II [10].

In the present study, we followed the effect of pronase incubation on the spectral characteristics of both the field-sensitive and the nonfield-sensitive carotenoid pools to investigate whether or not the two pools are affected by pronase digestion in the same way.

Chromatophore preparations of *Rps. capsulata*, wild type, were incubated with a pronase from *Streptomyces griseus* (Sigma, type VI, 20 μg/mg protein) at 37°C. The digestion was stopped by

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Abbreviations: LHC, light-harvesting complex; Mops, 4-morpholinepropanesulfonic acid.

10-fold dilution at room temperature of the samples taken from the reaction vessel after different incubation times (cessation of enzymatic activity was substantiated by the fact that the position of the long-wavelength band did not shift further to the blue during repeated measurement in the following 20 min).

In previous studies, we showed that the isobestic points of the difference spectrum induced by continuous illumination can be used to establish the wavelengths of the peak centers of the field-sensitive pool only [4]. Since the light-induced red shift of the isosbestic point, amounting to about 1 nm [4], is small in comparison with the wavelength distance between the electrochromically responding pool (E) and the nonresponding pool (N), amounting to more than 10 nm in this species, the isosbestic point is a good approximation of the band center of the field-sensitive population.

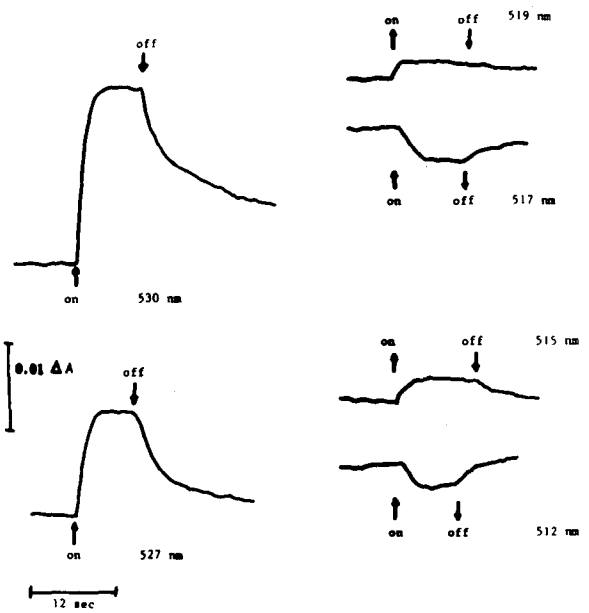

Fig. 1. Light-induced changes as affected by pronase treatment. Chromatophores (7.5 μg/ml BChl) of *Rps. capsulata* wild type were suspended (and prepared) in a medium containing 2 mM Mops at pH 7 and 20 μM $MgCl_2$ with a total K^+ concentration of 2 mM. Other details of preparation and measurement are essentially as described in Ref. 4. ΔA was measured at the wavelength of the maximum of the difference spectrum and at wavelengths close to the supposed isosbestic point. Upper traces: control, without pronase. Lower traces: after 21 min of pronase incubation.

ing to the long-wavelength band, was graphically interpolated, taking measurements of light-induced absorbance changes (ΔA) at two wavelengths, respectively, higher and lower than the supposed location of the isosbestic point. Two examples of such measurements are shown in Fig. 1. The position of the isosbestic point could be calculted with an error of 0.1 nm [4], at least during the first half-hour of the treatment when ΔA is still sufficiently large.

The observed pronase-induced blue shifts of the isosbestic point ($\Delta\lambda_i$) correspond to the blue shift ($\Delta\lambda_E$) of the field-sensitive pool multiplied by the pronase-affected fraction of this pool (F_E). Thus, $\Delta\lambda_i$ gives a measure for the pronase effect on the field-sensitive pool only.

It should be remarked that the whole light-induced difference spectrum is blue shifted, i.e., the isosbestic points as well as the positions of the maxima and minima shift up to 7 nm to the blue (see Fig. 1). This implies that the maximal extent of the carotenoid absorbance change after pronase treatment should be measured at smaller wavelengths than in control experiments.

Furthermore, since mild pronase treatment did not seem to influence light-induced electron transport nor membrane permeability, Webster et al. [10] concluded that the digestion does not affect the field generated across the membrane.

The position of the center of the long-wavelength absorption band, centered around 510 nm, could be determined with the same precision (0.1 nm). The pronase-induced wavelength shift, $\Delta\lambda_o$, of the center of the redmost absorption band is the average of the shifts of the two populations, weighted by their respective size:

$$\Delta\lambda_o = \Delta\lambda_E F_E \alpha_E + \Delta\lambda_N F_N \alpha_N$$

where $\Delta\lambda_j$ is the independent blue shift of each population induced by pronase, α_j the size of the pool and F_j the fraction of each pool affected by the pronase. Comparison of $\Delta\lambda_i$ and $\Delta\lambda_o$ will tell us whether or not the two pools are affected in the same way.

In Table I, we can see that during the first 10 min of the digestion the isosbestic point shifts much faster to the blue than the top of the long-wavelength band, implying that the carotenoids

TABLE I

DIFFERENTIAL EFFECT OF PRONASE ON THE CAROTENOID SHIFT AND OVERALL ABSORBANCE

Time (min before or after addition of pronase)	−5	10	23	44	62
Top overall spectrum (λ_o) (nm)	510.0	509.5	507.1	506.5	505.4
Isosbestic point (λ_i) (nm)	519.4	517.5	513.7	513.0	512.0
$\frac{\Delta\lambda_o}{\Delta\lambda_i}$		0.26	0.63	0.83	1.10

which respond to the electrical field are more sensitive to pronase treatment than the field-insensitive pool. In this respect, we can introduce for a time t_i after adding the pronase the parameter $\Delta\lambda_o/\Delta\lambda_i$ (t_i), which gives an indication of the size of the different pools of carotenoids affected by the pronase during the time interval $(t_i - t_{i-1})$. If at a certain stage of the treatment only one of the pools (let us say the light-sensitive pool) is affected, the parameter $\Delta\lambda_o/\Delta\lambda_i$ (t_i) clearly is a measure of the size of this pool. Indeed, at the beginning, only a small percentage (25–35%) of the carotenoids is shifting, a fraction very similar to the estimated size of the field-sensitive pool in this strain [11]. For large incubation times, the parameter increases, implying that progressively more of the nonfield-sensitive pool is attacked by the pronase. In some instances this parameter became larger than unity, indicating that during the time interval considered the nonfield-sensitive population shifted over a longer distance than the field-sensitive one.

Another feature of the spectral changes is that during the first 30 min of the treatment, the bandwidth of the absorption bands of the carotenoid spectrum apparently decreases, which supports the conclusion that the underlying absorption bands, initially separated by 10 nm, 'approach' each other.

We also observed irreversible blue shifts of about 1 nm in the control samples kept in the same temperature bath, but in this case, remarkably, shifts of the same size were noted for both λ_i and λ_o, indicating that all carotenoids are similarly affected by raising the temperature. Similar irreversible blue shifts attributed to ageing or other membrane deterioration have been observed before [10,12].

It has been proposed recently [12] that the spectral features of the carotenoids imbedded in the LHC would be caused by interaction with a polar group of the chlorophyll or a polar amino acid group in these complexes. In this context, our results can be explained in two ways:

(1) The site of interaction of the field-sensitive carotenoid with the LHC is located closer to the surface of the membrane, making it more susceptible to the attack of pronase.

(2) The pronase, due to its specificity, preferentially attacks groups in the neighborhood of the field-sensitive carotenoids.

The second hypothesis seems unlikely, since proteinase K (Sigma, type XI, from *Tritirachium album*) which is an unspecific pronase [13] and trypsin produce similar differential effects on the two carotenoid pools. Moreover, results obtained after incubation with phospholipase A_2 (from *Naja naja* venom, Sigma) and with phospholipase D from peanuts are all very similar (Swysen et al., unpublished results). The field-sensitive carotenoid most probably lies close to the surface, as it is highly improbable that the enzymes tested, which attack proteins, and phospholipids, respectively, at widely different sites, all have drastic effects at some different locus where the carotenoids are buried. Furthermore, it is interesting to note that Kakitani et al. [12] could explain the spectral differences between the two pools by placing the charged group responsible for the 'permanent field' [14] of the field-sensitive carotenoids near the top of the molecule and close to the center for the bulk carotenoids.

The crucial question as to which electrical potential differences the carotenoids respond comes into consideration here [15,16]. It is clear now that the field-sensitive pigments (or at least that part of the molecules which will determine their spectral characteristics) are imbedded in a protein complex and are probably located close to the membrane surface. If the solvent environment of the carotenoids in the LHC protein has a higher dielectric constant than the dielectric constant of the lipid core of the membrane, electrical fields will be more localized within this region than in the lipid region.

This favors the hypothesis that the carotenoids in the LHC can respond to local fields [15] (see also Ref. 17). The occurrence of respective hydrophilic and hydrophobic amino acid residues in the carotenoid surrounding remains to be investigated. Furthermore, since the field-sensitive carotenoids seem to be situated close to the membrane surface, they may also respond to charge redistribution in this part of the membrane (see also Ref. 18).

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