Further Evidence for Carotenoids Engaged in a Metastable State in Photosynthesis

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During photosynthesis a metastable state is formed by superfluous excitation energy. Only carotenoids are responsible for the metastable state. Especially no chlorophyll-a is involved in this state. This result was obtained by using the repetitive ultra-short-flash technique in combination with the high frequency modulated measuring light technique.

During photosynthesis a metastable state with a half life of $\sim 3 \mu\text{s}$ has been observed which probably represents a valve for superfluous excitation energy¹.

The difference spectrum caused by the formation of this state shows three pronounced negative peaks in the blue spectral range (at 430, 460 and 490 nm). Therefore probably a carotenoid is involved. Because of the negative peak at 430 nm the participation of chlorophyll-a could not be excluded. But in this case there must occur additionally absorption changes in the red spectral range between 660 and 705 nm where chlorophyll-a in vivo has its second absorption band but not the carotenoids.

Measurement of fast and small absorption changes within microseconds can, however, not be carried out by the normal ultra-short-flash photometry apparatus¹ in the red spectral range because of the $\cong 10^4$ times larger chlorophyll fluorescence at the same wavelengths. This fluorescence can, however, be eliminated from interference with absorption changes by a special apparatus allowing time resolution of about $1 \mu\text{s}$ ². With this apparatus we succeeded in extending the difference spectrum in question into the red region. No absorption changes of $\sim 3 \mu\text{s}$ life time take place in the red. Therefore only carotenoids are responsible for the observed metastable state. This has already been reported in¹. The experimental documents and details on these measurements in the red region are presented in this paper.

Methods and Material

Formation and deactivation of the metastable state in spinach chloroplasts were recorded by the repetitive flash photometry with high frequency modulated detecting light². This apparatus was capable to separate completely fast absorption changes of $\Delta I/I = 5 \cdot 10^{-5}$ from $5 \cdot 10^4$ times larger fluorescence signals.

The excitation of photosynthesis was performed by ultra short flashes¹. With a multi-channel averager (Fabri-Tek Model 1072) the extremely small signal-to-noise ratio was enhanced by the factor 200.

Because of the limited time resolution of the averager ($1 \mu\text{s}$) it was suitable to use chloroplasts in glycerol. In such a suspension the life time of the metastable state is two times larger ($t_{1/2} \cong 7 \mu\text{s}$) than in an air-saturated aqueous suspension ($t_{1/2} \cong 3 \mu\text{s}$)¹.

The spinach chloroplasts were prepared as described in¹. For the measurements they were suspended in glycerol from Merck (redistilled p.a., density 1,23).

Results

Firstly, measurements were performed at the wavelengths 430 and 515 nm (see Fig. 1) at which the formation and deactivation of the metastable state are indicated by characteristic absorption changes¹. Fig. 2 shows that these absorption changes of chloroplasts suspended in glycerol ($t_{1/2} \cong 7 \mu\text{s}$) give the same amplitude ratio as the absorption changes of chloroplasts in an air-saturated aqueous suspension ($t_{1/2} \cong 3 \mu\text{s}$).

In the red spectral range between 660 and 705 nm where chlorophyll-a in vivo absorbs, however, no absorption changes with a half life of $7 \mu\text{s}$ could be observed (see Figs. 1 and 2). In this range there are only absorption changes observable with slow decay times $\gg 7 \mu\text{s}$. These absorption changes have already been attributed to the photoactive chlorophyll-a_I^{3, 4} and chlorophyll-a_{II}⁵.

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² H.-E. BUCHWALD and H. RÜPPEL, Nature [London] 220, 57 [1968]; H.-E. BUCHWALD, Thesis, Berlin 1969.

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⁴ B. RUMBERG and H. T. WITT, Z. Naturforsch. 19 b, 693 [1964].

⁵ G. DÖRING, G. RENGGER, J. VATER, and H. T. WITT, Z. Naturforsch. 24 b, 1139 [1969].

Fig. 1. Time course of the absorption changes at $\lambda=430$, 520 and 685 nm in a suspension of spinach chloroplasts in glycerol. Chlorophyll content: 10^{-6} M. Water content: about $5 \cdot 10^{-2}$ M. Temperature: 20 °C. Optical path: 78 mm. $\lambda > 610$ nm (2 mm RG 1), duration $4 \cdot 10^{-7}$ s, repetitive ultra-short-flash technique, frequency 10 Hz, 41.160 flashes were fired per measurement. Measuring light: interference filters $\lambda=430$ nm ($\Delta\lambda=20$ nm), 520 nm (15 nm) resp. 685 nm (13 nm). Electrical bandwidth: 1 MHz.

Fig. 2 shows that in the whole range 660 to 705 nm possible absorption changes with $t_{1/2} \cong 7 \mu\text{s}$ are smaller than 20% of the blue peak at 430 nm. Because the red difference bands of other chlorophyll-a-reactions (e. g. triplet formation in vitro⁶)

Fig. 2. Measuring points: Fast absorption changes indicating the metastable state in a suspension of spinach chloroplasts in glycerol ($t_{1/2} \cong 7 \mu\text{s}$). Measuring light: interference filters, half width: about 15 nm. For further details see Fig. 1. Solid line: Spectrum of the absorption changes indicating the metastable state in an air-saturated aqueous suspension of spinach chloroplasts ($t_{1/2} \cong 3 \mu\text{s}$)¹.

are at least as large as the blue bands we estimate the possible participation of chlorophyll-a in the 430 nm band as being smaller than 20% of the total amplitude.

Discussion

The negative peaks at 430, 460 and 490 nm (see spectrum Fig. 2) could be caused by the reaction of 1. chlorophyll-a (430 nm), 2. chlorophyll-b (460 nm), 3. carotenoids (all three wavelengths) and 4. flavins (all three wavelengths).

Chlorophyll-b is out of the question because the metastable state is formed also in algae containing no chlorophyll-b⁷.

Flavins in vivo have an absorption spectrum with three peaks (respectively shoulders) between 400 and 500 nm⁸. However, the excited metastable state of flavins has no absorption band at wavelengths above 500 nm⁹. Therefore the flavins are out of the question, too.

Only chlorophyll-a (Chl) and carotenoids (Car) are left for discussion. Our results show that chlorophyll-a forms if at all maximal 20% of the amplitude at 430 nm, that means

$$\frac{\Delta A_{\text{Chl}}}{\Delta A_{\text{Car}}} < \frac{1}{4}.$$

With the carotenoid absorption coefficient in vitro¹³ and the change of the chlorophyll absorption coefficient during triplet formation in vitro⁶ at 430 nm it follows a concentration ratio which is accidental the same as the ratio of the absorption changes:

$$\frac{C_{\text{Chl}}}{C_{\text{Car}}} < \frac{1}{4}.$$

Thus the concentration of chlorophyll-a which, if at all, is converted to the metastable state must be smaller than 25% of that of the carotenoids.

⁶ R. LIVINGSTON, in: Handbuch der Pflanzenphysiologie, Vol. V/1, p. 830, Ed. W. RUHLAND et al., Springer-Verlag, Berlin-Göttingen-Heidelberg 1960; K. SEIFERT and H. T. WITT, Naturwissenschaften 55, 222 [1968].

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As the excitation of the metastable state is possible even with red light only ($\lambda > 610$ nm)¹ the metastable state is undoubtedly formed via chlorophyll-a absorption (bulk absorption):

If the chlorophyll-a is involved in the metastable state itself than only a complex between chlorophyll-a and carotenoids would be possible because of the uniform kinetics of deactivation¹:

In this case, however, it would be expected a concentration ratio $C_{\text{Chl}}/C_{\text{Car}} = 1$.

As this is contrary to the above estimation it can be followed now that there is no chlorophyll-a at all involved in the metastable state itself. Only the excitation energy is transferred from chlorophyll-a to carotenoids:

Our conclusion that only carotenoids participate in the metastable state is supported additionally by the following facts (see also l. c.¹):

⁺ Abbreviations: Chl, Chl* = chlorophyll-a in its ground state and excited state respectively. Car, Car[•] = carotenoid in its ground state and metastable state respectively. (Chl-Car)[•] = metastable complex consisting of chlorophyll-a and carotenoid.

1. The negative difference bands (see Fig. 2) have their maximum just at those wavelengths where the carotenoids *in vivo* absorb.

Extracting¹¹ and photo-bleaching of chloroplasts¹² result in the fact that two of the carotenoid absorption maxima *in vivo* are at 460 and 490 nm and that the third maximum is not located at wavelengths above 500 nm. This third maximum is probably at 430 nm, but it could not be resolved because of the strong chlorophyll-absorption.

2. The distance of 30 nm between the negative difference bands corresponds exactly to the vibrational structure in the absorption spectrum of the carotenoids¹³.
3. The positive absorption change at 520 nm (Fig. 2) reflects the metastable state absorption of carotenoids as it was shown by experiments *in vitro*¹⁴.

The metastable state is formed in different species of algae indicated by very similar difference spectra⁷. These algae have totally different compositions of carotenoids¹⁵. Therefore probably several types of carotenoids are involved in the formation of the metastable state.

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