

UDC 581.174.1:576.343/582.738=20

CYTOCHEMICAL LOCALIZATION OF THE ACTIVITY OF PHOTOSYSTEM II IN BEAN ETIOCHLOROPLASTS

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Received November 20, 1987

Photoreduction of thiocarbamyl nitroblue tetrazolium (TCNBT) was used to detect ultrastructurally the activity of photosystem II in the thylakoids of etiochloroplasts in greening bean leaves. To increase the retention of TCNBT formazan and consequently the contrast in the thylakoids, the samples were incubated in TCNBT and then treated by the OsO_4 -thiocarbohydrazide- OsO_4 method.

Dark deposits of TCNBT formazan appeared at first in the granal initials of the etiochloroplasts illuminated for 3 hours. The deposits were at the beginning scarce, but became more abundant after 6 and more hours of greening of the leaves. In young chloroplasts maintained in light for 24 or 48 hours the formazan deposits were located mostly in the partitions of the grana, although some scattered granules were attached also to the stroma thylakoids. There were no formazan deposits in the plastids of samples incubated only in darkness or in light with the addition of dichlorophenyl-dimethylurea.

Introduction

Tetrazolium dyes are photoreduced in photosynthetic tissue to blue coloured insoluble formazan, which makes them applicable to light microscope cytochemistry (Downton and Pyliotis 1970, Robertson and Earle 1987). The photoreduction is inhibited by dichlorophenyl-dimethylurea (DCMU). Therefore, it is supposed that tetrazolium dyes react with a compound of the photosynthetic membrane located near the photosystem II. The reduced thiocarbamyl nitroblue tetrazolium (TCNBT) reacts with OsO_4 and is thus detectable in electron

microscope as dark granular material attached to the thylakoids (Marty 1977, Vaughn et al. 1983). Preliminary electron microscope investigations, carried out by the author, have shown that the contrast of formazan deposits is much enhanced when — after the usual incubation in TCNBT — the tissue is treated *en bloc* by the OsO_4 -thiocarbohydrazide- OsO_4 (OTO) procedure (Aoki and Tavassoli 1981).

In the paper presented here the development of the activity of photosystem II in bean etioplasts is studied by photoreduction of TCNBT, followed by the OTO method. These investigations complement a paper published earlier (Wrischer 1978), in which in bean etioplasts the activity of photosystem I was examined by photo-oxidation of diaminobenzidine.

Material and Methods

Bean seedlings (*Phaseolus vulgaris* L. cv. Starozagorski) were grown in vermiculite for 8 days in darkness at 25°C. Primary leaves were examined either in darkness or after an illumination period of 1, 2, 3, 6, 24, and 48 hours (white light of 2 fluorescent tubes 20 W, 4000 lux at ground level). Pieces of leaves were fixed for 60 minutes in 2% formaldehyde (pH 7.2) in 0.05 M phosphate buffer. After rinsing in phosphate buffer (with addition of 5% sucrose) for 30 minutes the material was transferred to the incubation medium containing 1 mg/ml TCNBT (thiocarbonyl mitrotetrazolium blue chloride, Serva, Heidelberg) in the same buffer. The incubation was first in darkness (60 minutes) and then in light (4000 lux, 60 minutes). Control samples were held either constantly in darkness in TCNBT medium or they were incubated in light in TCNBT medium containing 1×10^{-5} M DCMU (dichlorophenyldimethylurea, Serva, Heidelberg). After rinsing in buffer the samples were either postfixed for 60 minutes in buffered 1% OsO_4 , or they were treated as follows: 1% OsO_4 , in phosphate buffer (10 minutes), rinsing in buffer (20 minutes), 1% TCH (thiocarbohydrazide, Serva, Heidelberg) (5 minutes), rinsing in buffer (20 minutes), 1% OsO_4 (10 minutes). After dehydration the material was embedded in Araldite or in Spurr's low viscosity embedding medium. The sections were examined in the electron microscope without additional staining.

Results

When etiolated leaves had been illuminated, the prolamellar bodies of the etioplasts were almost immediately transformed into irregular loose tubular coils. After several hours in light these structures disappeared completely. The single thylakoids of the etioplasts began to multiply and form grana after about 3 hours of illumination. At the same time rapid greening of the leaves occurred. The leaves, which had been in light for 24 or 48 hours, already contained young chloroplasts with well developed grana.

The deposits of the reduced TCNBT appeared neither in the single thylakoids nor in the prolamellar bodies of etioplasts. The reaction also remained negative in leaves when illuminated for 1 or 2 hours. After 3 hours in light the photoreduction of TCNBT began in etioplasts simultaneously with the formation of granal initials. In partitions

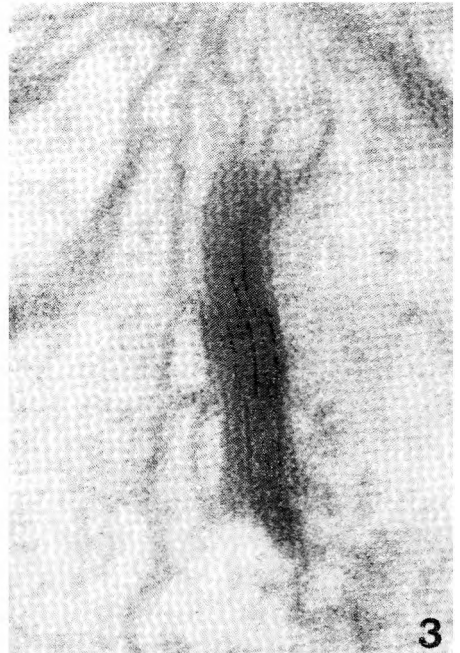
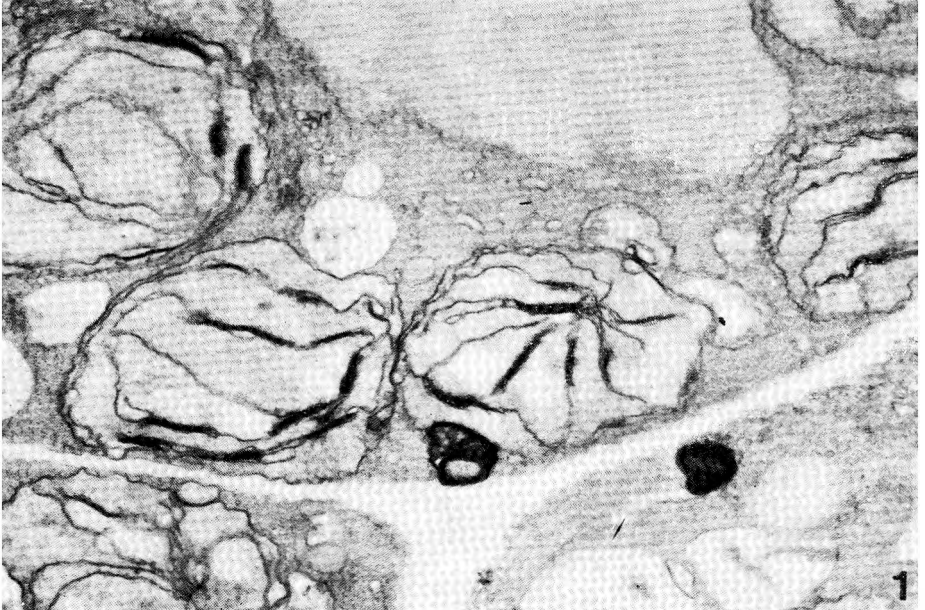


Fig. 1. Etiochloroplasts from an etiolated leaf maintained 6 hours in light. The small grana contain some dark TCNBT formazan. 16,000 : 1.

Fig. 2. Detail of Fig. 1 at higher magnification. In the partitions of the granum dark deposits of reduced TCNBT are present. 140,000 : 1.

Fig. 3. Part of an etiochloroplast from an etiolated leaf illuminated for 6 hours. Deposits of TCNBT formazan are located in the partitions of the granum. 120,000 : 1.

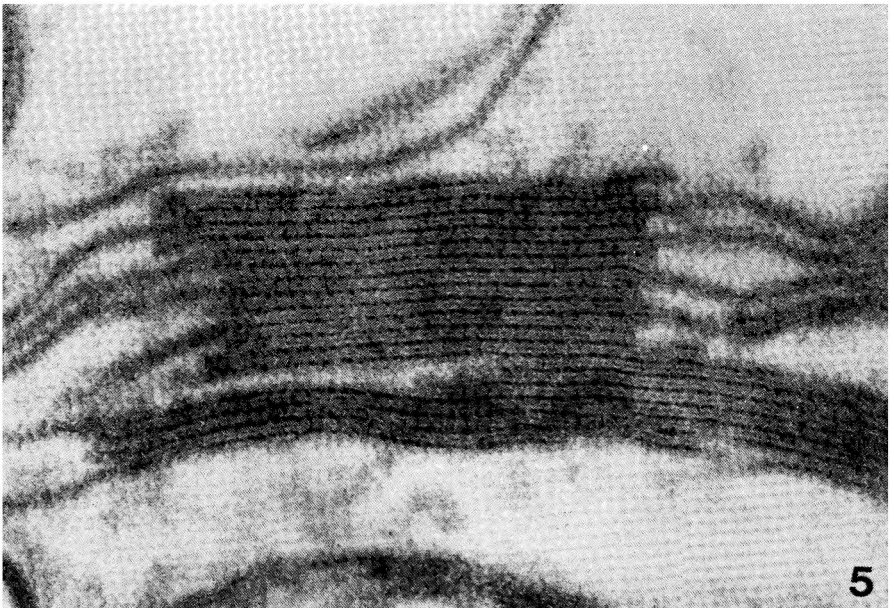
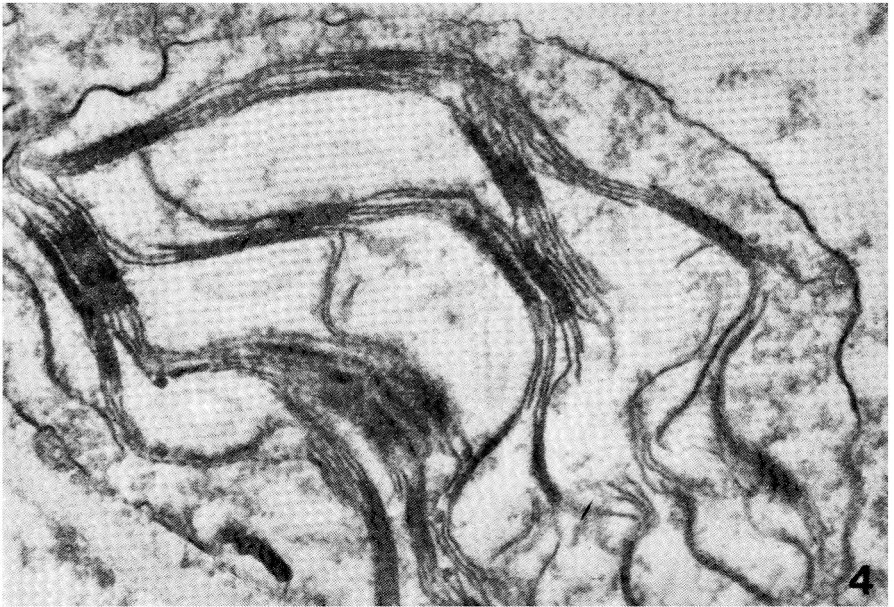


Fig. 4. Young chloroplast from an etiolated leaf after 24 hours of greening in light. The grana are dark due to the accumulated reduced TCNBT. 26,000 : 1.

Fig. 5. Detail of Fig. 4. at higher magnification showing dark deposits of TCNBT formazan in the partitions of the grana. 130,000 : 1.

of these grana TCNBT formazan accumulated as dark granular material. At first, the accumulation of formazan was scarce, but after prolonged illumination (6 or more hours) it became more abundant (Figs. 1, 2, 3). In young chloroplasts maintained in light for 24 or 48 hours most of the TCNBT formazan was located in partitions of the grana, with scattered granules adhering also to the stroma thylakoids (Figs. 4, 5). Plastid envelopes and tubular coils of the disorganized prolamellar bodies always remained TCNBT negative.

Control experiments showed that formazan was absent from samples incubated in TCNBT media containing DCMU. The reaction was also negative in the material incubated in darkness only. The samples treated with the OTO method contained much more dark deposits in the grana than those postfixed only in OsO_4 . The OTO method was especially useful in the study of the early stages of etiochloroplast development, as it made possible the detection of faint formazan accumulations in the granal initials.

Discussion

As reported in a previous paper (Wrischer 1978), the activity of photosystem I, monitored by photooxidation of diaminobenzidine, could be observed in single thylakoids of bean etiochloroplasts already after 1 hour of illumination. The activity of photosystem II, detected by photoreduction of TCNBT, began somewhat later, and was observed in etiochloroplasts which already contained some granal initials. This delay in the start of the activity of photosystem II is connected with the increased O_2 evolution that begins after fusion of the thylakoids into grana (Strasser and Butler 1976).

In chloroplasts of leaves illuminated for 1 or 2 days TCNBT formazan was restricted mostly to the granal partitions. It is supposed that TCNBT reacts with components of the photosynthetic electron transport system located close to the photosystem II and facing the outer side of the thylakoids (Vaughn et al. 1983). The site of accumulation of formazan is consistent with the generally accepted model of the separated position of the two photosystems on the photosynthetic membrane: photosystem II reaction is centered mostly in the grana thylakoids and photosystem I reaction is centered in the stroma thylakoids and stroma exposed grana thylakoids (Staelin and Arntzen 1983). The data obtained previously with diaminobenzidine agree with this model as well. Its reaction products accumulated mostly in the stroma thylakoids and peripheral grana thylakoids (Wrischer 1978). The photooxidation of diaminobenzidine and the photoreduction of TCNBT are thus valuable cytochemical procedures which make possible the *in situ* localization of the photosynthetic activity in the thylakoids.

The OTO method has been applied by Aoki and Tavassoli (1981) to increase the contrast and structural preservation of actin filaments in animal cells. It shortens the treatment with OsO_4 , which is known to destroy fragile protein structures in the cells (Bullock 1984). In the case of TCNBT formazan the OTO method increased the contrast of the accumulated granular material, and at the same time improved its attachment to the thylakoids.

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The author wishes to thank Prof. Dr. Z. Devidé for valuable comments on the manuscript.

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SAŽETAK

CITOKEMIJSKA LOKALIZACIJA AKTIVNOSTI FOTOSISTEMA II U ETIOKLOROPLASTIMA GRAHA

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Koristeći se fotoredukcijom tiokarbamil-nitrotetrazolijskog modrila (TCNBT = thiocarbamyl nitrotetrazolium blue . chloride) praćena je aktivnost fotosistema II u tilakoidima etiokloroplasta tijekom ozelenjavanja etioliranih listova graha. Kontrast taloga reduciranog TCNBT povećan je metodom OTO (OsO_4 -tiokarbohidrazid- OsO_4).

Tamni talog TCNBT formazana pojavljuje se najprije u začecima grana u etiokloroplastima etioliranih listova koji su 3 sata osvjetljavani. Ispočetka je talog vrlo nježan, no nakon što su listovi bili 6 i više sati na svjetlosti, postaje intenzivniji. U mladim kloroplastima listova, koji su osvjetljavani 24 ili 48 sati, talog formazana leži pretežno u područjima grana, i to između tilakoida (intertilakoidno). Pojedinačnih tamnih granula ima međutim mjestimično i na stroma tilakoidima. U uzorcima, koji su bili inkubirani ili u tami ili na svjetlosti uz dodatak diklorofenildimetilureje (DCMU = dichlorophenyldimethylurea) talog formazana nije nađen.

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