Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1665 (2004) 57–64

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

A dynamic phase microscopic study of optical characteristics of individual chloroplasts

V.P. Tychinsky[a], A.V. Kretushev[a], T.V. Vyshenskaya[b], A.N. Tikhonov[b,*]

[a]*Moscow State Institute for Radioengineering, Electronics and Automation (TU), prosp. Vernadskogo 78, 119454 Moscow, Russia*
[b]*Department of Biophysics, Faculty of Physics, M.V. Lomonosov Moscow State University, 119992 Moscow, Russia*

Received 3 March 2004; received in revised form 16 June 2004; accepted 25 June 2004
Available online 23 July 2004

Abstract

Dynamic phase microscopy (DPM) allows the monitoring of optical path difference (or phase height), $h(x,y,t) \approx \int n(x,y,z,t)dz$, an integral refractive index projection of the medium, $n(x,y,z,t)$, in optically transparent biological specimens at high spatial and temporal resolutions. In this study, DPM was used for the analysis of fluctuations in the optical characteristics of individual bean chloroplasts in various metabolic states. A "phase image" of an individual chloroplast, which represents a three-dimensional plot of the "phase height", was obtained for the first time, and the frequency spectra of the fluctuations of $h(x,y,t)$ were investigated. The fluctuation patterns, i.e., the intensity and the frequency spectra of phase height fluctuations in bean chloroplasts (Class B) were found to depend on their metabolic state. Under conditions of noncyclic (or pseudocyclic) electron transport, the fluctuations displayed characteristic frequencies in the range of 0.25–0.6 Hz and were space–time-correlated in the chloroplast domains with the cross sizes of ~2 μm. The fluctuation intensity decreased in the presence of uncouplers (nigericin and valinomycin, 20 μM). A stronger (in comparison with 20 μM valinomycin) effect of 20 μM nigericin suggests that the light-induced generation of the transmembrane pH difference (ΔpH) makes the main contribution to the increment of space-correlated fluctuations of $h(x,y,t)$. Studies of chloroplasts incubated in media of various osmolarity (50–500 mM sucrose) have shown that structural changes in thylakoids are among other factors responsible for phase height fluctuations.

Keywords: Chloroplast; Dynamic phase microscopy; Membrane potential

1. Introduction

The structural and functional organization of the photosynthetic systems of higher plant chloroplasts and cyanobacteria are rather well studied. The main achievements in this field include determination of the sequence of the light and dark stages of photosynthesis and elucidation of molecular mechanisms of key reactions of electron and proton transport in chloroplasts [1]. The spatial structures of pigment–protein complexes and reaction centers of Photosystems I and II (with resolutions of 2.5 and 3.8 Å, respectively) of the cyanobacterium *Synechococcus elongatus* [2,3], of the cytochrome *bf* complex [4] and of the ATP synthase [5,6] complex have been established using X-ray diffraction analysis. Other currently central problems in the biochemistry and biophysics of photosynthesis include elucidation of regulatory mechanisms of various bioenergetic processes in native photosynthetic systems and adaptation of these photosynthetic systems to the changing conditions of the environment [7–11]. Study of light-induced changes in various structural and functional states of individual chloroplasts in a real-time mode is yet another important and interesting field of research.

Valuable information about the dynamics of structural changes occurring in individual chloroplasts can be derived from optical measurements. In the present study, we applied the method of dynamic phase microscopy (DPM) [12,13],

Abbreviations: DPM, dynamic phase microscopy; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

* Corresponding author. Tel.: +7 95 9392973; fax: +7 95 9391195.
E-mail address: an_tikhonov@newmail.ru (A.N. Tikhonov).

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.06.014

which consists in recording of interference images of tested objects in coherent light. A salient advantage of DPM is the possibility to detect even very small (~0.1 nm) time-dependent changes in the optical path difference with prospective measurements of phase heights [$h(x,y,t)$] over classical spatial resolution [14,15]. Previous studies have shown that certain optical characteristics (e.g., refractive indices of isolated mitochondria) of some thin unstained biological specimens vary considerably depending on the metabolic state of the system under study. The use of DPM has made it possible to establish a correlation between the changes in the phase heights of individual isolated mitochondria and their metabolic states [16,17] and to interpret this phenomenon in terms of the electrooptical effect [18].

This study is the first attempt to apply DPM to the analysis of fluctuations in the optical characteristics of individual bean chloroplasts depending on their metabolic state. It was found that phase height fluctuations are correlated in space and time; some of their components manifest characteristic frequencies in the range of 0.2–0.6 Hz.

2. Materials and methods

2.1. Chloroplast preparations

Chloroplasts (Class B) were isolated from 2 to 3-week-old bean seedlings as described previously [19,20]. The incubation medium contained 2 mM KH_2PO_4, 2 mM $MgCl_2$, 10 mM HEPES (pH 7.8) and 200 mM sucrose. Isolated chloroplasts (2 mg/ml chlorophyll) were stored at 77 K in the presence of a cryoprotector (10% glycerol). One or two hours before the experiment, the chloroplasts were defrozen portionwise and stored at 4 °C. Immediately before optical measurements, aliquots of dense chloroplast suspensions were diluted with the incubation buffer (1:20) supplemented with 20 μM methylviologen, an artificial mediator of electron transfer from Photosystem I to oxygen. The osmolarity of the incubation medium was varied by changing sucrose concentration (50–500 mM).

2.2. Optical measurements

The DPM method [12,13] allows one to obtain images of quasi-static objects on the display of the microscope as three-dimensional spatial surfaces $<h(x,y,t)>$ (the so-called "topograms") with pseudocolored elevation numbers. Here, $h(x,y,t)$ is the phase height at a point with the coordinates (x,y) in the image plane measured at a definite moment of time (t). Hereinafter, all the values used to describe the parameter $h(x,y,t)$ (e.g., the height profile $h(x,t)$ along a certain scan line (y=const) and the distribution of fluctuation intensities $I(x,t)$ along the scan line) will be defined by the adjective "phase" in order to distinguish from the corresponding parameters for the actual geometrical height $H(x,y,t)$. The dependence $h(x,y,t) \approx \Delta n(x,y,t) \cdot H(x,y)$, where $\Delta n(x,y,t)$ is the difference between the refractive indices of the object and the surrounding medium, is a link between the phase and geometric heights. The condition $h(x,y,t) \ll H(x,y,t)$ is usually met for transparent objects in aqueous media.

Phase height measurements were performed on an "Airyscan" microscope [12,13]. Its optical layout represents a modification of the Linnik microinterferometer and utilizes a He–Ne laser (1 mW, λ=633 nm) as a coherent radiation source. Linear-periodic modulation of the reference wave phase was achieved through the use of a mirror fitted with a piezoelectric actuator. Interference signals and their analog–digital conversion into local phase values were recorded with the help of a coordinate-sensitive photodetector (a dissector image tube LI-620) and an electronic unit. The tested object was placed into a cell with a polished silicon substrate and a thin cover glass. The measurements were performed using a 50*/0.75 lens. The size of the scanning field area was 200×200 μm. The accuracy of measurements of the optical path difference (h_{min}) determined by the signal-to-noise ratio was equal to 0.1 nm.

The dynamic parameters of the tested object were measured by continuous scanning of phase height profiles $h(x,t)$ along a line (further referred to as a "scan line") set on the image (a topogram) of an individual chloroplast (y=const). The resulting matrix (further referred to as a "track chart") contained a uniform sampling of $h(x,t+p\tau)$ values at a point x of the scan line (y=const). Here, τ is the line-to-line delay time and p=0,1,2,3... is the line number. In our study, the periodicity of the sampling frequency and the image input rate were determined by the modulation frequency of 1 kHz (or 1 ms/pixel).

Treatment of track chart charts with the help of a special computer program has made it possible to present the information about the dynamic processes contained in the $h(x,t)$ matrix as the following set of functions:

(1) local fluctuation intensity $I(x)$ (nm^2) of the phase height along the scan line;
(2) spectral portrait $\rho(F,x)$ (nm^2/Hz) (distribution of average spectral density reflecting the predominance of characteristic frequency components along the scan line);
(3) phase height fluctuation spectrum $\rho(F,x)$ (nm^2/Hz) localized at a fixed point of the scan line. Here, $\rho(F,x)$ is the spectral fluctuation density linked with a local intensity by the formula $I(x)=\int\rho(F,x)dF$, where F is the frequency.

3. Results and discussion

3.1. Visualization of single chloroplasts by DPM method

A typical phase image (a topogram) of an individual chloroplast surface $h(x,y)$ obtained with the help of DPM is shown in Fig. 1. This three-dimensional image (Fig. 1, top)

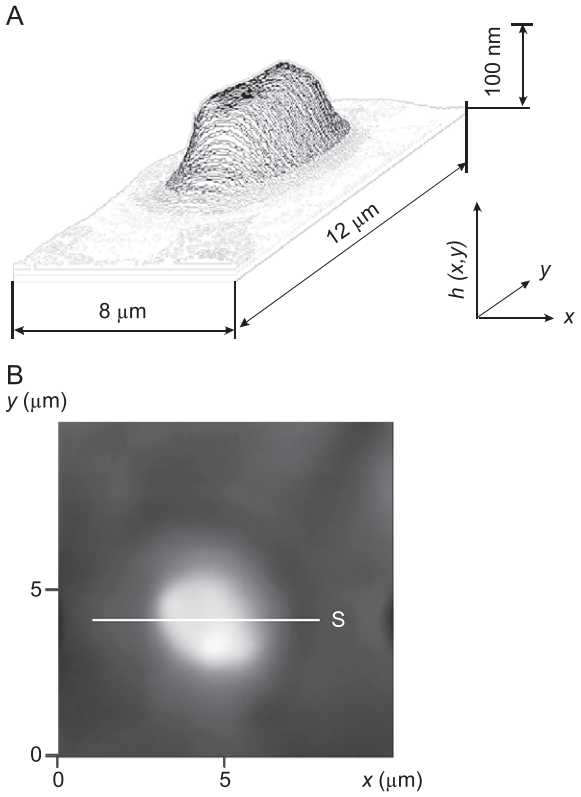

Fig. 1. The typical phase images $h(x,y)$ of a chloroplast. (A) A three-dimensional image of a chloroplast as a distribution of the phase height in the x–y coordinates; (B) A phase image (a topogram) of a chloroplast in the x–y coordinates. The horizontal line shows the position of the scan line.

represents an optical path difference proportional to the "projection" of the refractive index at a point (x,y) in the direction of axis z. The lower part of Fig. 1 shows an alternative pseudocolored phase image of a chloroplast in the plane x–y. The horizontal line shows the position of the scan line S used for a spatial-temporal analysis of the phase height of an individual chloroplast in this part of the topogram. The phase height $h(x)$ was measured in the transversal section of the chloroplast along the scan line S parallel to the axis x. In the plane x–y, the chloroplast image had the shape of an ellipse with the transversal sizes of ~4.5×3 μm or a circle with a diameter of ~3 μm depending on the specific localization of the tested object relative to the viewing laser beam. The transversal sizes of a single chloroplast varied from 3.0 to 4.5 μm at the phase height of 80–120 nm, depending on the specific position of the tested object in control chloroplast preparations. The measurements of phase height fluctuations were performed using periodic ($T \approx 20$ ms) scanning of phase height profiles along the scan line S (for details see Section 2.2).

3.2. Effect of medium osmolarity on phase height profiles of chloroplasts

The variability of the internal volume of chloroplast thylakoids is known to depend critically on the osmolarity of the incubation medium [20]. In hypotonic solutions, thylakoids show a tendency to swell, while in hypertonic media (e.g., at higher sucrose concentrations [20]) they are more prone to shrink. These observations were corroborated by the results of our DPM measurements. For the sake of comparison, the typical profiles of the phase heights of chloroplasts measured in media of low (100 mM sucrose) and high (500 mM sucrose) osmolarity are shown in Fig. 2A. It can be seen that for chloroplasts suspended in low-sucrose media the $h(x)$ function has a greater width and height than that for chloroplasts suspended in high-sucrose media, presumably due to the light-induced swelling of chloroplasts with an increase in their phase heights.

3.3. Effects of uncouplers on phase height profiles of chloroplasts

The characteristic sizes of the phase portraits of chloroplasts measured by DPM appeared to be highly sensitive to the uncouplers present in the incubation medium. It was found that nigericin (20 μM), which is known to prevent the generation of the transmembrane pH difference in thylakoids (ΔpH), caused a pronounced (down to 60 nm) decrement of the phase heights of chloroplasts without any effect on the transversal sizes of the profiles at the half-height level of $h(x)$ (Fig. 2B). An addition of an equimolar amount of valinomycin able to suppress the generation of the electrical component ($\Delta\Psi$) of the proton potential ($\Delta\mu_{H+}$) in the presence of 30 mM KCl did not

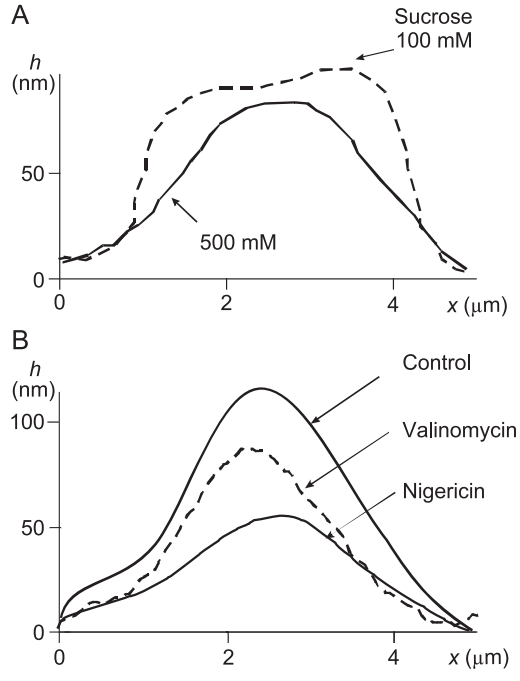

Fig. 2. The effects of uncouplers and medium osmolarity on phase heights of chloroplasts. (A) Typical phase height profiles of chloroplasts measured in low (100 mM sucrose, dotted line) and high (500 mM sucrose, solid line) osmolarity media. (B) A decrement of the phase height of a chloroplast after addition of 20 μM nigericin (solid line) or 20 μM valinomycin (dotted line).

induce any significant decrease in the transversal sizes of the chloroplasts or in their phase heights either (Fig. 2B, dotted line). The lack of a significant effect of valinomycin can be attributed to the fact that $\Delta\Psi$ does not induce any noticeable changes in the $\Delta\mu_{H+}$ value in chloroplasts. These findings are consistent with the well-established fact that the crucial role in the generation of $\Delta\mu_{H+}$ in chloroplast thylakoid membranes is ascribed to ΔpH [1,10,11].

3.4. Light-induced changes in phase heights of chloroplasts

In our method, the measuring beam of a helium–neon laser served simultaneously as an active light flux, which induced light-dependent reactions of electron and proton transport in chloroplasts. According to our estimates, at the incident light density of $\sim 10^{17}$ quanta$\times$cm^{-2} s^{-1} the average radiating power did not exceed 0.3 μW/chloroplast, but was high enough to initiate the photosynthesis. It seemed therefore important to follow the changes in the phase heights of individual chloroplasts in the course of their irradiation. The methodology of our optical measurements was such that the chloroplasts were exposed to irradiation by the laser beam at the very early stages of the experiment due to the necessity to search the tested object in the visual field of the microscope (the "dead time" was ≈60 s). Kinetic measurements of light-induced changes in phase heights were preceded by experiments performed in accordance with the following protocol. Preliminary irradiation of chloroplasts was carried out simultaneously with setting-up procedures (e.g., a search for chloroplasts in the visual field area of the microscope). The duration of the light exposure (t_1) in this step did not exceed 60–90 s. After switching-off, the chloroplasts were adapted to darkness for a definite period of time (t_{ad}), after which the light was switched on again and the changes in $h(x,y,t)$ were recorded in the region (x,y).

The kinetics of changes in the phase height $h(x,t)$ of an individual chloroplast in response to irradiation followed by dark adaptation (t_{ad}=60 s) is shown in Fig. 3. It can be seen that within the first few seconds of light exposure, the $h(x,t)$ value was lower than before switching-off. In experiments with incubation of chloroplasts in an isotonic medium (200 mM sucrose), the $h(x,t)$ value showed a tendency to decrease (on the average, from 100 to 80 nm) (Fig. 3a), while after incubation in a hypertonic medium (500 mM sucrose) the phase height of chloroplasts decreased from 60 to 40 nm (data not shown). The switching-on after a dark pause was accompanied by a lag pause (≈10 s) with a subsequent slow increase of $h(x,t)$ up to the quasi-stationary level. Apparently, the decrement of the phase height in the dark stage is due to the dissipation of the proton gradient in chloroplast thylakoids. Indeed, in chloroplasts containing 20 μM nigericin the light-induced changes in $h(x,t)$ were absent (see Fig. 3b).

There were no significant changes in the phase heights of control chloroplasts after short-term dark adaptation either

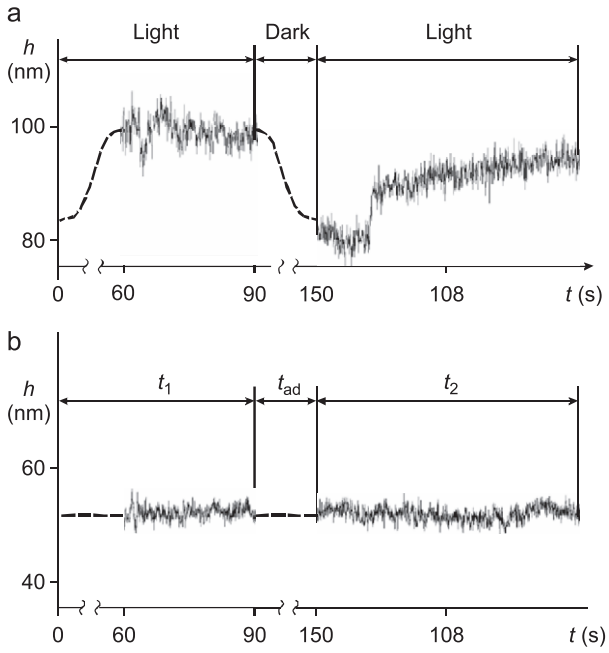

Fig. 3. The photoinduced changes in the phase heights of individual chloroplasts: (a) kinetics of changes in the phase heights $h(x,y,t)$ of control chloroplasts (a characteristic decrement of the phase height by 20 nm after dark adaptation; (t_{ad}=1 min); (b) lack of response of an individual chloroplast to dark adaptation after addition of 20 μM nigericin.

(t_{ad}≤10–20 s). The relatively slow decrements and increments in the phase heights of chloroplasts in the light and dark stages, respectively, can be attributed to the changes in their refractive indices due to the dissipation of the proton gradient in the dark and generation of ΔpH in the light, which, in its turn, caused slow structural rearrangements in chloroplast thylakoids. The characteristic times of these reactions measured in bean chloroplasts under similar conditions were equal to ≈10–20 s. It is of note that an addition of 2 mM Mg-ADP to the incubation medium was not accompanied by any significant changes in the phase heights following dark adaptation (t_{ad}=10–90 s) (data not shown). These data suggest that ATP synthesis is not concomitant with space-correlated changes in the chloroplast structure normally observed in control preparations (e.g., in the absence of exogenous Mg-ADP).

3.5. Phase height fluctuations in individual chloroplasts

An important feature of the DPM method used in this study is that it offers a unique opportunity for studying dynamic characteristics of individual chloroplasts. As can be seen from Fig. 3a, the values of the phase heights $h(x,y,t)$ measured in the specific domain of a chloroplast with the coordinates (x,y) display significant time-dependent fluctuations. Considering that an analysis of phase height fluctuations is an important tool for elucidating the nature of the aforementioned phenomenon, we studied the correlation between the irradiation conditions and the composition of the incubation media, on the one hand, and the

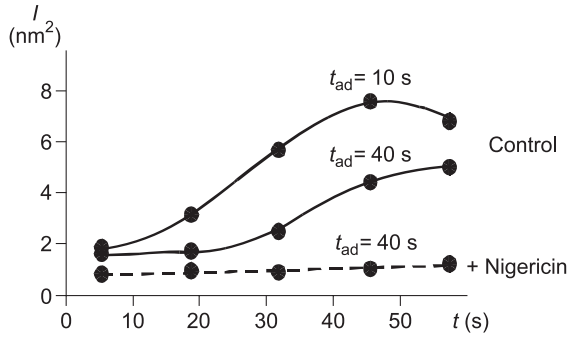

Fig. 4. The time-dependent changes in the fluctuation intensities $I(x,t)$ after dark adaptation of chloroplasts (solid lines, t_{ad}=10 or 40 s, as indicated). In the presence of nigericin (t_{ad}=40 s), the fluctuation intensity remained unchanged and did not exceed 1 nm^2 (dotted line).

intensity and the spectrum of phase height fluctuations in chloroplasts, on the other hand.

While following the kinetics of light-induced changes in $h(x,t)$ (Fig. 3a), we observed that the fluctuation intensity measured during the first few seconds of irradiation of dark-adapted chloroplasts was much lower than that recorded in the quasi-stationary state, i.e., after a relatively long-term (60–90 s) irradiation. The fluctuation intensity increased with time, reaching a maximum after 30–40 s. The value of $I(x,y,t)$ (for definition see Section 2.2) was used as a characteristic intensity of fluctuations around point (x,y) in the time interval $(t, t+\tau)$. The time-dependent changes in $I(x,t)$ for dark-adapted chloroplasts (t_{ad}=10 and 40 s) are shown in Fig. 4. As can be seen, the fluctuation intensity increased in a time-dependent manner after switching-on in both cases. However, after short-term dark adaptation, the initial value of $I(x,t)$ was increased in comparison with prolonged adaptation. No effect of dark adaptation on the phase height and fluctuation intensity was observed after addition of 20 μM nigericin to the incubation medium (see Fig. 4, dotted line).

The intensities and fluctuation spectra measured in both stationary state (prior to switching-off) and after dark adaptation (t_{ad}=60 s) differed noticeably from one another as could be evidenced from the spectral portraits which represented Fourier transforms of the track chart of $h(x,t)$ (for details see Section 2.2). A typical spectral portrait is shown in Fig. 5a. Here, the contrast of the lines in an image correlates with the $\rho(F,x)$ value, which reflects the fluctuation density at the frequency F at a point with the scan line coordinate x. The spectral portrait of an individual chloroplast in the quasi-stationary state contains contrasting components with $F \approx 0.35$ and 0.55 Hz (Fig. 5a). The extent of the contrasting components with F=0.55 and 0.35 Hz (Fig. 5d and e) in the horizontal sections of the spectral

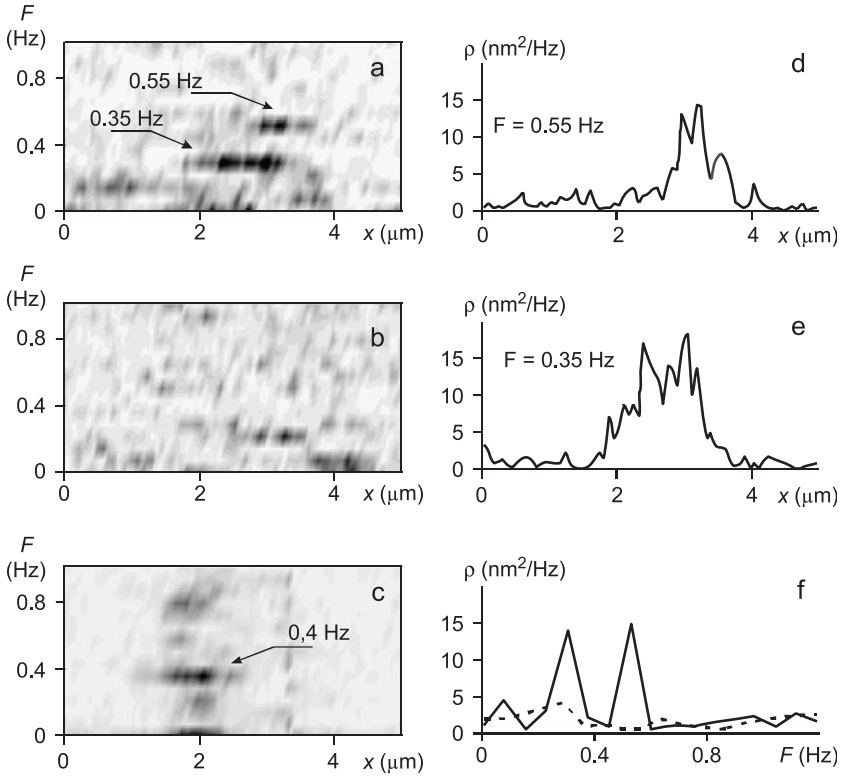

Fig. 5. A light-induced response of an individual chloroplast to dark adaptation. (a) A typical spectral portrait characterized by the presence contrasting components with $F \approx 0.35$ and 0.55 Hz and extension of ~1 and 2 μm recorded in the quasi-stationary state; (b) a spectral portrait recorded within the first few seconds following 60-s dark adaptation; (c) a spectral portrait containing a contrasting component with $F \approx 0.4$ Hz recorded after 60-s dark adaptation; (d, e) changes in the spectral densities of the components with $F \approx 0.55$ and 0.35 Hz located along the scan line; (f) a fluctuation spectrum containing contrasting components with F=0.55 and 0.35 Hz recorded in the quasi-stationary state at a point ρ=3 μm. The dotted line indicates a fluctuation spectrum recorded within the first few seconds after dark adaptation.

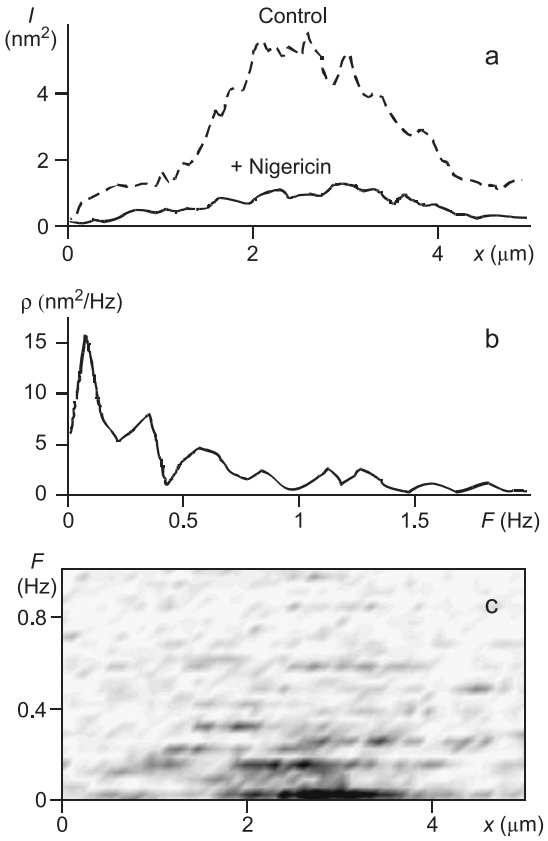

Fig. 6. Effects of nigericin (20 μM) on a fluctuation intensity profile (a), spectral fluctuation density (b) and a spectral portrait (c). The fluctuation intensity in the presence of nigericin (solid line) was much lower than in control chloroplasts in the quasi-stationary state (dotted line); the fluctuation spectrum contained no contrasting components (the components with F<0.2 Hz indicate a slow drift); the spectral portrait did not exhibit any space–time correlation of fluctuation intensity with the exception of the drift component.

portrait were equal to about 1 and 2 μm, respectively; their maxima were shifted by ~0.5 μm relatively to one another. An analysis of the longitudinal sections of these components (Fig. 5d and e) and the distribution of the spectral density $\rho(x)$ (nm^2/Hz) revealed the presence of a fine structure with the characteristic sizes of ~100–200 nm. The fluctuation spectrum reflecting the spectral density at a point x=3 μm is shown in Fig. 5f. It can be seen that the contrasting components with F=0.55 and 0.35 Hz exceed background noises nearly tenfold. The dark phase (1 min) was followed by a practically complete disappearance of the contrasting components with F=0.55 and 0.35 Hz from both the fluctuation spectrum (Fig. 5f, dotted line) and the spectral portrait (Fig. 5b). The 0.4 Hz component appeared in the spectral portrait of the same chloroplast as late as 60 s after termination of the dark pause (Fig. 5c). It is of note that in chloroplasts incubated in hypertonic media (500 mM sucrose), the component with F=0.55 Hz was usually absent, while the relatively intense component with F=0.25–0.4 Hz remained in the spectrum (data not shown).

Our studies have demonstrated that the composition of the incubation medium affects not only the phase heights of the chloroplasts (see Fig. 2), but also the spectra and intensities of their fluctuations (Fig. 6). In the presence of nigericin (solid line), which causes the dissipation of ΔpH, the fluctuation intensity is much less pronounced than in the quasi-stationary state (Fig. 6a, dotted line-control). The spectral portrait (Fig. 6c) and the fluctuation spectrum (Fig. 6b) do not display any contrasting components characteristic of control chloroplasts. At the same time, in the presence of 30 mM KCl, valinomycin (20 μM), which is known to suppress the electric component of the transmembrane proton potential without any effect on ΔpH, did

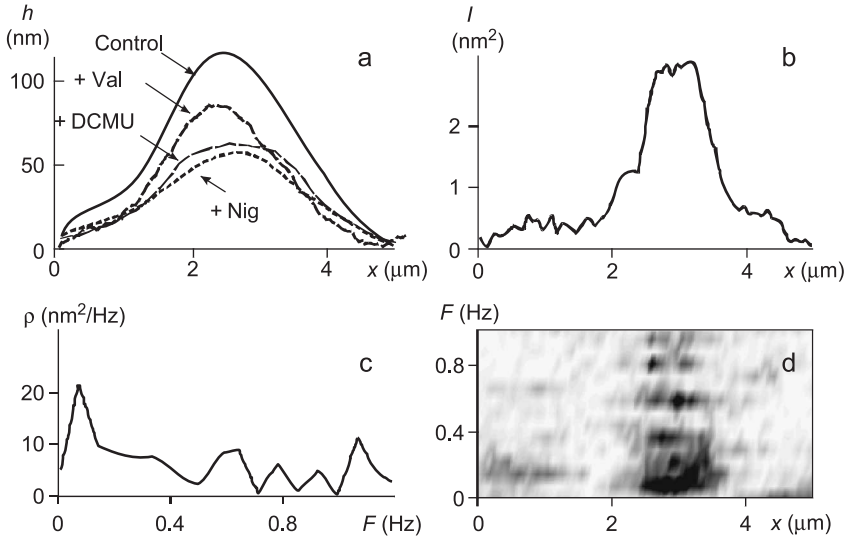

Fig. 7. The effects of valinomycin on phase height fluctuations in chloroplasts. (a) Phase height profiles in control chloroplasts and effects of valinomycin (20 μM, Val), nigericin (20 μM, Nig) and 20 μM DCMU; (b) the fluctuation intensity in the presence of 20 μM valinomycin; (c) a phase height fluctuation spectrum of a chloroplast in the presence of 20 μM valinomycin; (d) a spectral portrait (in the presence of 20 μM valinomycin) containing weakly contrasting components with F=0.1–1 Hz.

not provoke any increment of light-induced fluctuations in dark-adapted chloroplasts. Immediately after dark adaptation, the fluctuations were at the ambient noise level; however, their intensity increased after a while with a subsequent reduction down to the control level within the first 30–40 s after irradiation.

Fig. 7a demonstrates phase height profiles recorded after addition of valinomycin (20 μm), nigericin (20 μm) or 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (20 μm) to the incubation medium as well as in control chloroplasts. In the presence of valinomycin, the fluctuation intensity was recorded in a relatively narrow (Δx=1.5 μm) segment of the scan line (see Fig. 7b). The spectral portrait (see Fig. 7d) and its longitudinal section (Fig. 7c) contain weakly contrasting components with F=0.1–1 Hz (see Fig. 7). The lack of valinomycin effect can be attributed to the fact that the electric potential difference $\Delta\Psi$ does not make any significant contribution to the generation of the transmembrane difference of electrochemical potentials of hydrogen ions in chloroplast thylakoids. It may thus be concluded that the concentration component of the proton potential, ΔpH, is the most plausible factor responsible for the increasing intensity of phase height fluctuations in light-exposed chloroplasts. In the presence of the Photosystem II inhibitor DCMU, which represents a blocker of noncyclic (pseudocyclic) electron transport in chloroplasts, the intensity of phase height fluctuations was minimum. In this case, the spectral portrait of an individual chloroplast contained no contrast components characteristic of control samples (data not shown).

A question arises as to whether the above-described phenomena result from artefacts, e.g., due to heating of chloroplasts or dielectrophoresis. The data available suggest that such side effects cannot adequately be explained by light-induced changes in the phase heights or increasing fluctuation intensities in light-exposed chloroplasts. Indeed, if heating were the main reason for the increase in the fluctuation intensity, this effect would have been observed independently of the metabolic state of the chloroplasts. However, our data suggest that in the presence of nigericin the intensity of phase height fluctuations decreased in comparison with control.

4. Conclusion

Our pioneering studies of individual isolated bean chloroplasts have shown that the geometrical parameters of phase images of chloroplasts recorded by the DPM method correlate with the well-known data on the structural organization of chloroplasts. Some variability in the heights and widths of phase height profiles can be attributed to the ambiguous orientation of thylakoids with respect to the optical axis and the structural heterogeneity of the chloroplasts themselves. The measurements of static and dynamic characteristics of phase portraits of chloroplasts under different experimental conditions (e.g., osmolarity, effects of nigericin, valinomycin and the electron transport inhibitor DCMU, etc.) indicate that the phase heights and fluctuation intensities of individual chloroplasts increase as a result of generation of the transmembrane pH difference (ΔpH) in thylakoids. The origin of this phenomenon is not yet clearly understood. It may be assumed, however, that the quasi-static increase in the phase height is related to the swelling of chloroplasts in the course of their illumination. This hypothesis is consistent with the fact that the $h(x,y,t)$ value diminishes with an increase in the osmolarity of the incubation medium.

Space–time-correlated phase height fluctuations are the most original and interesting findings of this study as can be evidenced from the presence of characteristic contrasting components with $F \approx 0.25$–0.6 Hz and their extension along the scan line of ≤2 μm. This finding testifies to a cooperative effect which may involve dozens of thylakoids. The nature of these oscillations is nevertheless obscure. Suffice it to say that similar oscillations with $F \geq 0.3$ Hz, which may involve larger areas, can also be observed in mitochondria [16–18]. However, in contrast to chloroplasts, a crucial role in the appearance of fluctuations in mitochondria is played by the electric component of the membrane potential which is considered to be the main component of the proton potential in mitochondria.

Acknowledgements

The authors are grateful to Dr. V.V. Ptushenko for the generous supply of bean chloroplasts and Dr. B.V. Trubitsin for his valuable assistance in conducting spin label measurements of ΔpH. This work was partly supported by the grants of the Rostock University (Germany), the Russian Foundation for Basic Researches (03-04-48981 and 04-04-49132), INTAS (99-1086 and 01-483), Program "Universities of Russia" (01.04.081).

References

[1] R.E. Blankenship, Molecular Mechanisms of Photosynthesis, Blackwell Science, 2002.
[2] A. Zouni, H.-T. Witt, J. Kern, P. Fromme, N. Krauß, W. Saenger, P. Orth, Crystal structure of photosystem II from *Synechococcus elongatus* at 3.8 Å resolution, Nature 409 (2001) 739–743.
[3] P. Jordan, P. Fromme, H.-T. Witt, O. Klukas, W. Saenger, N. Krauß, Three-dimensional structure of cyanobacterial photosystem I at 2.5 Å resolution, Nature 411 (2001) 909–917.
[4] G. Kurisi, H. Zhang, J.L. Smith, W.A. Cramer, Structure of the cytochrome b_6f complex of oxygenic photosynthesis: tuning the cavity, Science 302 (2003) 1009–1014.
[5] J.P. Abrahams, A.G.W. Leslie, R. Lutter, J.E. Walker, Structure at 2.8 Å resolution of F1-ATPase from bovine heart mitochondria, Nature 370 (1994) 621–628.
[6] D. Stock, A.G.W. Leslie, J.E. Walker, Molecular architecture of the rotary motor in ATP synthase, Science 286 (1999) 1700–1705.

[7] G. Noctor, C.H. Foyer, Homeostasis of adenylate status during photosynthesis in a fluctuating environment, J. Exp. Bot. 51 (2000) 347–356.
[8] J. Allen, Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain, Trends Plant Sci. 8 (2003) 15–19.
[9] T. Pfannschmidt, Chloroplast redox signals: how photosynthesis controls its own genes, Trends Plant Sci. 8 (2003) 33–41.
[10] L.A. Blumenfeld, A.N. Tikhonov, Biophysical Thermodynamics of Intracellular Processes. Molecular Machines of the Living Cell, Springer-Verlag, New York, 1994.
[11] D.M. Kramer, C.A. Sacksteder, J.A. Cruz, How acidic is the lumen? Photosynth. Res. 60 (1999) 151–163.
[12] V.P. Tychinsky, Coherent phase microscopy of intracellular processes, Usp. Fiz. Nauk (Adv. Phys. Sci.) 171 (2001) 649–662 (Rus.).
[13] D.G. Weiss, V.P. Tychinsky, W. Steffen, A. Budde, Digital Light microscopy techniques for the study of living cytoplasm, in: D.-P. Haeder (Ed.), Image Analysis: Methods and Applications, 2nd ed., CRC Press, Boca Raton, 2000, pp. 209–240.
[14] V.P. Tychinsky, G.E. Kufal, T.V. Vyshenskaya, E.V. Perevedentseva, S.L. Nikandrov, Measurements of submicron structures using a laser phase-contrast microscope Airyscan, Kvant. Elektron. (Quantum Electron.) 24 (1997) 754–758 (Rus.).
[15] A.V. Kretushev, V.P. Tychinsky, Superresolution in phase images, Kvant. Elektron. (Quantum Electron.) 32 (2002) 66–70 (Rus.).
[16] V.P. Tychinsky, D. Weiss, T.V. Vyshenskaya, L.S. Yaguzhinsky, S.L. Nikandrov, Cooperative processes in mitochondria: measurement by dynamic phase microscopy, Biofizika (Biophysics) 45 (2000) 870–877 (Rus.).
[17] T.V. Vyshenskaya, A.V. Kretushev, E.G. Smirnova, L.S. Yaguzhinsky, V.P. Tychinsky, D.G. Weiss, Quasiperiodic changes in the phase height of an individual mitochondrion induced by operation of membrane proton pumps, Biol. Membr. 19 (2002) 491–498 (Rus.).
[18] V.P. Tychinsky, A.V. Kretushev, T.V. Vyshenskaya, Membrane potential-dependent electrooptical effect in mitochondria, Eur. Biophys. J. (in press).
[19] A.N. Tikhonov, G.B. Khomutov, E.K. Ruuge, L.A. Blumenfeld, Electron transport control in chloroplasts. Effects of photosynthetic control monitored by the intrathylakoid pH, Biochim. Biophys. Acta 637 (1981) 321–333.
[20] B.V. Trubitsin, A.N. Tikhonov, Determination of a transmembrane pH difference in chloroplasts with a spin label tempamine, J. Magnetic Res. 163 (2003) 257–269.