Analysis of chlorophyll fluorescence decays of isolated thylakoids in various stages of greening

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Abstract

The decay of chlorophyll (Chl) fluorescence of etiochloroplasts isolated in various stages of greening of cucumber cotyledons was analysed in order to get structural information on a photosynthetic apparatus. Two model decays, multiexponential and stretched exponential, were applied in the analysis. The quality of fit in these two models was different in various stages of chloroplast greening. The two-exponent model did not provide a good fit at early greening stages. To improve the fit it was necessary to introduce an additional third component which became very low at later stages. However, chloroplasts in the early stage of greening could also be described by a stretched exponential with parameters indicating rather planar (two-dimensional) arrangement of donor and acceptor molecules. The chloroplasts treated by DCMU and/or photooxidized by strong irradiance exhibit a similar character of fractal decay as untreated samples but in the multiexponential model the exact values of lifetimes and amplitudes of components vary. This suggests that the structure of investigated system does not dramatically change as a result of these two types of treatment.

Additional key words: chloroplast; *Cucumis sativus*; 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; etiochloroplast; fractal model; photooxidation.

Introduction

Both the photochemical resistance and spectral properties of thylakoids differ at

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Abbreviations: Chl - chlorophyll; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; LHC - light-harvesting complex; PS2 - photosystem 2.
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various stages of development (Franck *et al.* 1995, Więckowski and Waloszek 1995, Frąckowiak *et al.* 1997, Myśliwa-Kurdziel *et al.* 1997). It happens because the pigment-protein complexes associated with core LHC as well as reaction centres are synthesised prior to peripheral LHC (Shibasaka *et al.* 1993), and because the ratio of concentrations of various pigments, their mutual orientations and distances change with the greening time (Więckowski and Waloszek 1995, Frąckowiak *et al.* 1997).

The main part of Chl *a* fluorescence emitted at room temperature originates from photosystem 2 (PS2) (Govindjee *et al.* 1993). The decay of Chl fluorescence is very complex. Results of its analysis depend both on the techniques used for measurements and on the assumptions on system structure and intermolecular interactions (Holzwarth 1987, Yamazaki *et al.* 1990, Kowalczyk *et al.* 1996a). The complex nature of fluorescence decay may be caused either by microheterogeneity of the system and/or by energy transfer between chromophores.

If there exist several pools of pigment-protein complexes characterised by different kinetics of various radiative and radiationless paths of deexcitation, the decay of fluorescence of the whole system can be described by the multiexponential model. These deexcitations can result from several processes such as the excitation energy transfer inside of every pool and between different pools as well as the reversible electron transfer in the reaction centres. Traditionally, the decay of Chl fluorescence of greening chloroplasts is analysed in terms of three exponential components with the decay times: slow - of several thousands of ps, medium - of about 450 ps, and fast - of about 100 ps (Karukstis and Sauer 1983, Govindjee *et al.* 1993, Myśliwa-Kurdziel *et al.* 1997). The origin of these three components is explained differently by various authors (Karukstis and Sauer 1983, Govindjee *et al.* 1993). The exact values of decay times and the amplitudes of these components change with the greening of etiolated chloroplasts (Karukstis and Sauer 1983, Myśliwa-Kurdziel *et al.* 1997) and with the redox state of the reaction centre (Govindjee *et al.* 1993).

Taking into account that the chromophores are located in the flexible biological structure of thylakoids, the Chl fluorescence should be modelled in terms of decay time distributions (Gaussian or Lorenzian), around the most probable values for a given type of chromophore (Govindjee *et al.* 1993, Whitten *et al.* 1997).

Pigment density may be very high in some parts of the antenna system leading to very strong interactions—therefore both the incoherent (Förster-type) and coherent (exciton-type) energy transfer is possible (Hess *et al.* 1995, Knox 1996). Excitation energy transfer occurs with various efficiencies. Dau and Sauer (1996) assumed an ultrafast energy transfer inside both core and peripheral LHC. The slower excitation energy transfer occurs, according to these authors, between these two pools of chromophores. In open reaction centres the primary charge separation can be followed by a radical pair recombination producing excited antenna states. Taking into account all the above processes, Dau and Sauer (1996) predict four lifetime components.

The structures of the LHC of bacteria are better known than those of the green plants. Therefore, it can be inferred only by comparison that in peripheral LHC the distances between adjacent Chl molecules are about 1 nm, and the distance between core and peripheral LHC complexes is about 3 nm (Hess *et al.* 1995). The peripheral LHC of higher plants exhibits a trimeric structure, which is formed during greening

when the number of Chl *a*/Chl *b* complexes is sufficiently high (Kuttkat *et al.* 1995). The composite kinetics of time resolved absorption and fluorescence emission spectra suggest that even the pools of chromophores belonging exclusively to core LHC or to peripheral LHC are heterogeneous. In the peripheral LHC a part of Chl *a* molecules is located close to Chl *b* whereas other molecules are close to lutein (Yamazaki *et al.* 1990). The energy transfer between adjacent molecules of Chl *b* and Chl *a* occurs faster than the equilibration of excitation between five Chl *b* molecules present in the peripheral LHC complex. The decay analysis using three exponential components is only an approximation of real situation occurring in photosynthetic apparatus. All biophysical models involving energy transfer lead to the stretched exponential model of Chl fluorescence decay (Yamazaki *et al.* 1990). One of the parameters describing the decay provides some information on fractal dimension and therefore on the structure of the system. This description suggests that the analyses of Chl fluorescence decays in terms of multiexponential decays, even taking into account decay time distributions, as well as in terms of stretched exponential are in some extent formal, mathematical procedures. Each model reflects, however, certain properties of the investigated system.

Recently, such approach was applied to the study of green bacteria cells immobilised in a polymer (Kowalczyk *et al.* 1996a). The decays of Chl fluorescence were analysed in terms of fractal model, assuming that every emitter is surrounded by energy acceptors. This model describes the decays of aggregated bacteriochlorophylls *c* and *a* better than the multiexponential model, whereas the latter is more appropriate in case of molecules isolated from the energy transfer chain. In model systems containing Chl (Kowalczyk *et al.* 1996b) the fractal model fits better to decays of the aggregated than deaggregated pigment molecules.

In this paper we analyse the decay of Chl fluorescence of cucumber etiochloroplast thylakoids isolated after 4, 6, and 24 h of seedlings irradiation in terms of two models:

(*1*) a multiexponential model

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \qquad (1)$$

with a constraint $\sum_i \alpha_i \tau_i = 1$ where τ_i is a decay time of i-th component of the heterogeneous system and $\alpha_i \tau_i$ describes total amount of photons emitted by i-th component, and

(*2*) a stretched exponential model

$$F(t) = A \exp\left(-t/\tau_D - \beta(t/\tau_D)^{\gamma}\right) \qquad (2)$$

where τ_D is decay time of donor molecule, the power γ = d/s depends on arrangement of acceptor molecules described by space dimension (d) and type of interaction (s). For dipole-dipole interaction, s = 6. This class of model functions describe systems where energy transfer takes place (Förster 1949, Blumen *et al.* 1982), quenching (Van

der Auveraer *et al.* 1989) or deactivation from an excited state surface with a pinhole sink (Bagchi and Fleming 1990).

We expect that the stretched exponential model fits better to donor fluorescence in case of some regular arrangement of acceptor molecules in the vicinity of the fluorescent donor. This kind of analysis can provide some information about the arrangement of acceptor molecules and the type of intermolecular interactions (Yamazaki *et al.* 1990, Van der Auweraer *et al.* 1994, Kowalczyk *et al.* 1996b). Similar formulas as Eq. 2 describe the decay of Chl fluorescence assuming random walk of excitation in case of quasi-linear (1-D), planar (2-D) or spatial (3-D) random arrangements of acceptors (Roy and Blumen 1968, Yamazaki *et al.* 1990, Van der Auweraer *et al.* 1994).

for 1-D $$F(t) = \exp[-t/\tau_D - 2\,(5/6)n_A\,R_0\,(t/\tau_D)^{1/6}] \quad (3a)$$

for 2-D: $$F(t) = \exp[-t/\tau_D - (2/3)n_A\,R_0^2\,(t/\tau_D)^{1/3}] \quad (3b)$$

for 3-D: $$F(t) = \exp[-t/\tau_D - (3/4)^{3/2}\,n_A\,R_0^3\,(t/\tau_D)^{1/2} \quad (3c)$$

where τ_D is the lifetime of donor without acceptor, n_A represents the numerical densities of acceptor per unit length (3a), area (3b), or volume (3c). R_0 is the critical energy transfer distance at which the rate constant for the energy transfer is equal to that for the fluorescence of the donor in the absence of acceptors.

The comparison of Eqs. (2) and (3) shows that it is not possible to distinguish between random walk and fractal models only on the basis of Chl fluorescence decay analysis, but it is possible to find if the acceptor and donor molecules are arranged in a planar (two-dimensional), linear (one-dimensional), or spatial (three-dimensional) system. The aim of the paper is to determine a type of an arrangement of donor and acceptor molecules on the basis of stretched exponential (fractal) analysis of the Chl fluorescence decays.

Materials and methods

Thylakoid membranes were isolated from cotyledons of cucumber (*Cucumis sativus* L. cv. Wisconsin). The seedlings were grown on liquid nutrition medium in the dark for 6 d and then were irradiated (fluorescent tubes, *ca.* 120 μmol m^{-2} s^{-1} of photosynthetically active photons, PAR) for 4, 6, or 24 h as described in Więckowski *et al.* (1989). Chl (*a*+*b*) concentration was kept around 20 g m^{-3} and was estimated as described by Lichtenthaler (1987). Strong irradiance [incandescent lamp, *ca.* 3.5 mmol m^{-2} s^{-1} (PAR) for 15 min] caused photooxidation of about 20, 10, or 4 % Chl and 50, 25 or 10 % of carotenoid contents in the samples obtained after 4, 6, or 24 h of greening, respectively (Więckowski and Waloszek 1993). Thylakoid isolation as well as a procedure of sample photooxidation was described in Więckowski and Majewska (1990) and Więckowski and Waloszek (1993). Two independent samples, originating from different cultures, were investigated in each case. The samples:

intact, treated with 0.1 mM dichlorophenyl dimethyl urea (DCMU), photooxidized, and photooxidized followed by addition of DCMU, were measured three times in consecutive runs of a phase fluorimeter.

Measurements were done using the *ISS K2* (*Champaign*, USA) phase fluorimeter. The excitation beam was modulated with frequency $\omega/2\pi$ = 2,...,200 MHz. The excitation wavelength of 437 nm was selected by a monochromator from a xenon arc. Because of rather low intensity of emitted Chl fluorescence the whole emission of thylakoids was collected using an edge filter transmitting $\lambda > 600$ nm.

The parameters describing the fluorescence decays are estimated by minimising global χ^2 using non-linear least squares global analysis program based on an algorithm of Marquardt (1963):

$$\chi^2_g = \sum_l \left(\sum_\omega \left(\frac{m_\omega - m^c_\omega}{\delta m_\omega} \right) + \sum_\omega \left(\frac{\phi_\omega - \phi^c_\omega}{\delta \phi_\omega} \right)^2 \right)$$

where the index l sums over q experiments, and the index ω sums over the appropriate frequencies from the range 2-200 MHz. m_ω and ϕ_ω (m^c_ω and ϕ^c_ω) denote the observed (and calculated) values of the modulation and phase shift corresponding to the modulation frequency ω. The weighting factors δm_ω and $\delta\phi_\omega$ are, in general, unknown and were taken as 0.005 and 0.200, respectively. This choice is based on the accumulated experience (Lakowicz and Gryczyński 1991). As the real weighting factors may vary from experiment to experiment, the reasonable conclusions can be drawn only if the given set of data is fitted to different models.

The values of the integrals necessary for calculation of m_ω and ϕ_ω were obtained analytically in case of exponential decays whereas for stretched exponential the routine based QUANC8 algorithm was employed (Forsythe *et al.* 1977). Values collected during three consecutive experiments with the same sample were analysed globally with all parameters linked throughout the set.

The free parameters in case of exponential decays (Eq. 1) were: amplitudes α_i and decay times τ_i with a constraint: $\Sigma\alpha_i\ \tau_i = 1$. Instead of amplitudes, the fraction of photons $f_i = \alpha_i\ \tau_i$ corresponding to the decay time τ_i is presented in Table 1. The parameters of the stretched exponential model are β, τ, and γ (Eq. 2). Because of singularities in derivatives with respect to γ, this parameter was not included into Marquardt search but was kept constant at the preselected values 0.2, 0.3, and 0.4.

Whole Chl fluorescence emitted by the samples was analysed together, even if the emission from PS2 is predominant at room temperature (Govindjee *et al.* 1993).

Results and discussion

Table 1 shows the Chl fluorescence decay times and corresponding photon fractions obtained from the analysis of measured Chl fluorescence decays as a sum of three and two exponential functions as well as a stretched exponential. In most cases the emitting molecules could be divided into three pools as it was proposed by Karukstis

and Sauer (1983). According to them, the slow component is related to pigment uncoupled from energy transfer system, the medium one is due to heterogeneity of PS2 antenna system, whereas the fast component is emitted by antenna closely coupled with reaction centres. The four-exponential model (not shown) does not improve the fit compared to the three-exponential model. In most cases the fourth component has unrealistically low amplitude. It probably represents only some background effect. Therefore the analysis in terms of three components seems to be related to intrinsic nature of the sample. It is in agreement with Karukstis and Sauer (1983) and Govindjee *et al.* (1993).

Table 1. The analysis of chlorophyll fluorescence decays in terms of bi- and triple-exponential (Eq. 1) and stretched exponential (Eq. 2) models of isolated thylakoids after 4, 6, or 24 h of greening of cucumber seedlings. The samples are: untreated after preparation (*1*), treated with DCMU (*2*), photooxidized (*3*), or photooxidized and treated with DCMU (*4*). The parameter γ was kept constant at 0.3.

Greening time [h]	Sample	τ_1 [ns]	f_1	τ_2 [ns]	f_2	τ_3 [ns]	f_3	χ^2	τ [ns]	β	γ	χ^2
4	*1*			3.2	0.49	0.49	0.51	11.2	12.5	6.1	0.3	3.2
		5.1	0.25	1.4	0.48	0.23	0.27	2.7				
	2			3.1	0.45	0.67	0.55	9.5	4.2	3.2	0.3	14.8
		5.8	0.15	1.6	0.54	0.45	0.31	5.2				
	3			3.0	0.44	0.51	0.56	21.0	6.9	5.0	0.3	17.5
		9.0	0.10	1.7	0.52	0.34	0.38	9.8				
	4			3.0	0.45	0.48	0.55	14.5	9.5	5.8	0.3	8.8
		12.9	0.06	2.0	0.50	0.38	0.44	4.3				
6	*1*			3.5	0.44	0.44	0.56	21.5	40.0	101.0	0.3	12.2
		12.1	0.08	2.3	0.44	0.37	0.48	10.9				
	2			2.8	0.44	0.57	0.56	9.5	4.6	3.8	0.3	13.2
		7.0	0.09	1.9	0.48	0.46	0.43	5.8				
	3			2.5	0.42	0.44	0.58	9.3	7.2	5.5	0.3	10.6
		5.1	0.09	1.8	0.42	0.38	0.49	7.8				
	4			2.6	0.44	0.57	0.56	6.6	4.4	3.7	0.3	11.5
		3.6	0.23	1.3	0.41	0.42	0.35	5.5				
24	*1*			4.5	0.15	0.24	0.85	29.4	400.0	32.0	0.3	56
		110.0	0.03	2.8	0.15	0.23	0.82	14.5				
	2			3.0	0.21	0.35	0.79	38.2	60.0	15.4	0.3	49.4
		290.0	0.03	2.2	0.21	0.34	0.76	25.2				
	3			2.8	0.22	0.26	0.78	36.3	110.0	19.8	0.3	47
		250.0	0.03	2.2	0.22	0.25	0.75	23.1				
	4			2.9	0.22	0.30	0.78	22.0	1500.0	39.6	0.3	26.1
		90.0	0.02	2.3	0.23	0.29	0.75	13.0				

Yet the occurrence of two or three types of energetically isolated chromophores with definite values of Chl fluorescence decay times seems not to be the realistic molecular model of the complex structure of the photosynthetic apparatus. Discrete

component analysis of Chl fluorescence decay assumes that all emitting fluorophores decay with well definite lifetimes.

Inclusion of energy transfer into the model leads to decay described as a stretched exponential. To compare the fitting of both models one has to introduce to both of them the same number of parameters. Therefore we compared the goodness-of-fit of decays of the two-exponential model with that of the stretched exponential model because in such case both models are described by the same number of parameters (f, τ_1, τ_2, and β, τ, γ) and therefore the comparison is fair if it is done for the same set of values (Table 1). The analysis into two components gives, as expected, in most cases worse fits than the analysis in terms of three components. We expected that for shorter time of greening most Chls would not be included into antenna structure (Myśliwa-Kurdziel *et al.* 1997). As a result at this early stage, multiexponential model would fit better than in further growth stages. The result, however, is opposite. For 4 h of greening the stretched exponential model fitted, in most cases, better than the biexponential one whereas for longer times of greening the multiexponential model was more suitable. It confirms that the structure of thylakoids in the beginning of greening is different that from that at later stages of development.

For the fractal model, the acceptor concentrations should be sufficiently small to avoid multiple occupation of the same region in space and the donor-donor transfer should be absent. The simulation of dipole-dipole interaction in the two-dimensional fractal model (Van der Auweraer *et al.* 1994) shows that the model can be used when in the circular area with the radius of Förster critical radius R_0 is located from 0.2 to 3 acceptors. The R_0 of Chl in an ether solution is about 7 nm. When the surrounding causes an almost complete overlap of the absorption and emission bands of acceptor and donor, the R_0 reaches about 8.4 nm (Knox 1975). The coaxial alignment increases the R_0 value up to 11.3 nm (Knox 1975).

We can assume that the critical radius R_0 is close to 10 nm in antenna complexes, and therefore the number of acceptor molecules which can quench the donor fluorescence could be higher than predicted in the fractal model, especially for peripheral LHC complexes where the distance between pigment molecules is about 1 nm. Various types of interactions, such as the Förster-like type, dipolar and quadrupolar, and the exchange-like type can also occur, which is reflected by the change of Eq. 1 into an exponential-logarithmic pattern (Roy and Blumen 1968). If these interactions are not uniform within the antenna system, an even more complicated formula is expected.

The exact structure of LHC complexes in plants is not yet known (Holtzwarth 1995), therefore we can not decide *a priori* if this structure may be approximated by the quasi-linear, two-dimensional, or three-dimensional models leading to fluorescence decays described by Eqs. 3a-c. Comparison of Eq. 2 with Eq. 3 shows that in such complicated systems as LHCs we are not able to distinguish unambiguously between the random walk model of excitation and the fractal model because several properties of the system such as configuration of acceptors and type of interaction are not exactly known. The most probable model of the structure of antenna systems (Hess *et al.* 1995, Holtzwarth 1995) is planar arrangement in a membrane. Therefore the two-dimensional approximation can be the most suitable. To check this hypothesis we

varied the parameter γ trying to fit the experimental data to the stretched exponential model (Table 2). The outcome of the analysis was judged by both the goodness-of-fit and the recovered value of donor lifetime (the lifetime above 20 ns was considered

Table 2. The analysis of chlorophyll fluorescence decays in terms of the stretched exponential (Eq. 2) model of thylakoids isolated from cucumber seedlings. The parameter γ is kept constant at values of 0.2, 0.3, 0.4. The sample designations are as in Table 1.

Greening time [h]	Sample	τ [ns]	β	γ	χ^2
4	*1*	500.0	8.0	0.4	5.5
		12.5	6.1	0.3	3.2
		6.0	5.2	0.2	6.4
	2	7.8	4.3	0.4	10.1
		4.2	3.2	0.3	14.8
		3.2	3.3	0.2	19.7
	3	41.0	10.9	0.4	13.2
		6.9	5.0	0.3	17.5
		4.1	4.6	0.2	23.2
	4	150.0	19.0	0.4	6.4
		9.5	5.8	0.3	8.8
		4.6	4.9	0.2	14.2
6	*1*	1500.0	50.0	0.4	48.0
		40.0	10.1	0.3	12.2
		8.2	6.4	0.2	18.0
	2	9.5	5.3	0.4	10.0
		4.6	3.8	0.3	13.2
		3.5	4.0	0.2	16.9
	3	150.0	19.8	0.4	8.9
		7.2	5.5	0.3	10.6
		4.0	5.0	0.2	13.2
	4	8.5	5.0	0.4	8.9
		4.4	3.7	0.3	11.5
		3.2	3.8	0.2	14.2
24	*1*	89.2	27.2	0.4	103.0
		415.0	32.0	0.3	57.0
		78.0	16.0	0.2	38.4
	2	600.0	46.0	0.4	53.0
		60.0	15.4	0.3	49.4
		5.0	7.4	0.2	56.0
	3	200.0	24.0	0.4	87.0
		110.0	19.8	0.3	47.0
		13.3	10.4	0.2	42.0
	4	150.0	26.9	0.4	54.0
		1500.0	39.6	0.3	26.1
		7.4	8.6	0.2	30.8

unreasonable). Table 2 shows that for 4 h of greening in most cases the γ value 0.3 gives a reasonable τ_D and low χ^2. After 6 h of greening the exponent was 0.3-0.4. In a sample after 24 h of greening the fractal model was not adequate. The value of about 0.3, as follows from Eq. 3a, describes the random walk in the two-dimensional system. The value 1.3/6 ($\approx$0.2) was obtained from fractal analysis for the dye adsorbed on the vesicle surface, similarly as found for several dyes by Yamazaki *et al.* (1990). Similar values of parameter γ were also predicted for a self-avoiding random walk at the dipole-dipole interactions (Mc Kenzie 1976). When we assume the random walk mechanism then the system seems to be more planar at 4 h of greening than at 6 h of greening. In the second case, the three-dimensional structure should be more appropriate. In a fractal model the change of the parameter γ can be due not only to the change of fractal dimension d related to different arrangement of acceptors, but also to the change of interactions. Most likely both the arrangement of acceptor and type of interactions are changed during greening. It was unexpected that the samples treated by DCMU, strong irradiance, or both would exhibit a similar type of decay curves but exact values of lifetime and amplitude of components were changed. Also values of τ_D for analysis of the multiexponential model (Table 1) are not identical for all four types of sample. This study suggests that neither DCMU nor strong irradiance change in fundamental way the structure of the antenna system as well as their intermolecular interactions.

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