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A DIFFUSIONAL ANALYSIS OF THE TEMPERATURE SENSITIVITY OF THE Mg^{2+}-INDUCED RISE OF CHLOROPHYLL FLUORESCENCE FROM PEA THYLAKOID MEMBRANES

B.T. RUBIN, J. BARBER, G. PAILLOTIN *, W.S. CHOW and Y. YAMAMOTO

ARC Photosynthesis Research Group, Department of Pure and Applied Biology, Imperial College, London SW7 2BB (U.K.)

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The kinetics of the chlorophyll fluorescence rise induced by adding 20 mM $MgCl_2$ to a suspension of isolated pea chloroplasts treated with 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (DCMU) have been examined experimentally and theoretically as a function of temperature. The application of similarity arguments and particle aggregation theory to the experimental results suggests that at the first approximation, the salt-induced time-dependent fluorescence changes may be described by the diffusion-controlled lateral movement of Photosystem II pigment-protein complexes. From an analysis of the temperature dependence of the fluorescence changes, estimates obtained for the lateral diffusion coefficients were $1.85 \cdot 10^{-12}$–$3.08 \cdot 10^{-11}$ cm^2/s over the temperature range $10°C \leqslant T \leqslant 30°C$.

Introduction

Recently, it has been advocated that the lateral diffusion of pigment-protein complexes occurs in the lipid matrix of isolated thylakoid membranes of higher plant chloroplasts in response to changes in the ionic conditions of the suspending medium [1]. It is suggested that as a consequence of this reorganization, changes in the chlorophyll fluorescence yield and in the degree of membrane-membrane appression are observed. Experimental evidence and theoretical considerations relevant to the general concept have been presented in a series of recent papers [2–8]. Essentially, it is argued that manipulation of the electrolyte level in the suspending medium, so as to minimize the double layer repulsive forces acting between adjacent membrane surfaces and between surfaces of exposed portions of integral proteins within the same membrane, allows van der Waals' interactions to occur which facilitate protein aggregation and thus the development of discrete pigment-protein domains. It is visualized that the production of separate domains for PS I and PS II pigment-protein complexes occurs and that this process gives rise to the characteristic increase in chlorophyll fluorescence emission, the associated decrease in excitation transfer from PS II to PS I and also the surface charge heterogeneity responsible for granal and stromal lamellae formation. General evidence in support of the concept of a domain-formation process may be drawn from observations of lateral diffusion of proteins and lipids in biological membranes [9–17] and the formation of enriched regions of a given membrane component [18–21].

In this paper we have measured the kinetics of the salt-induced increase in chlorophyll fluorescence at various temperatures so as to explore in greater detail the connection between changes in protein particle

* Present address: Commisariat a L'energie Atomique, Service de Biophysique, Departement de Biologie, Centre D'etudes Nucleaires de Saclay, 91191 Gif-Sur-Yvette Cedex, France

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; PS, photosystem; Chl, Chlorophyll.

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distribution and changes in fluorescence emission in relation to diffusion-controlled formation of protein-rich domains. The approach adopted has permitted the determination of lateral diffusion coefficients and has provided a basis for relating the physical properties of the thylakoid membrane to other membrane systems.

Materials and Methods

Envelope-free chloroplasts from pea leaves were prepared as described in Ref. 7 in the unstacked form by including 0.5 mM EDTA in the washing medium. For experiments, an aliquot of the ice-cold stock chloroplast suspension was diluted into a medium containing 100 mM sorbitol, 50 μM EDTA, 10 μM DCMU, 1 mM Hepes, 1 mM KOH (pH 7.6, HCl) and 10 μg Chl/ml. The mixture was equilibrated at a given temperature for several minutes before the measurement of chlorophyll fluorescence as described in Ref. 7. Upon the addition of 20 mM $MgCl_2$, the time course of the chlorophyll fluorescence increase was recorded for each temperature. $MgCl_2$ (40 μl) was injected through a syringe needle into a 2 ml volume of suspension in a cuvette. Formation of bubbles was minimized by positioning the end of the needle just beneath the surface of the suspension. The concentration of $MgCl_2$ (20 mM) added to a medium containing a low concentration of monovalent cation (1 mM) ensured a rapid transition from minimum to maximum electrostatic screening of surface charges [2, 8]. The $MgCl_2$-induced chlorophyll fluorescence increase was normalized according to the maximum change in individual samples.

It was found that the mixing time was shorter than the recorder response time which was about 0.15 s. Because of the time lag in the system, the time $\tau = 0$ in Fig. 3 was taken to be the time when the fluorescence signal began to rise from the steady level occurring prior to the addition of $MgCl_2$. Typically, $\tau = 0$ was 0.3 s and 0.4 s at 29.6 and 14.0°C, respectively, from the beginning of the injection of $MgCl_2$, the latter being monitored concurrently by changes in light scattering.

Temperature of the chloroplast sample was regulated to within 0.1°C by flowing water through an insulated cuvette jacket connected to a Grant Instruments thermostatically controlled circulator. The aliquot of $MgCl_2$ to be injected was also pre-equilibrated to the appropriate temperature.

Theory

The family of curves in Fig. 1 suggests the possibility that the kinetics of salt-induced fluorescence changes at different temperatures may be attributed to a single physical process. To test this hypothesis we employ similarity arguments. If a single mechanism is operative, all curves in Fig. 1 should collapse to one universal curve. Thus, the functional dependence of the kinetics of the salt-induced fluorescence rise may be written generally as:

$$F_i = F(\tau/\tau_i^*) \tag{1}$$

where F is the relative fluorescence level, τ the time and τ_i^* a characteristic time of the process; the subscript i indicates the particular curve $i = 1, 2, ..., 6$ corresponding to the temperature T_i. For convenience, we designate the T_6 curve as the universal curve and relate the characteristic time of each curve τ_i^* to the characteristic time of the universal curve τ_6^* by the formula:

$$\tau_i^* = \tau_6^*/\mu_i \tag{2}$$

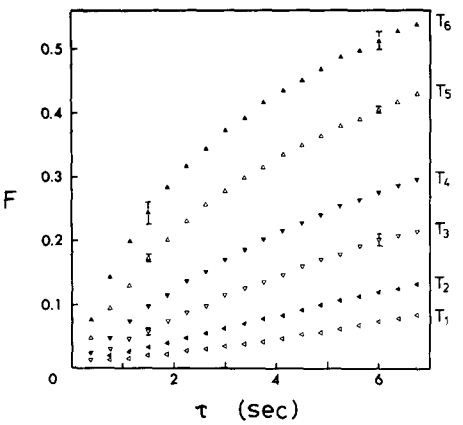

Fig. 1. Raw experimental data of relative fluorescence level F as a function of time τ at six different temperatures: T_1 = 10.1, T_2 = 14.0, T_3 = 19.0, T_4 = 22.5, T_5 = 26.7, T_6 = 29.6°C. All results are normalized to the maximum relative fluorescence level which for each temperature occurs at times $\tau_M > 300$ s. Each data point represents the mean of four values. Representative standard errors are indicated.

TABLE I

μ_i is the similarity coefficient. D_i is the two-dimensional lateral diffusion coefficient of PS II protein particle complexes undergoing translational motion in a finite diffusion space. See text for details.

i	T_i (°C)	μ_i	D_i (cm²/s) (×10¹²)
1	10.1	0.060	1.85
2	14.0	0.100	3.08
3	19.0	0.187	5.75
4	22.5	0.300	9.23
5	26.7	0.600	18.45
6	29.6	1.000	30.75

where μ_i is the similarity coefficient and the identity $\mu_6 \equiv 1$ holds. Using Eqn. 2, Eqn. 1 becomes:

$$F_i = F(\mu_i \tau / \tau_6^*) = F_6(\mu_i \tau) \quad (3)$$

The similarity condition expressed in Eqn. 3 requires that values of the relative fluorescence level F_i obtained at a low temperature, say T_1, correspond on an extended time scale to the same values of F_6 obtained at the higher temperature T_6 for shorter times. It is readily seen that for the μ_i values given in Table I all the experimental curves collapse to a single universal curve shown in Fig. 2. Hence, it may be supposed that the observed fluorescence rise depends on a single process such as diffusion or another process characterized by a single rate constant. In order to model the universal curve of Fig. 2, we adopt the mechanistic implications of previous experimental and theoretical considerations [1–8,22,23] which link salt-induced fluorescence changes to changes in spatial distribution of PS I and PS II protein complexes occurring in thylakoid membranes. Under conditions when the reaction centres are closed, the extent of PS II particle fluorescence may depend on the PS II-PS I interparticle distance which in turn is thought to regulate the degree of exciton transfer or 'spillover' between PS II donor and PS I acceptor particles. It has been argued [1–8] that the distribution of protein particle complexes in thylakoid membranes is sensitive to the electrostatic screening properties of cations at suitable concentrations. In the unstacked state it is thought that electrostatic repulsive forces acting between negatively charged membrane protein complexes give rise to random intermixing of the membrane components. It is presumed that as a consequence of this the average distance for PS II-PS I pairs is smaller than that which occurs in the natural stacked state. Thus, the relative fluorescence level in the unstacked condition is low and the degree of spillover is high [1]. The addition of suitable screening cations at the appropriate salt concentration 'instantaneously' triggers a reduction in electrostatic repulsion [1,6,8] which facilitates the onset of protein aggregation and domain formation. In the screened condition, PS I particles are thought to bear a relatively greater negative charge than the PS II particles although the effective charge on both is significantly reduced from that in the unstacked condition due to the presence of added cations. The interaction between PS II-PS I particle complexes remains essentially repulsive. In the case of PS II-PS II particle interactions, the electrostatic repulsive component of the potential energy is sufficiently reduced so that aggregates of PS II particles begin to form as a consequence of van der Waals' attractive forces acting between PS II particles both in plane and between adjacent membrane surfaces which come together to form the grana partition. During the process of PS II particle aggregation, PS II particles within a growing aggregate experience steric particle

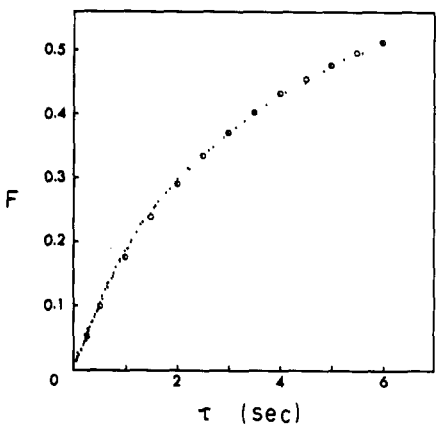

Fig. 2. Universal curve of relative fluorescence level F vs. time τ. Application of similarity arguments using Eqn. 3 and the values of the similarity coefficient from Table I demonstrate that the experimental points (·) for each temperature T_i lie on the single universal curve $T = T_6$. Theoretical points (o) generated by Eqn. 11 are also indicated.

shielding which would be expected to decrease exciton transfer between shielded donor PS II particles in the aggregate and acceptor PS I particles. Hence, the physics of the process envisaged suggest, that as a first approximation, the relative increase in fluorescence level associated with the salt-induced transition from the low- to the high-fluorescence state may be described primarily by the kinetics of PS II particle aggregation. Hence, we employ Schmoluchowski's theory of coagulation [24,25]. Let c_n be the number of aggregates per unit area of membrane containing n PS II particles per aggregate (i.e., an n aggregate), then:

$$dc_n/d\tau = (1/2)\, k \sum_{m=1}^{n-1} c_m c_{n-m} - k c_n c \tag{4}$$

where

$$c = \sum_{m=1}^{\infty} c_m \tag{5}$$

and k is the collision kernel or aggregation coefficient. The solution of Eqn. 4 and 5 is given [24,25] as:

$$c = c_0/(1 + c_0 k\tau/2) \tag{6}$$

$$c_n = c_0(c_0 k\tau/2)^{n-1}/(1 + c_0 k\tau/2)^{n+1} \tag{7}$$

where c_0 is the value of c at the instant of time $\tau = 0$. The fraction p_n of the n aggregate is:

$$p_n = c_n/c = (c_0 k\tau/2)^{n-1}/(1 + c_0 k\tau/2)^n \tag{8}$$

Let us call f_n the chlorophyll fluorescence yield of an n aggregate and let us further suppose that at the instant of time $\tau = 0$, $p_n = 0$ except for $n = 1$ ($p_1 = 1$), then:

$$F = \sum_{n=2}^{\infty} (f_n - f_1)\, p_n \tag{9}$$

Introducing the simplifying assumption, $f_n \simeq f_2$ for $n \geqslant 2$, and finding the sum of the infinite series reduces Eqn. 9 to the form:

$$F = (f_2 - f_1) \sum_{n=2}^{\infty} p_n$$

$$= \{(f_2 - f_1)\, c_0 k\tau/2\}/(1 + c_0 k\tau/2) \tag{10}$$

Discussion

Comparison of Eqn. 10 with the experimental universal curve displayed in Fig. 2 reveals that a good fit is obtained with

$$F = F_6 = 0.223\tau/(1 + 0.268\tau) \tag{11}$$

Hence

$$(f_2 - f_1) = 0.832 \tag{12}$$

$$c_0 k = 0.536 \tag{13}$$

If the PS II particle aggregation is considered to be a two-dimensional diffusion-limited process, then k may be identified as the two-dimensional collision kernel. The two-dimensional collision kernel for identical particles undergoing aggregation in a finite circular region of radius r ($\bar{R} \leqslant r \leqslant R^*$) subject to the perfect absorbing condition at $\bar{R}$, zero flux at R^* and constant uniform particle distribution at time $\tau = 0$ may be obtained from the general formulation given by Buas [26]. His treatment is based upon a derivation which relates the mean first collision time (i.e., the mean time it takes for a diffusing particle to strike first an 'absorbing' target) to the collision kernel. Thus, in two dimensions:

$$k = 4\pi D/s \tag{14}$$

and the corresponding mean first collision time $\hat{\tau}$ given by Adam and Delbruck [27] has the approximate form:

$$\hat{\tau} = (R^*)^2 s/2D \tag{15}$$

where

$$s = \ln(R^*/\bar{R}) - 1/2 \tag{16}$$

Here D is the diffusion coefficient, $\bar{R}$ is the radius of the absorber and R^* is the outer radius of the diffusion space. Combining Eqns. 13 and 14 the diffusion coefficient of the universal curve is:

$$D = D_6 = 0.536s/4\pi c_0 \tag{17}$$

To obtain the diffusion coefficients D_i at the given

temperatures T_i, we note that the characteristic time τ_i^* given in Eqn. 1 may now be identified as the mean first collision time $\hat{\tau}_i$ at the corresponding temperature. Thus,

$$\tau_i^* \equiv \hat{\tau}_i = (R^*)^2 s/2D_i \tag{18}$$

Utilizing Eqns. 2 and 18, the similarity coefficient is easily seen to be:

$$\mu_i = D_i/D_6 \tag{19}$$

Combining Eqns. 17 and 19 yields the result:

$$D_i = 0.536\, \mu_i s/4\pi c_0 \tag{20}$$

If δ is the average PS II-PS II interparticle spacing and each particle is located at the centre of a square, then $c_0 = 1/\delta^2$. Moreover, for a random distribution of protein particle complexes at time $\tau = 0$ (condition of the unstacked state prior to the addition of screening cations), the calculation of the mean distance between nearest neighbours gives $c_0 = 1/4\delta^2$ and Eqn. 20 becomes:

$$D_i = 17.06 \cdot 10^{-2} \mu_i \delta^2 \{\ln(R^*/\bar{R}) - 1/2\} \tag{21}$$

wherein Eqn. 16 has been employed. In order to estimate the numerical values of the diffusion coefficient a knowledge of the numerical values for μ_i, δ and the ratio $(R^*/\bar{R})$ is required. Freeze-fracture data suggest that the average interparticle PS II-PS II spacing in the unstacked state is $\delta \cong 100$ nm [28]. The boundary radius R^* for a typical pea thylakoid granal disc may be in the range $200 < R^* < 400$ nm [29]. The particle capture radius $\bar{R}$ corresponding to a perfect absorber occurs in the range $2a \leqslant \bar{R} \leqslant 4a$ where a is the PS II particle radius. Taking $a \simeq 10$ nm, $\bar{R} \simeq 30$ nm and $R^* \simeq 300$ nm, we find $(R^*/\bar{R}) = 10$. Further, taking $\delta \simeq 100$ nm and utilizing the μ_i values in Table I, the values of D_i at each temperature T_i are computed by Eqn. 21 and tabulated in Table I. The results obtained for D_i are not inconsistent with other experimental evaluations of lateral protein diffusion coefficients at comparable temperatures [10,12,15, 16,19,30]. A plot of D_i vs. $1/T_i$ shown in Fig. 3 exhibits some non-linearity with an approximate effective activation energy in the range of 1 eV. The

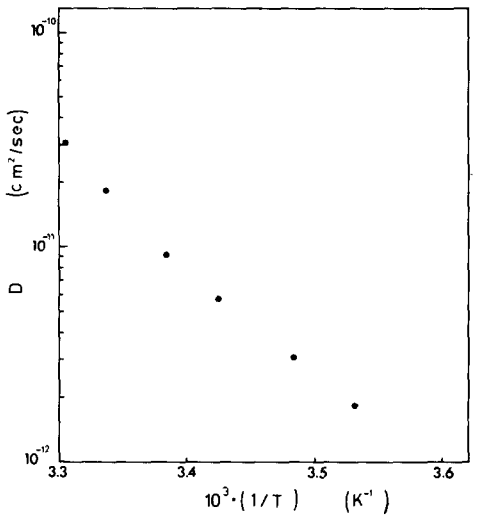

Fig. 3. A plot of the log of the diffusion coefficient as a function of reciprocal temperature.

apparently high activation energy may arise from several contributions to the potential energy barrier for the PS II aggregation process including electrostatic repulsion between charged particles and hydrodynamic interactions.

It may be concluded that within the limitations, an aggregation model can be used to analyze the kinetics of salt-induced chlorophyll fluorescence changes occurring in thylakoid membranes. The treatment therefore gives support to the concept that the salt-induced changes in PS II particle fluorescence yield, spillover of energy from PS II to PS I and associated stacking changes involve lateral protein diffusion.

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