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CONNECTION BETWEEN THE RATE OF COOLING AND FLUORESCENCE PROPERTIES AT 77 K OF ISOLATED CHLOROPLASTS

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Summary

Cooling of chloroplasts to −196°C can under certain circumstances lead to an erroneous analysis of energy distribution. After minimizing influences of sample geometry and effects of plastid concentration it is shown that externally induced membrane change leads to an increase in the ratio F_{740}/F_{687} of the fluorescence emission spectrum. Similar alterations can be observed by variation of the rate of cooling the plastids to 77 K, especially if whole chloroplasts are used. The differences in emission ratios are indicative also of changes in initial energy distribution between the photosystems, given here by the value α_N. This is inferred from experiments with either osmotically induced thylakoid disturbances or those effected through a slow cooling process. The circumstances and the significance of these observations are discussed.

Introduction

Fluorescence measurements at 77 K provide an excellent tool to discriminate between the various pigment-complexes within the light-harvesting apparatus of chloroplasts [1—3]. Such determinations are, however, sensitive to a number of artefacts which tend to affect quantitative parameters if no adequate correction is made [3,4]. In a previous communication [5] I presented evidence that the rate of cooling of the sample to 77 K can induce changes in the physical arrangement of the pigments within the thylakoid membrane which manifests itself as a shift in initial energy distribution between pigment system PS I (fraction α) and PS II (fraction β). This notion was disputed by Butler and Strasser [6] who showed that α and β are seemingly independent of the rate of cooling. They implied that the previously observed effects [5] might be caused

either by reabsorption, differences in scattering between the various types of samples used, alterations in geometry of sample optics or a combination of all of these.

The present paper addresses itself again to the basic question whether cooling can induce membrane alterations and, thus, lead to an erroneous picture of initial energy distribution within the pigment systems. Data ascertaining the notion that changes in the physical state of the membrane are indicated by the fluorescence emission ratios F_{740}/F_{687} are presented first. Next follows an experimental comparison which shows that variations in fluorescence analogous to those induced by changes in hypotonicity of the plastid suspension can also be obtained through variations in the rate of cooling to liquid N_2 temperature. A repetition of the measurements of energy distribution under various circumstances of cooling confirms the earlier interpretation that a definite correlation exists between membrane 'integrity', defined here operationally as identical to the reference state i.e. the membrane state at room temperature, and the actual values of α and β [5]. The data lead to the conclusion that spectral differences as well as alterations in α seen after variation of the cooling rate are in all probability linked to a disturbance of the pigment arrangement within the thylakoid caused by the freezing process.

Material and Methods

Plastids were isolated from spinach. Intact chloroplasts were prepared either according to Nakatani and Barber [7] or according to Leech [8] using a stepwise sucrose gradient (46–50%).

Class I chloroplasts were prepared in the usual manner [9]. The medium contained 0.4 M sucrose, 1 mM $MgCl_2$, if noted, 50 mM NaCl, 10 mM Tricine, pH 8. The suspension was washed once in the same buffer. It's concentration was adjusted to 1 mg chl/ml and stored in the dark at ice temperature.

For preparation of the measuring sample, a piece of cheesecloth was dipped into the suspension and squeezed gently almost to dryness. Chloroplasts adsorbed to the cotton were thus retained while all liquid within the meshwork was removed. This permitted a direct contact between liquid nitrogen and the chloroplasts during subsequent cooling. The chloroplasts-impregnated cheesecloth was stretched and mounted as a double layer on two interlocking polypropylene rings [3]. It was quickly cooled down by immersing it into liquid nitrogen in a vertical position.

The rate of cooling was lowered in two different ways. The above sample type was either frozen in an open tin can swimming on liquid N_2, or the cheesecloth prepared as before was mounted firmly between plexiglas discs. These discs constitute a thermal barrier and through variation of their thickness a differential cooling rate can be achieved without a change in overall optical geometry. For determination of α_N, the chlorophyll content was up to 20 $\mu g \cdot cm^{-2}$ exposed area; in all other experiments it was 1—2 $\mu g \cdot cm^{-2}$ exposed area.

The instrumental setup for the fluorescence measurements was as follows: Excitation light was provided by a tungsten-halogen lamp. The light was filtered through 2 Kg-1 heat absorbing filters, in series with a 450—500 nm special interference filter with steep flanks (Spectro-Film Inc.), and guided

through one arm of a multiple arm fiber optic directly to the sample, resting on the flat translucent bottom of a Dewar flask with liquid nitrogen.

For measurements of emission spectra, the fluorescence was sampled through the second arm of a double arm fiber optic, 360 degrees to the exciting light, and passed through a monochromator to a photomultiplier (Hamamatsu R-361) from whence it was processed in the usual manner [5].

For the determination of α_N a triple arm fiber optic similar to that of Strasser and Butler [10] was used, one arm serving for excitation as described above, the other two to guide the emission to two photomultipliers (Hamamatsu R-361) being shielded by either a 691 nm or a 746 nm interference filter. (Schott-DIL). The signals were fed after suitable amplification and noise suppression into the memory of a Nicolet 1090 A-Explorer digital oscilloscope from whence they were recorded either separately or on a x-y plot against each other. The data obtained are corrected for residual light leakage and photomultiplier sensitivity.

α_N was either calculated according to the equation

$$\alpha_N = 1 - \frac{(F_v/F_m)_{740}}{(F_v/F_m)_{690}}$$

from the individual kinetic traces or extrapolated from the x-y plot exactly as given by Butler and Strasser [6].

Results

The determination of α is based on kinetic measurements in various regions of the chloroplast emission spectrum. The spectrum itself already gives reliable information about energy transfer between pigments under two conditions. The first is, that structural changes leading to alterations in energy transfer to or between fluorescing species are the cause of spectral variations, i.e. all other de-excitation processes competing with fluorescence are unaffected. The second requirement is, that proper corrections for self-absorption and sample geometry, possible only within limits, can be made. Under these circumstances the ratios between the three principal emission bands are a good indicator of the state of photosystem interaction. This notion was first discussed by Govindjee [11] who presented evidence in its favour. The experiments of Murata on membrane changes induced by Mg^{2+} [12] show also connected differences in emission ratios of chloroplasts at liquid N_2 temperature. The magnesium effect is accompanied by changes not only in α and β, giving the initial energy distribution, but also by an alteration of the probability of energy transfer between the photosystems [13]. It provides the first evidence that emission peak ratios and light energy distribution within the pigment systems are connected, if the aforementioned conditions are met.

In order to assess and minimize the influence of self absorption on the spectral quality a series of measurements was taken with samples of increasing chlorophyll content/unit exposed area. The rationale for this experiment is the observation that self-absorption of fluorescence emission tends to increase the ratio of F_{740}/F_{687}, since only the emission at 687 nm is reabsorbed by chlorophyll of neighbouring plastids. This was first shown by Govindjee and Yang

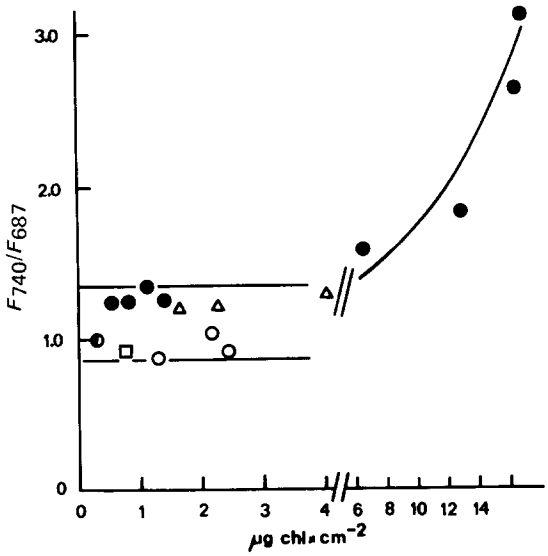

Fig. 1. Effect of plastid concentrations, in μg chlorophyll · cm^{-2} exposed sample area, on the ratio of fluorescence emission F_{740}/F_{687}. Class I chloroplasts, excited at 450—500 nm, quickly frozen within 15 min of preparation. Data of a set of experiments of this type, each denoted by a different symbol, are shown for comparison and for demonstration of the spread between the various preparations (delimitid through the horizontal lines). Every point is the average of three independent measurements during each experiment. The same symbol refers to data from a single experiment.

[14]. The data of Fig. 1 show, that up to 4 μg chlorophyll/cm^2 exposed area the effect of self-absorption among particles is neglegible in the cheesecloth samples used. (There is presently no way to attack the question of reabsorption within a single particle.) Also indicated in the figure is the spread of ratios (F_{740}/F_{687}) obtained with different whole and class I chloroplast preparations. They all center around 1, the value already reported by Govindjee [11] as indicating samples with reduced reabsorption of fluorescence.

The data given in Table I support the notion that the emission ratios, especially F_{740}/F_{687}, might act also as an indicator of membrane 'integrity'. In

TABLE I

INFLUENCE OF HYPOTONICITY ON THE FLUORESCENCE EMISSION RATIOS OF CHLOROPLASTS

Intact chloroplasts according to Leech [8] were diluted 1 : 15 with 0.1 M Tricine buffer, pH 7.8, concentrated by centrifugation and resuspension of the pellet in 0.1 M sucrose/0.1 M Tricine buffer (pH 7.8) and stored for the time indicated at 4°C. Control, 0.24 μg chl · cm^{-2} exposed area; hypotonically suspended probes, 0.2 μg chl · cm^{-2} exposed area.

Minutes in hypotonic medium	F_{740}/F_{687}	F_{740}/F_{697}
Control	1	0.98
15	1.56	1.8-
30	1.62	2.00
45	1.83	2.16
60	2.10	2.80

this experiment intact chloroplasts were broken by resuspension in hypotonic medium and the emission ratios taken at chl concentrations which rule out self-absorption among the plastid particles. The ensuing osmotic alteration of membrane structure, shown earlier by electron microscopy [15], is accompanied by an increase of F_{740}/F_{687} and F_{740}/F_{695}.

It was previously stated [3,5] that a variation in the rate of cooling also induces membrane alterations and thus causes differences in the emission peak ratios. This can be shown in a simple way. If class I chloroplasts, mounted on cheesecloth as described, were fast frozen by direct immersion in liquid nitrogen, values of 1.35 were obtained for the ratio F_{740}/F_{687} and 1.29 for F_{740}/F_{697}. If, however, identical probes of the same plastid preparation at an identical concentration of 1.1 μg chl $\cdot$ cm^{-2} exposed area were allowed to cool slowly by placing them in a tin can swimming on liquid nitrogen, these ratios increased to 1.67 and 1.55, respectively. This experiment is analogous to those reported by Cho and Govindjee [4] for *Chlorella*.

Supporting results also indicating membrane alteration through the cooling process were obtained, if the experiment was varied in the following way. The chloroplast-soaked cheesecloth was placed between plexiglas discs of different thickness. The thicker the plexiglas disc, the slower should be the cooling rate due to the relatively low thermal conductivity. The data of Table II show these marked differences in the emission ratio obtained. This experiment rules out that the observed variations are caused by differences in optical geometry between the cheesecloth and suspension samples used in previous experiments [5] and suggested as explanation [6].

If the peak ratios are indicators of variations in photosystem interaction similar differences should be observed in the measured values of α and β. Table III gives value of α_N, determined for plastid preparation disintegrating either through aging or by osmotic shock [15] through both the geometric method of Butler and Strasser [6] and through calculation from kinetic data (see Materials and Methods). Dense samples of up to 20 μg chl $\cdot$ cm^{-2} exposed area were used in these experiments. In the case of Nakatani-Barber type chloroplasts, prolonged suspension suffices to increase α_N with a simultaneous decline in the degree of intactness. Similarily, a dilution of whole chloroplasts from a stepwise gradient, being suspended in strongly hypertonic medium (50% sucrose), into a hypotonic medium leads to an alteration of structure visible in the loss of intactness and an increase of α_N. The changes in α are small though,

TABLE II

INFLUENCE OF DIFFERENTIAL FREEZING ON EMISSION PEAK RATIOS OF CHLOROPLASTS

The plexiglas cover method was used. The chloroplasts, prepared without addition of $MgCl_2$, were stored several hours before use in the dark. The data, except the 1 mm plexiglass values, are averages of three separate determinations. Chlorophyll concentration was 1 μg/cm^2 exposed area. n.d., not determined.

	F_{740}/F_{687}	F_{740}/F_{697}
Directly frozen	1.82 ± 0.17	1.58 ± 0.04
1 mm plexiglas	2.52 (n.d.)	2.18 (n.d.)
2 mm plexiglas	3.11 ± 0.31	2.17 ± 0.05

TABLE III

VARIATIONS OF α_N UNDER VARIOUS CONDITIONS OF HYPOTONICITY

The α_N values given are averages of three independent determinations. Percent intact chloroplasts were measured with the ferricyanide method of Heber and Santarius [16].

Chloroplast type	Minutes in 0.3 M sorbitol (pH 7.5) *	Minutes after dilution **	α_N	% intact chloroplasts
Nakatani and Barber [7]	20	—	0.617 ± 0.04	84
	140	—	0.672 ± 0.024	0
Leech [8]	—	control	0.603 ± 0.054	100
	—	115	0.721 ± 0.08	0

* Resuspension and storage medium [7].
** As in Table I.

about 10%, compared to the drastic change in intactness, which, however, is greatly influenced by other parameters besides thylakoid structure. It has to be mentioned that this finding should not be confused with the notion of Butler and Strasser [6] that α_N is independent of varations in peak ratios caused by reabsorption among particles. This I could fully confirm in my experiments. I does not imply, however, that variations in membrane structure, contributing to differences in peak ratios in the absence of particle self-absorption, are irrelevant with regard to α_N.

A comparison with variations in the value of α_N induced by decreasing the rate of cooling with the 'tin can' method is given in Table IV. The thylakoid system of whole chloroplasts or good class I plastids gives with the quick freeze method an average value of $\alpha_N = 0.613$. This corresponds to an absolute α value of 0.29 using the approximation $\alpha_N = \alpha/(\alpha + \beta \cdot 0.27)$, where 0.27 represents the average value of the transfer term $\phi_{T(II \to I)(M)}$ as given by Butler and Strasser [6]. The variances between different preparations, indicating physiological differences in the material are very likely due to the inhomogeneity of the leaf

TABLE IV

VARIATION OF α_N INDUCED BY THE RATE OF COOLING

Experiment	Type of chloroplasts	Independent samples/ determination	$\bar{\alpha}_N$ quickly frozen	$\bar{\alpha}_N$ slowly frozen	$\bar{\alpha}_N$ thawed and refrozen
1	intact, Nakatani	3	0.629 ± 0.052	0.758 ± 0.055	0.726 ± 0.071
2	intact, Nakatani	6	0.580 ± 0.082	0.658 ± 0.073	0.671 ± 0.1
3	intact, Leech	5	0.600 ± 0.08	0.742 ± 0.048	—
4	intact, Leech	3	0.646 (n.d.)	0.787 ± 0.076	—
5	intact, Leech	4	0.645 ± 0.08	0.788 ± 0.035	—
6	intact, Leech	6	0.595 ± 0.065	0.772 ± 0.017	—
7	class I *	5	0.640 ± 0.019	0.748 ± 0.025	0.744 ± 0.020
8	class I *	4	0.535 ± 0.041	—	0.679 ± 0.033
9	class I *	5	0.625 ± 0.024	0.705 ± 0.033	—
10	class I *	4	0.634 ± 0.080	0.707 ± 0.07	—
Overall average			0.613 ± 0.036	0.741 ± 0.043	0.705 ± 0.036

* Frozen for measurement within 15 min after preparation.

material used. Table IV shows clearly that the decrease of the cooling rate by the 'tin can method' leads in every circumstance to an increase in α_N. The corresponding α value is 0.42 on average. This result is in strict disagreement with the data presented by Butler and Strasser [6] who found no difference in α_N when varying the rate of cooling.

In some experiments, also incorporated into Table IV, the samples of the 'quick freeze' determination were thawed out slowly, left in a moist chamber in the dark for 90 min and were quickly refrozen. The rationale of the experiments being that membrane alteration occurs during thawing [17] which should show up as an altered α_N in a subsequent measurement. While many samples gave kinetic traces without distinguishable F_0 and F_v states, those which did (about 40%) always gave α_N values of 0.7 in the range of those obtained with slowly cooled samples, again in contrast to the data of Butler and Strasser [6].

Discussion

The above data indicate, first of all, a close connection between the state of the thylakoid membrane and α. They show secondly that under certain circumstances cooling alters the photosystem arrangement within it, probably by physical distortion or even disruption [18]. Thirdly, I infer from the results that the emission ratio F_{740}/F_{687} can serve as an indicator of 'integrity' as defined before.

These conclusions fit well into the present framework of knowledge. The photosynthetic pigment systems comprise large complexes of chlorophyll. The physical arrangement of individual complexes determines the extent and probability of energy transfer. An alteration of the underlying and surrounding membrane matrix leads to a variation of transfer properties. A number of factors acting this way are known, e.g. Mg^{2+} [12], pH [19,20] and light [21–23]. Stresses during cooling to liquid nitrogen temperature also alter membrane properties [24,25]. This can be deduced from extensive cryobiological work on cells and mitochondria [26–29] and should hold true also for the equally complex membrane system of chloroplasts. The data presented above agree with the notion that an effect of the rate of cooling on the probabilities of initial energy distribution between the pigment complexes (i.e. α and β) should be and indeed was noticed.

This aspect was, however, disputed by Butler and Strasser [6]. Their data indicate that the rate of cooling was of no importance to the calculated values of α and β. However, their results are in agreement with the above findings if one assumes that they were taken with chloroplasts whose membrane system was already damaged before freezing, i.e. class II rather than class I plastids. Then, the thylakoid changes induced by cooling are small compared to the extent of damage already present and thus not noticable.

Thus, in conclusion, the experiments presented support the notion that the rate of cooling influences the membrane structure of chloroplasts and consequently the fluorescence parameters at 77 K. The important consequences of this finding have been adequately stated by Butler and Strasser [6].

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