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Effect of Chiral Aggregate of Lutein on Photosynthetic Electron Transport Activity in Spinach Thylakoid, and Interaction between Lutein and Thylakoid Membrane[a)]

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Summary

A herical chiral aggregate of lutein, considered to be an electron conductor, inhibits photosynthetic electron transport (photoreduction) activities in PS I and PS II. Lutein dispersed in thylakoid suspension was incorporated into thylakoid membrane to form the same chiral aggregate as that originally contained in thylakoid. Such a chiral aggregate exhibited different CD patterns from the ordinary one in aqueous system. The residual lutein in the suspension will form the ordinary chiral aggregate which adheres on to the membrane to make up by-path of electron transport. The results indicate an apparent inhibition of photoreduction activities in presence of added lutein.

Introduction

In our earlier reports[7,8,9,10,11,12)] we have elucidated the following facts regarding lutein, a major carotenoid in green leaves, dispersed in aqueous solutions of surfactant or protein. 1) Lutein dispersed in various aqueous solutions loses fine structure and shows appreciable low absorbance, and its absorption maximum (λmax) shifts to shorter wavelength region by about 60 nm. These phenomena indicate formation of a molecular aggregate of lutein larger in size than the wavelength of visible light[4)]. 2) In the same dispersion systems lutein acquires strong CD of split type, whose typical spectra have peak, trough and cross over point at 376, 408 and 386 nm, respectively. When conformation of the molecular aggregate is considered on the basis of optical rotation theory[5)], two transition moments of different lutein molecules in near position are not placed on the same plane and occupy a definite space orientation not parallel with each other. Many chromophores in lutein molecules must associate side-by-side although slightly twisted with respect to each other to form "chiral herical conformation"[10)]. 3) Bradley et al.[3)] suggested that a right handed helix of polyene aggregate with ten of the chromophores in a pitch showed a CD spectrum of split type similar to those observed for lutein. CD spectrum of lutein, furthermore, resembles that of α-helix structure of proteins in terms of pattern. It is deduced from these facts that lutein aggregate shall take right handed herical conformation. 4) Under electron micrograph[13)], lutein aggregate showed a long and slender form.

From the above facts lutein aggregate is deduced to have a possible conformation of so called chiral card pack[13)]. Hydroxyl groups in both terminal ionone rings of lutein are oriented outside the helical structure to increase hydrophilic properties of the aggregate. Inside the aggregate, since conjugated polyenes combine side-by-side, a dipole-dipole interaction between π electrons of adjacent lutein molecules is expected to occur. If this interaction occurs, electrons may easily migrate inside the aggregate. Thus lutein aggregate has the possibility

a) Biochemical Studies on Carotenoids. Part XVII. For Part XVI, see reference (13).
Abbreviations ; Circular Dichroism (CD), Photosystem (PS), 2,6-Dichloroindophenol (DCIP)

of working as electron conductor.

In this paper, it is examined on the basis of the above considerations whether lutein added to spinach thylakoid has effects on photoreduction activity, and further the added lutein interacts with thylakoid.

Materials and Methods

Lutein preparation

Crystalline lutein was prepared from spinach leaves[6].

Preparation of spinach thylakoid[2]

Spinach leaves (70 g) are homogenized with a mixer in 150 ml of 20 mM Tris buffer, pH 7.2, containing 10 mM NaCl and 400 mM sucrose for 15 sec at low speed. The slurry is filtered through four layers of gauze. The filtrate is centrifuged at 1,500 ×g for 7 min. The pellet is suspended in 70 ml of grinding solution and homogenized with a teflon homogenizer. The homogenate is centrifuged at 200 ×g for 1 min. The next centrifugation of the supernatant, at 1,800 ×g for 7 min, collects chloroplasts as a pellet. This pellet is resuspended in 20 mM Tris buffer, pH 7.2, and homogenized with a teflon homogenizer for 30 sec. The homogenate is centrifuged at 20,000 ×g for 20 min to precipitate thylakoid which is rehomogenized in 20 mM Tris buffer, pH 7.2, with teflon homogenizer for 20 sec to obtain thylakoid suspension. All procedures mentioned above were carried out at 0°C.

Determonation of chlorophylls and carotenoids in thylakoid

The chlorophyll content of spinach thylakoid was determined essentially by Arnon's method[1]. Carotenoids in thylakoid were determined by HPLC method[14].

Assays of $NADP^+$ photoreduction and DCIP photoreduction

$NADP^+$ photoreduction by H_2O (TPR) is followed at 340 nm in 3 ml of reaction mixture containing 30 mM Tris buffer, pH 8.3, 40 mM NaCl, 0.17 mM $NADP^+$, saturated amounts of crude spinach ferredoxin and thylakoid suspension of about 40 μM chlorophyll. $NADP^+$ photoreduction by PS I is also followed at 340 nm in 3 ml of reaction mixture containing 70 μM DCIP, 3 mM ascorbate and 10 μM DCMU in addition to components in TPR. PS II activity was assayed by photoreduction of DCIP, which was followed at 600 nm. Three ml of reaction mixture contained 30 mM Tris buffer, pH 7.2, 40 μM DCIP and thylakoid of about 40 μM chlorophyll. Illumination of the reaction mixture was carried out with red light through Toshiba VR 65 filter from a Xenon lamp. These assays were carried out using a Hitachi 356 two-wavelength double beam spectrophotometer at room temperature.

Absorption and CD spectrum

Absorption and CD spectra were measured with a Shimadzu Multipurpose Model 3,000 spectrophotometer, and a JASCO J-500A spectropolarimeter, respectively, at room temperature.

Results

Effect of lutein on photoreduction activity in spinach thylakoid

Lutein when added with spinach thylakoid inhibits photoreduction activities of the thylakoid. One example shown in Fig. 1 is time courses of the activity of TPR in presence of lutein. In TPR or PS I, photoreduction activity was not inhibited by lutein below 10 μM, but began to be inhibited above this lutein concentration to reach finally to about 50% inhibition at 40 μM of lutein. In PS II, photoreduction activity of DCIP was inhibited at low concentration of lutein to reach about 70% inhibition at 40 μM (Fig. 2). But the extent of this inhibition varied according to the ratios between concentrations of thylakoid, DCIP and lutein.

CD of thylakoid

In order to search a mode of the inhibitory effects of lutein on photoreduction activity, spectroscopy should be used to examine any interaction between thylakoid and lutein. Thylakoid suspension, immediately after preparation, showed CD spectra which have four troughs at

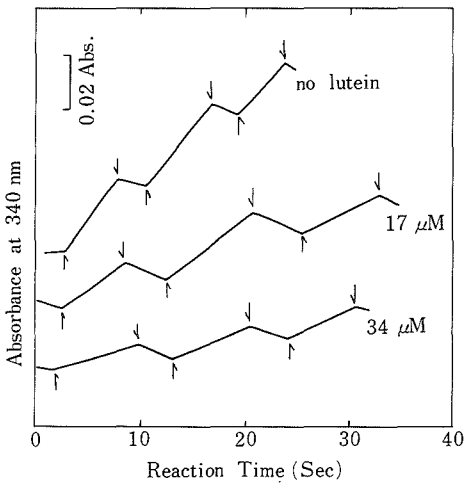

Fig. 1 Effect of lutein on photosynthetic $NADP^+$ reaction activity of spinach thylakoid.
Chl. (thylakoid) conc., 40 μM. Arrows indicate "ON" ↑ and "OFF" ↓ of illumination switch. 0.2 ml of lutein ethanol solution is added into 2.8 ml of reaction mixture at the end of addition.

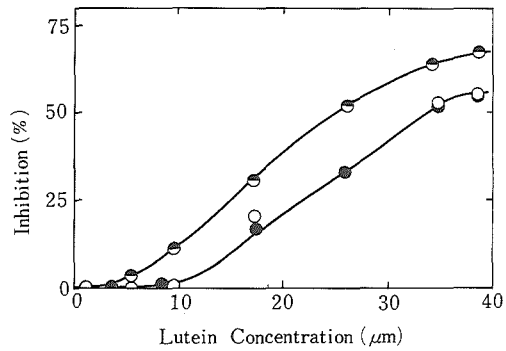

Fig. 2 Effects of lutein concentrations on photoreduction activities in three photosystems.
Chl. conc., 35 μM. TPR, ○—○; PS I, ●—●; PS II, ◐—◐.

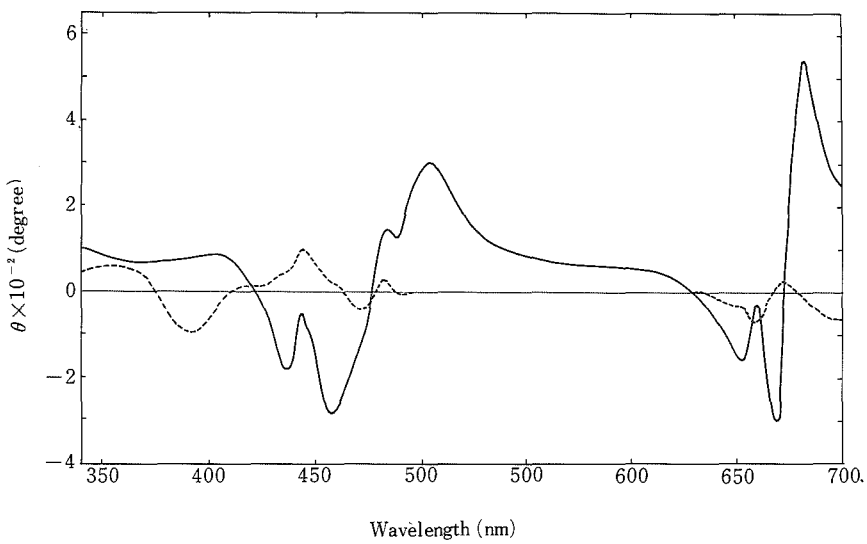

Fig. 3 CD spectra of spinach thylakoid dispersed in 29 mM Tris buffer, pH 7.2.
Solid line, thylakoid immediately after preparation. Dotted line, thylakoid on standing for 3 days at 0°C after preparation. Chl. conc., 37.5 μM.

435, 457, 655 and 670 nm, and two major peaks at 505 and 690 nm depending on chlorophylls and carotenoids (Fig.·3). When thylakoid is allowed to stand at 1°C for 2 days or at 25°C and above for some hours, however, CD spectrum patterns change drastically to show very low ellipticities especially of both peaks at 690 and 505 nm. During this time, CD spectrum pattern of thylakoid suspension in the range of 320 to 400 nm is modified to be similar to the ordinary CD pattern of lutein aggregate[10]. The molar ellipticities ((θ)) of some of the peaks and troughs calculated on the basis of lutein quantities in thylakoid (Table 1) were much

Table 1 Photosynthetic pigments in spinach thylakoid

Pigment	Concentration in thylakoid suspension*1	Concentration in reaction mixture
Total chlorophyll	266.0 μg/ml	35.0 μM
β-carotene	2.9	0.64
Lutein	4.1	0.83
Antheraxanthin	0.6	0.13
Violaxanthin	1.8	0.35
Neoxanthin	0.4	0.09

*1 One example of analyses in thylakoid pigments at an arbitrary concentration.

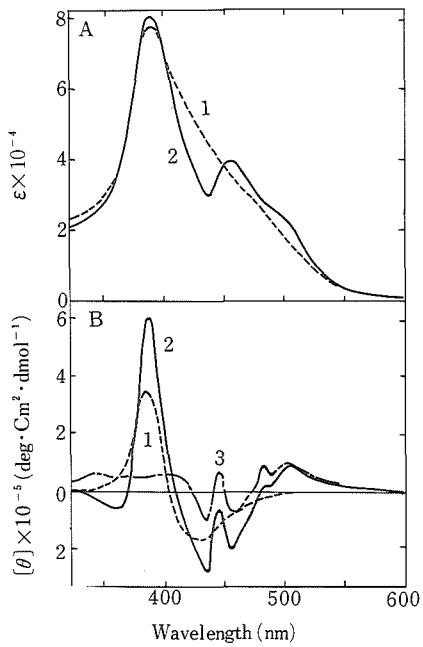

Fig. 4 Differential absorption and CD spectra of lutein in thylakoid suspension.
Chl. conc., 40 μM. Lutein, 25 μM. Curve 1, lutein in 20 mM. Tris buffer, pH 7.2. Curve 2, lutein in thylakoid suspension in the above buffer. Curve 3, thylakoid suspension in the above buffer. At differential absorbance determination, reference cell contains thylakoid suspension.

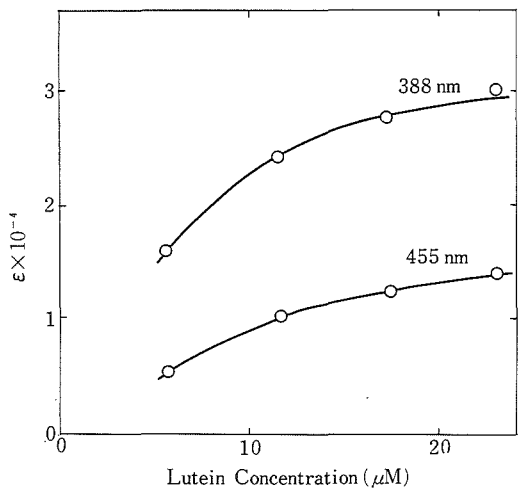

Fig. 5 Effect of lutein concentration on molar extinction in the presence of thylakoid.
Chl. conc., 35 μM.

larger than those of lutein aggregate so far obtained in aqueous systems (Fig. 9). The above results show that carotenoids and chlorophylls in thylakoid membrane in vitro are considerably in an unstable state. Therefore, it is very important to establish that lutein exists in thylakoid as a chiral aggregate.

Interaction of lutein aggregate with thylakoid

Effects of lutein on absorption and CD spectra of spinach thylakoid were examined with fresh thylakoid with attention to treatment conditions mentioned above. Differential absorption and CD spectra of reaction mixtures for determination of photoreduction activity containing 25 μM of lutein are shown in Fig. 4. As both absorption and CD spectra of reaction mixtures containing lutein were almost the same as those of simple mixtures composed of only thylakoid and lutein, the latter simple mixtures were used in all subsequent experiments. Differential absorption spectra (Fig. 4A) of lutein added into thylakoid suspension indicates a clear new peak at 455 nm besides the usual peak at 388 nm, while the CD spectra of lutein shows the usual peak at 385 nm of the aggregate in addition to both peaks and troughs observed in thylakoid suspension. However, the usual CD trough of lutein observed in aqueous systems from 397 to 405 nm becomes obscure for the trough seems to be combined with that of thylakoid at 435 nm. The molar ellipticities of troughs at 435 and 457 nm observed in case of thylakoid only become large with addition of lutein (Fig. 4B).

Effects of added lutein quantities on both molar extinction (ε) and molar ellipticity (θ) are shown in Fig. 5 and 6, respectively. For calculation of both these quantities, total lutein content, namely that in thylakoid and added lutein, was taken into consideration. Molar extinctions at both 388 and 455 nm decreased with decrease of ratios of added lutein to thylakoid

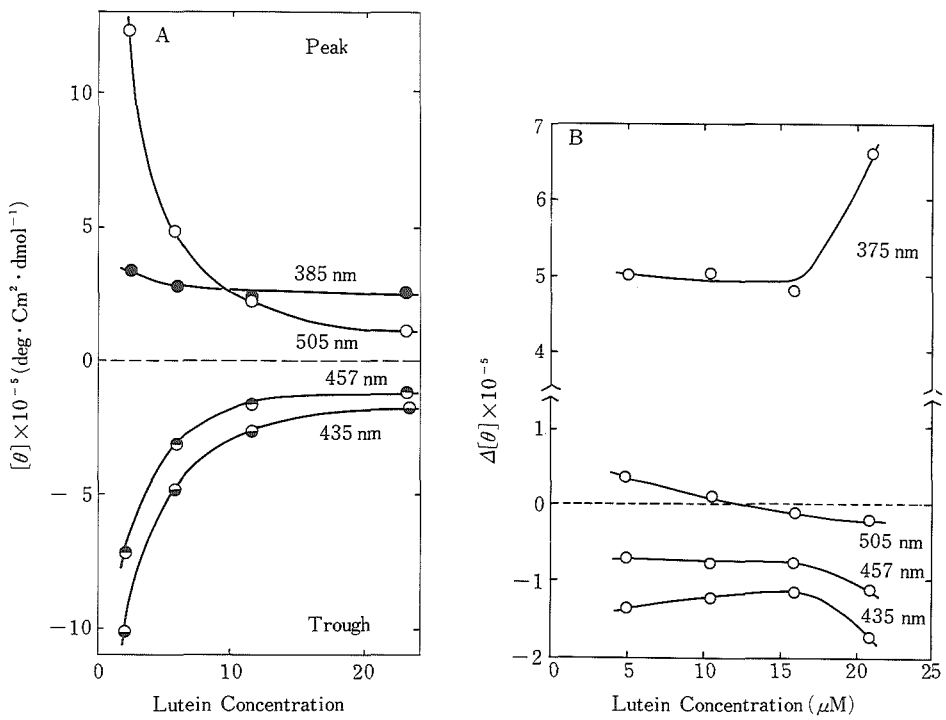

Fig. 6 Effect of lutein concentrations on molar ellipticities (A) and differential molar ellipticities (B) in the presence of thylakoid.
Chl. conc., 35 μM.

(Fig. 5 and 8A). Fig. 6 describes variations of molar ellipticities of both peaks and troughs of added lutein. Values of (θ), except for that in 385 nm, are much large in the low concentrations, because the values depend strongly on thylakoid. When lutein more than 10 μM is added to thylakoid suspension containing 35 μM chlorophyll, values of (θ) at each wavelength falls to almost a constant value. Lutein begins to inhibit photoreduction activities of TPS and PS I above 10 μM. The increase of CD intensities at both 435 and 457 nm in the thylakoid suspension resulting from addition of lutein are evident from a plot (Fig. 6B) of differential molar ellipticities $(\Delta(\theta))$, obtained from differences of (θ) of thylakoid suspension in the presence and absence of lutein against lutein concentration. Increase of lutein concentration up to 15 μM does not result in change of $\Delta(\theta)$ at 375, 435 and 457 nm. This fact indicates that ellipticities, not (θ), increase in proportion to added lutein quantities. Because above 15 μM of lutein, introduction of lutein into thylakoid is saturated, residual quantities of added lutein will form the ordinary chiral aggregate[10] in aqueous system to increase $\Delta(\theta)$ of the peak and the troughs at the three wavelengths. On the other hand, $\Delta(\theta)$ of peak at 505 nm was little affected by lutein concentration. This indicates that CD peak at 505 nm probably

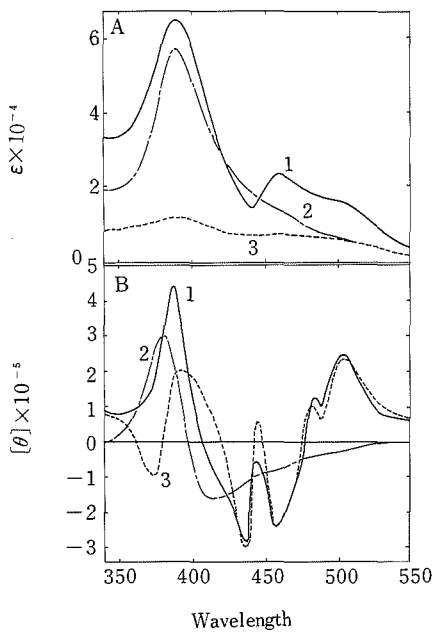

Fig. 7 Differential absorption and CD spectra of lutein dispersed in thylakoid suspension in 20 mM Tris buffer, pH 7.2.
Chl. conc., 36.7 μM. Lutein added, 10 μM.
Curve 1, lutein in thylakoid suspension.
Curve 2, supernatant after centrifugation at 8,000 rpm for 30 min.
Curve 3, precipitate resuspended in the same buffer.

Table 2 Lutein Quantities binding to Thylakoid

Lutein added	Lutein binding to thylakoid*
7.3 μM	2.7 μM
14.6	4.2
29.2	6.7

* Total chlorophyll, 36.7 μM.

arises from pigments other than lutein, for example chlorophylls.

Next lutein quantities bound to thylakoid was determined, because added lutein interacts with thylakoid. When reaction mixture was centrifuged at 8,000 rpm for 30 min to remove thylakoid, differential absorption spectra of supernatant lost the sub-peak at 455 nm which appeared only in the presence of thylakoid (Fig. 4), and further absorbance of the main peak at 388 nm exhibited slight decrease (Fig. 7A). Lutein quantities bound to thylakoid were obtained from this decrease of absorbance at 388 nm after removing thylakoid (Table 2). Evidently added lutein binds to thylakoid in proportion to the lutein concentration. In CD spectra, the supernatant shows ordinary pattern of lutein aggregate in aqueous system which has peak at 376 nm and one slightly broad trough at 408 nm (Fig. 7B, curve 2). The precipitated suspension in 20 mM Tris buffer, pH 7.2, shows a specific CD spectrum which has a trough at 375 nm and a peak at 392 nm other than those shown in thylakoid (Fig. 7B, curve 3). This phenomenon indicates that lutein bound to thylakoid forms a different chiral aggregate from the ordinary one by interaction with thylakoid membrane, and an aliquot of added lutein forms a chiral aggregate similar to that of inherent lutein in thylakoid. Further, differential absorption spectra and CD spectra of thylakoid suspension in the presence of lutein were examined at various thylakoid concentrations (Fig. 8). These cases like the results

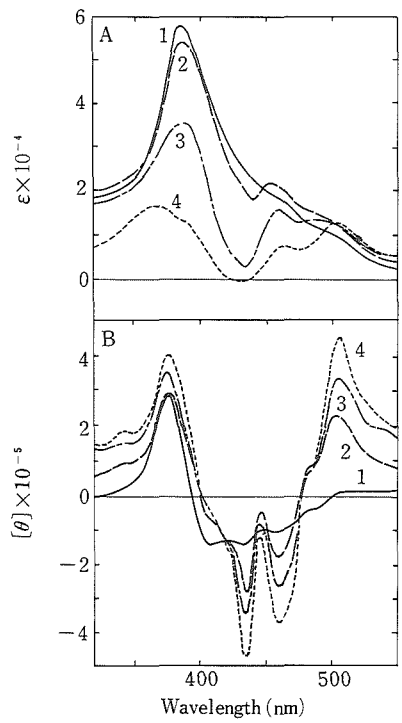

Fig. 8 Variation of absorption and CD spectrum patterns of thylakoid suspensions with different concentrations in the presence of lutein.
Lutein, 13.2 μM. Chl. conc., 1) 7.3 ; 2) 36.5 ; 3) 54.8 ; 4) 73.8 μM, respectively.

included in Fig. 4 give rise to (θ) of peak at 376 nm, depending on ordinary lutein aggregate, which has tendency to increase with thylakoid concentration in spite of constant concentration of added lutein (13.2 μM). Molar ellipticities of the other troughs at both 435 and 457 nm and peak at 505 nm increased in proportion to thylakoid concentration (Fig. 8B). In differ-

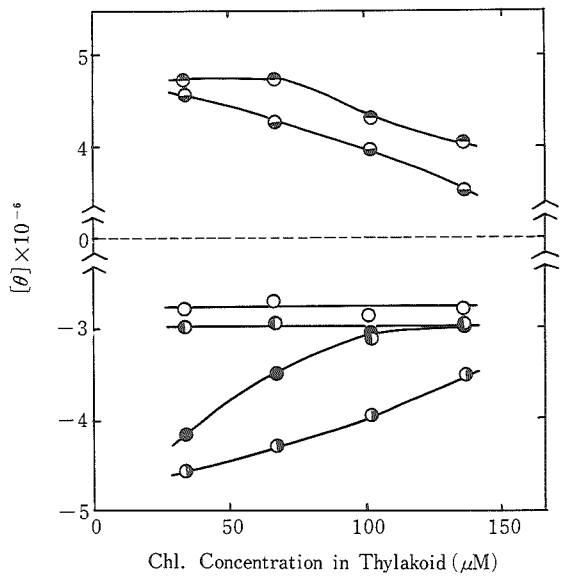

Fig. 9 Effect of thylakoid concentrations on molar ellipticities of lutein and other pigments in thylakoid, which are calculated on the basis of lutein quantities originally contained in thylakoid under presence or absence of added lutein.
Lutein conc., 13.2 μM. (θ) at 435 nm (trough) under presence and absence of lutein, ●——● and ○——○, respectively. (θ) at 475 nm (trough) under presence and absence of lutein, ◑——◑ and ◐——◐, respectively. (θ) at 505 nm (peak) under presence and absence of lutein, ⊖——⊖ and ⊖——⊖, respectively.

ential absorption spectra, other characteristic besided appearance of sub-peak at 455 nm are appearance of peak at 505 nm and a remarkable lowering of the absorbance at 388 nm in high concentration of thylakoid (Fig. 8A). These results may also indicate that lutein strongly interacts with thylakoid on its surface or in the inner site of its membrane.

In Fig. 9, (θ) at 435, 475 and 505 nm calculated on the basis of lutein concentrations (Table 1) originally contained in thylakoid were plotted against concentrations of thylakoid with or without addition of lutein. When thylakoid concentration was increased, (θ) of troughs at both 435 and 475 nm were almost constant in the absence of added lutein, while the intensities began to decrease gradually on addition of lutein (10.6 μM). This means that the effect of added lutein on thylakoid is weakened by increasing thylakoid concentration. Molar ellipticity of peak at 505 nm showed the same tendency of decreasing in both cases whether lutein was added or not. Thus, it is also considered that the peak at 505 nm will not be derived from lutein.

Discussion

Electron transport activities of PS in spinach thylakoid are inhibited by added lutein (Fig. 1 and 2). It is considered that this inhibition is responsible for a binding of lutein to thylakoid membrane surface followed by interference of the electron transport. It has been established by the author that lutein dispersed in aqueous systems formed a chiral aggregate with helical structure to act as an electron transport substance[11,12]. According to this point of view the inhibition of electron transport activity will cause adhesion of excess residual lutein on

thylakoid membrane to make up by-path of electron for $NADP^+$ (PS I) or DCIP (PS II).

Addition of lutein strongly affects absorption and CD spectrum patterns of thylakoid (Fig. 3), and at the same time those patterns of added lutein itself are also strongly modified (Fig. 4). The result, where ε of lutein decreases in the presence of thylakoid, means that excitation of π electrons of lutein is depressed by interaction with thylakoid membrane. Increase of CD intensities of troughs at both 435 and 457 nm, further, may indicate that an aliquot of added lutein is introduced into thylakoid to form a chiral aggregate different from ordinary one obtained in aqueous systems. Values of (θ) at 385, 435, 457 and 505 nm, though increased initially with added lutein, tend to a limiting value at each of the wavelengths (Fig. 6A). This is because of use of total lutein concentration for computation of (θ) and secondly once lutein quantity in thylakoid reaches a point of saturation at about 10 μM of lutein, no further change in (θ) occurs. Thus residual lutein molecules, a form of ordinary chiral aggregate, adhering to thylakoid surfaces inhibit electron transport activities (Fig. 2) by working its by-path.

Differential (θ) of troughs at 435 and 457 nm are almost constant up to about 15 μM lutein followed by increase of CD intensities with further increase of lutein concentrations (Fig. 6B). This suggests that lutein introduced into thylakoid acts together with lutein in thylakoid which makes up an inherent chiral aggregate of different conformation from that already observed in aqueous systems[10,12].

Further, because differential absorption spectral patterns of lutein aggregate under the presence of thylakoid are deformed (Fig. 8A), it is found that lutein strongly interacts with thylakoid on surface or at inner site of the membrane, and excitation of its π electrons is much repressed by the interaction.

It may be concluded from the facts above that lutein originally contained in thylakoid may possibly form a chiral aggregate, and luteins introduced into thylakoid will also form the same chiral aggregate. Thus it should be suggested that an inhibition of photoreduction activities in case of addition of lutein shown in Fig. 2 occurs by making up a by-path of electron transport on thylakoid membrane with lutein aggregates. Function and role of a chiral aggregate originally existent in thylakoid, however are not still obscure.

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References

1) Arnon D. I. : Plant Physiol. **24**, 1−15 (1949).
2) Barr R., M. D. Henninger, and F. L. Crane : Plant Physiol. **42**, 1246−1254 (1967).
3) Bradley D. F., I. Tinoco JR. and R. W. Woody : Biopolymers **1**, 239−267 (1963).
4) Duysens L. N. M. : Biochim. et Biophys Acta. **18**, 1−12 (1956).
5) Imabori K. : Protein, Nucleic Acid and Enzyme. **13**, 502−513 (1968) Kyoritsu Publish. Tokyo.
6) Takagi S. : Sci. Rep. Fac. Agr. Okayama Univ. **44**, 44−46 (1974).

7) TAKAGI S., M. SHIROISHI and T. TAKAGI: Agric. Biol. Chem. **44**, 2111−2117 (1980).
8) TAKAGI S., M. SHIROISHI and T. TAKAGI: Agric. Biol. Chem. **45**, 1159−1165 (1981).
9) TAKAGI S., K. TAKEDA and T. TAKAGI: Agric. Biol. Chem. **46**, 399−404 (1982).
10) TAKAGI S., K. TAKEDA, K. KAMEYAMA and T. TAKAGI: Agric. Biol. Chem. **46**, 2035−2040 (1982).
11) TAKAGI S., K. TAKEDA and M. SHIROISHI: Agric. Biol. Chem. **46**, 2217−2222 (1982).
12) TAKAGI S., and K. TAKEDA: Agric. Biol. Chem. **47**, 1435−1440 (1983).
13) TAKAGI S., T. YOSHIDA and T. TAKAGI: Sci. Rep. Agr. Okayama Univ. **65**, 47−52 (1985).
14) TAKAGI S.: Agric. Biol. Chem. **49**, 1211−1213 (1985).

ルテインのキラルな集合体がホウレンソウチラコイドの電子伝達活性に及ぼす影響とチラコイド膜との相互作用

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緑葉主要カロチノイドの1つのルテインが水系においてキラルな集合体を形成していることをすでに明らかにしている。集合体は3，3′水酸基を外側に出して親水性を獲得し，内部には共役ポリエンがややずれた形で腹と腹を接合させ充填された形となり，共役π電子間の双極子一双極子相互作用により集合体内部でπ電子の移動が容易におこり光合成系における電子伝導体として働く可能性があることを示して来ている。

ホウレンソウチラコイド懸濁液に外部から加えたルテインの一部はチラコイド内部に導入されてチラコイド内に「もとから在るキラル集合体」に組入れられる。残部のルテインは懸濁液中で通常の水系におけるキラル集合体を形成し，多分チラコイド膜に付着して電子伝達の by-path を作っている。添加したルテインはチラコイド膜と強い相互作用を示すものの，チラコイド内にはもとからルテインの充分量があるため低濃度添加では PS I 及び PS II 活性に影響せず，10 μM 以上のルテイン高濃度で膜表面への付着集合体による電子伝達の by-path 形成によって電子が系外へ流出するために活性阻害が観察される。