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Oku, Tatsuo
Laboratory of Biophysics, Department of Agriculture, Kyushu University

Tomita, Giiti
Laboratory of Biophysics, Department of Agriculture, Kyushu University

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Effects of nucleotides on the Hill reaction in aged chloroplasts

Tatsuo OKU and Giiti TOMITA*

Introducton

It is known that the aging of chloroplasts causes various changes in photosynthetic systems. Ke[13] reported that the transient absorption changes at 430 mμ in aged chloroplasts were induced by the flash illumination of red light. Witt et *al.*[27] studied the light-induced absorption changes at 705 mμ in aged chloroplasts, under the coupling of the photo-oxidation of ascorbate with phenazine methosulphate.

These studies are concerned with the optical properties of the photosynthetic pigments involved in the photochemical reaction system I

On the other hand, the aging by the storage of spinach chloroplasts *in* vitro at low temperature is known to bring about changes in the photochemical and physicochemical properties which are closely related to the photochemical reaction system II.

Vernon and Zaugg[25] demonstrated that aged chloroplasts from spinach leaves lost the ability for NADP photoreduction with water and the ability lost could be recovered by the presence of dichlorophenol indophenol and excess ascorbate.

The explanation for the loss of Hill activity was given by the release of unsaturated fatty acids from chloroplast membrane, during the aging process, by the hydrogenation of endogenous galactolipase.[15]

Avron *et al.*[4] and Stiller and Vennesland[23] reported that adenosine triphosphate (ATP), a product of photophosphorylation, depressed Hill activity of freshly prepared chloroplasts in an unknown manner.

We found that the presence of the suitable amount of an unsaturated fatty acid can eliminate the inhibition effect of ATP on the Hill reaction? This finding indicates the existence of the interference between the inhibition mechanisms of the Hill reaction by ATP and by an unsaturated fatty

* Institute of Biophysics, Faculty of Agriculture, Kyushu University, Fukuoka.

acid. This kind of interference seems to be a very important factor for Hill activity of aged chloroplasts.

The present investigation deals with the effects of various nucleotides on Hill activity of spinach chloroplasts aged for various periods in order to make clear the above-mentioned interference.

In the case of chloroplasts isolated from bean leaves, Hill activity is more rapidly lost by aging than in spinach chloroplasts.[1,14] For this reason, the effect of nucleotides on Hill activity of aged bean chloroplasts was also examined, comparing with that of aged spinach chloroplasts.

Experimental

Once-washed chloroplasts were prepared from spinach (*Spinacea oleracea* L.) and bean (*Phaseolus vulgaris* L. cv. EDOGAWA) leaves according to the usual procedure, using 0.4 M sucrose-O.01 M NaCl-0.025 M Tris-HCl buffer at pH 8.0 for homogenizing and washing.[5]

Spinach leaves were purchased from a local market. Bean leaves were grown in the greenhouse. In order to obtain aged chloroplasts, chloroplasts suspended in 0.035 M NaCl-0.003 M Tris-HCl buffer at pH 7.8 were stored at 4" in the dark and shaked occasionally.

The suspension was adjusted to 0.4 mg chlorophyll per ml. The reaction mixture for the measurement of Hill activity contained the following per 3 ml : KCl, 60 μmoles ; $MgCl_2$, 10 μmoles ; potassium ferricyanide, 4 μmoles ; Tris-HCl buffer, 100-200 μmoles to keep pH constant (7.8) ; chloroplasts equivalent to 40 μg of chlorophyll (obtained by the dilution of the chloroplast suspension with NaCl-Tris buffer after the desired aging time). The reaction was performed in a fused quartz cell with the dimension of 1 x 1 x 5 cm^3 at 20° ± 0.50. The reaction mixture was illuminated with a 300 W tungsten lamp through a water filter. Light intensity was approximately 80,000 lux. The reduced ferricyanide as the measure of Hill activity was determined spectrophotometrically at 510 mμ with o-phenanthroline according to the method of Avron and Shavit.[6] The concentration of chlorophyll was determined by the Arnon's method.")

The extraction of lipids was made as follows. The chloroplast suspension stored at 4" for 144 hrs was centrifuged at 20,000 × g for 20 min. Acetone was added to the supernatant solution to the final concentration of 80 %. After stirring, the mixture was centrifuged. The lipids in aqueous acetone solution were transferred to petroleum ether (b.p. 30~60°) and the organic solvent was evaporated in vacuum. The dried lipids were dissolved in a small volume of ethyl alcohol.

Results

Aging of spinach chloroplasts was made by the storage in the dark at low temperature, which brought about the decrease of Hill activity.

The variation of Hill activity with aging time is shown in Table 1, the second column. It can be seen that Hill activity decreases gradually with the increase of aging time in the region of short aging times but over 74 hrs rapidly decreases. Hill activity of chloroplasts aged for 145 hrs is about five per cent of that of fresh chloroplasts. In order to find the effect of ATP on Hill activity of chloroplasts at various aging periods, 5 μmoles of ATP were added to the chloroplast suspension after the aging treatment. Hill activity with the addition of ATP is given in the last column.

Table 1. Variation of ATP effect on Hill activity of spinach chloroplasts with aging time.

Aging time	Hill activity*	
(Hour)	$A^{(-)}$	$A^{(+)}$
0	303.2	146.4
51	254.3	186.8
74	226.8	195.8
101	44.4	94.0
121	28.8	81.0
145	13.6	50.9

* Expressed by μmoles of reduced ferricyanide/mg chlorophyll/hr. $A^{(+)}$ and $A^{(-)}$, Hill activities with and without addition of ATP (5 μmoles), respectively.

The drop of Hill activity of fresh chloroplasts by the addition of ATP has been reported by Avron *et al.*[4] and Stiller and Vennesland.[23]

The results in Table 1 tell us that, at shorter aging time, ATP behaves as an inhibitor for the Hill reaction but, at longer aging time, inversely as a stimulator. This situation can be clearly shown by a plot of $A^{(-)}$ (Hill activity without addition of ATP) $-A^{(+)}$ (Hill activity with addition of ATP) against aging time (Fig. 1).

The inhibition effect gradually decreases with the increase of aging time and finally increases to the saturation value of zero after passing through the minimum inhibition which appears at about 125 hr aging. The negative inhibition means the stimulation.

The saturation at the long aging time in $A^{(-)}-A^{(+)}$ *vs.* aging time curve

implys that the complete loss of Hill activity by aging can give no room for the stimulation effect of ATP.

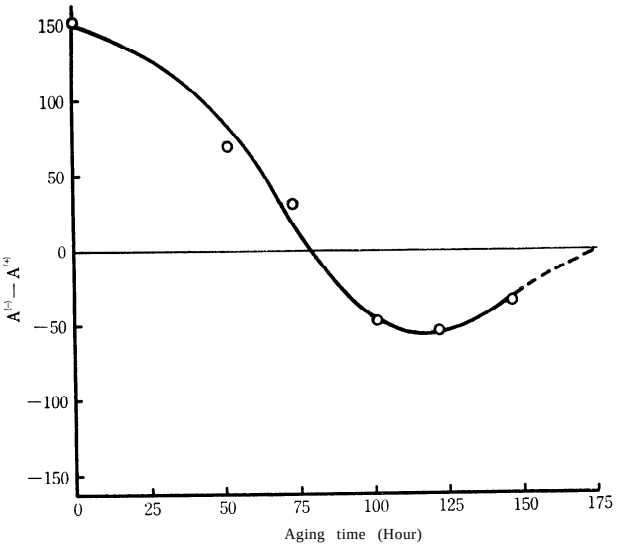

Fig. 1. Differences between Hill activities with and without addition of ATP as a function of aging time. $A^{(+)}$ and $A^{(-)}$, Hill activities with and without addition of ATP (5 μmoles), respectively.

Hill activity of aged chloroplasts increases with the increase of the concentration of ATP added to the reaction mixture so far as the ATP concentration is not exceedingly high. Furthermore, the ATP effect on Hill activity is not influenced by the presence of NH_4^+ ions which are known as an uncoupler of photophosphorylation and an inhibitor of ATPase. These results are shown in Table 2.

Table 2. Effect of concentration of ATP on Hill activity and effect of NH_4^+ ions.

Addition (μmole)	Hill activity*
None	13.59
ATP 2	15.98
〃 5	33.96
〃 8	50.91
〃 5 + NH_4Cl 45	33.96

* Expressed by μmoles of reduced ferricyanide/mg chlorophyll/hr. Chloroplasts were aged for 145 hrs.

As reported by many workers, chloroplasts prepared from bean leaves more rapidly lose Hill activity by the aging treatment than spinach chloroplasts. The aging for 6 hrs diminishes Hill activity to only 13 per cent of that of fresh chloroplasts. However, when ATP is added to the fresh and aged chloroplast suspension, Hill activity remarkably increases. The stimulation by ATP observed in fresh bean chloroplasts suggests that Hill activity is lost partly during the preparation of chloroplasts from bean leaves and recovered by the addition of ATP. The results are given in **Table 3.**

Table 3. Effect of ATP on Hill activity of fresh and aged bean chloroplasts.

Sample	Addition	Hill activity*	Stimulation (%)
Fresh chloroplasts	None	138.13	
	ATP	174.96	21.1
Aged chloroplasts	None	31.98	
	ATP	51.18	63.2

* Expressed by μmoles of reduced ferricyanide/mg chlorophyll/hr. Chloroplasts were aged for 6 hrs. Concentration of ATP, 6 μmoles.

Adenosine monophosphate (AMP) and adenosine diphosphate (ADP) also have the same stimulation effect as ATP. However, the degree of the effectiveness decreases in the order of ATP>ADP>AMP. Adenosine has no effect of the stimulation. The per cent of stimulation of Hill activity of aged spinach chloroplasts by the addition of various nucleotides is given in **Table 4.**

Table 4. Stimulation of Hill activity of aged spinach chloroplasts by the addition of various nucleotides.

Nucleotide	Hill activity*	Stimulation (%)
None	34.2	
Adenosine	34.2	0
AMP	58.8	71.3
ADP	76.8	124.6
ATP	117.0	342.1

* Expressed by μmoles of reduced ferricyanide/mg chlorophyll/hr. Chloroplasts were aged for 123 hrs. Concentrations of each nucleotide, 6 μmoles, respectively,

Hill activity is inhibited by the presence. of fatty acid. This inhibition is highly eliminated when chloroplasts are washed and fatty acids are removed. This suggests that the decrease of Hill activity may be derived from the adsorption of lipids or fatty acids on lamellae membrane.

An attempt was made to make clear the action of nucleotides on the stimulation of Hill activity. In order to find the effect of lipids on Hill activity, the lipid extract obtained from the 121 hr-aged chloroplast suspension was added to the fresh chloroplast suspension. The addition of lipids causes a large loss of Hill activity. However, when ATP or ADP is present, the inhibition effect of lipids is highly weakened (Table 5).

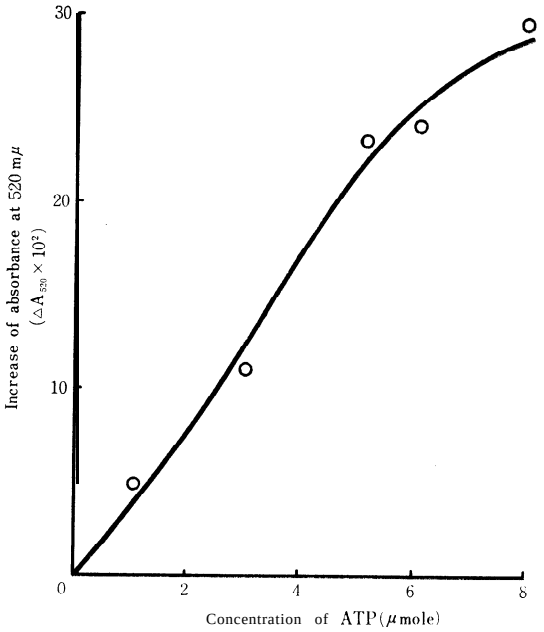

Fig. 2. Increase of absorbance at 520 mμ of lipid extract with concentration of ATP. The medium contains the following per 3.5 ml : KCl, 25 mM ; Tris-HCl buffer, pH 7.8, 0.04 M ; lipid extract, 0.2 mg.

The direct interaction between the lipids extracted from the chloroplast suspension and ATP was examined to clarify the mechanism of the action of nucleotides on aged or lipid-treated chloroplasts.

Upon the addition of lipids dissolved in ethyl alcohol to the medium containing KC1 and Tris-HCl buffer, the solution becomes turbid. The

absorbance at 520 mμ was measured as the measure of turbidity. As seen in Fig. 2, the absorbance increases with the increase of the concentration of ATP. This implys that the degree of the dispersion of lipids in the solution is lowered by the addition of ATP, owing to the formation of the colloidal particles.

Therefore, when nucleotides are added to the aged or lipid-treated chloroplasts, lipids adsorbed on lamellae membrane may be released to some extent into the medium as the colloidal particles. This is due to the mutual interaction between nucleotide and lipid. This situation may explain the restoration of Hill activity.

Table 5. Hill activity of lipid-treated chloroplasts in the presence of nuclotides.

Addition	Hill activity*
None	228.3
Lipid extract	8.5
Lipid extract + ADP	133.2
Lipid extract + ATP	112.0

* Expressed by μmoles of reduced ferricyanide/mg chlorophyll/hr. Lipid extract obtained from the 121 hr-aged chloroplast suspension was dissolved in a small volume of ethyl alcohol, and its suitable amount was added to the reaction medium. Concentrations of ADP and ATP, 6 μmoles, respectively.

Discussion

Photochemical reaction system II associated with the Hill reaction is sensitive to the chemical or physical treatment of chloroplasts,[7-9,17] whereas photochemical reaction system I associated with the electron flow is comparatively stable. When the chloroplast suspension is stored at 4" in the dark, lipids are liberated from chloroplasts and the concentration in the suspension medium increases with the increase of aging time, and the ability for oxygen-evolution decreases.

McCarty and Jagendorf[15] found the appearance of a free unsaturated fatty acid (linolenic acid) in the chloroplast suspension medium, during the aging process, with the endogenous enzymatic reaction.

The photosynthetic apparatus needs the participation of fatty acids as the essential components for the appearance of its activity.[2,10,24,25]

The effects of fatty acids on photosynthetic activities and conformation changes of chloroplasts or algal cells have been studied by various

workers. Fatty acids, when added from outside, are known to behave as an inhibitor for photosynthetic reactions such as the Hill reaction,[12] photophosphorylation[22,23] and carbon cycle reactions.[22] A light-induced increase in light scattering is caused by the addition of saturated fatty acids (lipoic acid, octanoic acid and methyl octanoate) to Chlorella cells.[11] The washing of the cells diminishes the light scattering. Chloroplasts treated with an unsaturated fatty acid also lose Hill activity. However, when washed, the activity recovers to some extent. The degree of the recovery depends on the concentration of an unsaturated fatty acid used? From the absorbance changes at 520 mμ in the chloroplast suspension, isolated chloroplasts are known to swell by the presence of an unsaturated fatty acid (oleate).[16]

In the present investigation, we found the considerable degree of the restoration of Hill activity of aged chloroplasts under the influence of nucleotides such as AMP, ADP and ATP.

Packer et al.[19~21] have reported that the presence of **ATP** is necessary as the energy source for yielding the reversible light-scattering responses in aged chloroplasts. In the present case, however, we can not consider that nucleotides added act as the energy source needed to restore Hill activity, since the increase of inorganic phosphate in the reaction medium can not be observed during the Hill reaction and NH_4^+ ions are not effective for the stimulation or inhibition of the Hill reaction, as shown in Table 4.

The addition of the lipid extract obtained from the aged chloroplast suspension to the fresh chloroplast suspension results in the remarkable inhibition of Hill activity. The presence of ADP or ATP diminishes the inhibition effect of the lipid extract as seen in Table 5. The buffer solution of lipids extracted from the chloroplast suspension becomes turbid in the presence of ATP as seen in Fig. 2.

These facts lead to the following conclusion. Chloroplasts lose their Hill activity with aging time. The loss of Hill activity may be mainly derived from the liberation of lipids from chloroplasts and the adsorption of free lipids on chloroplast lamellae. The addition of nucleotides to the aged chloroplast suspension may cause the removal of lipids adsorbed on lamellae membrane by the mutual interaction between nucleotide and lipid, leading to the stabilization of lamellae membrane as in the native state.

The stabilization of lamellae membrane may give rise to the restoration of Hill activity. This may be the main reason why nucleotides behave as an stimulator for chloroplasts aged for the long period or lipid-treated chloroplasts. The order of the effectiveness of the stimulation (ATP$>$ ADP$>$AMP) suggests that the negative charge in nucleotide molecule is essential to the interaction between nucleotide and fatty acid. The direct interaction of nucleotide with lamellae membrane may destruct the membrane structure and may induce the conformation changes in lamellae

structure. This mechanism may explain the inhibition of Hill activity observed by the addition of nucleotide in the case of chloroplasts freshly prepared or aged for the short period.

Further detailed mechanisms for the inhibition and stimulation effects of nucleotides on Hill activity remain to be made clear in the future investigation.

Summary

The interference between the effects of various nucleotides and lipids on the Hill reaction of chloroplasts was studied at the various stages of aging. The presence of nucleotides such as ATP, ADP and AMP inhibits not only Hill activity of freshly prepared chloroplasts without phosphorylating reagents but that of chloroplasts aged at low temperature. However, the nucleotides act as a stimulator for chloroplasts aged for the long period over about 75 hrs. Hill activity is lowered by the presence of the lipids extracted from the aged chloroplast suspension, but when the nucleotides are added, recovered to a large extent. The lowering of Hill activity due to the aging can be understood by the adsorption effect of free lipids (containing free fatty acids) released from chloroplasts on the lamellae membrane. For this reason, in the case of chloroplasts freshly prepared or aged for the short period, nucleotides may can directly attack to destruct the membrane structure of lamellae, leading to the inhibition of Hill activity.

In the case of chloroplasts aged for the long period, the free lipids may form the adsorption layer on the lamellae membrane enough to cause the inhibition of Hill activity. Such an adsorption layer may be removed to some extent by the addition of nucleotides, and the lamellae membrane may come back to more active state. The controlling of the adsorption of the lipids is understood to be derived from the direct interaction of nucleotide with fatty acid.

This expiains the observed inhibition and stimulation effects of nucleotides, depending on the aging period, on the Hill reaction of aged chloroplasts.

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