Stabilization of the Photosynthetic Activities of Isolated Spinach Chloroplasts during Prolonged Ageing

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Isolated spinach chloroplasts (type A complete) were used to study the changes in the photochemical activities upon storage in order to establish optimum conditions for prolonged storage. Chloroplasts stored at −5 °C were found to retain over 70% of their photosynthetic electron transport from H_2O even after 5 days. Increases in the level of state 2 electron transport (due to uncoupling) with concomitant loss of state 3 phosphorylation activity was observed during the initial period of ageing. Addition of 1% bovine serum albumin decreased the level of uncoupling and maintained the phosphorylation activity for a longer period. Chloroplasts stored at 77 °K maintained their phosphorylation capacity for a period of 10 days even after repeated freezing and thawing. Photosystem I activity was found to be more stable over the whole ageing period of 15 days. Loss of plastoquinone may be responsible for the decrease in electron transport between photosystems II and I.

Introduction

Rapid loss of Hill activity in isolated chloroplasts stored at room temperature has often been observed [1–4]. Constantopaulos and Kenyon [1] reported a 70% decrease of Hill activity in chloroplasts stored for 2 hours at room temperature with a concomittant increase in the level of free fatty acids. Krogmann and Jagendorf [3] also observed an inhibition of Hill reaction by fatty acids. However, attempts have not been too successful in stabilizing various photosynthetic activities for long periods. More recently Takaoki *et al.* [5] studied the stabilization of chloroplast membranes, devoid of their soluble stroma components by isolation in a salt medium, by storage in a sucrose medium in the presence of BSA. They showed over 70% activity even after 20 days of ageing at 4 °C; activity was measured as the quenching of atebrin fluorescence in illuminated chloroplasts which is a sensitive measure of their capacity to develop H^+ gradients. Other activities were not reported.

Abbreviations: Asc, sodium ascorbate; BQ, *p*-benzoquinone; BSA, bovine serum albumin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; DPIP, 2,6-dichlorophenolindophenol; FeCy, ferricyanide; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; MV, methylviologen; PQ, plastoquinone; PS I, PS II, photosystems I and II.

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In this paper we report a detailed study of the changes in PS I and II activities upon storage of isolated, unfixed chloroplasts which initially showed good coupling between phosphorylation and electron transport. Suitable conditions to preserve the photosynthetic control and phosphorylation capacity over a 10 – 15 day period are described.

Materials and Methods

Type A complete chloroplasts [6] were isolated from greenhouse grown spinach as described [7]. Chloroplasts were suspended in buffered medium (10 mM NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 50 mM HEPES and varying concentrations of sorbitol, all adjusted to pH 7.5) at a final chlorophyll concentration of 1 mg/ml, and then stored in the dark at the indicated temperatures. BSA where added was at 10 mg/ml. Oxygen evolution and uptake were measured [7, 8] under saturating orange-red light in a Rank Bros., Cambridge, O_2 electrode [9] at 15 °C. Where used the concentrations of FeCy (PS II + I), BQ (PS II) and MV (PS II + I) were 2.0, 1.0 and 0.4 mM, respectively. The PS I reaction was measured as O_2 uptake in the presence of DCMU, 1.0 μM; Na ascorbate, 2.0 mM; DPIP, 100 μM and MV, 0.4 mM.

Results

Changes in the various electron transport reactions in chloroplasts ageing at −5 °C are shown in Fig. 1. The overall Hill reaction with MV (PS II

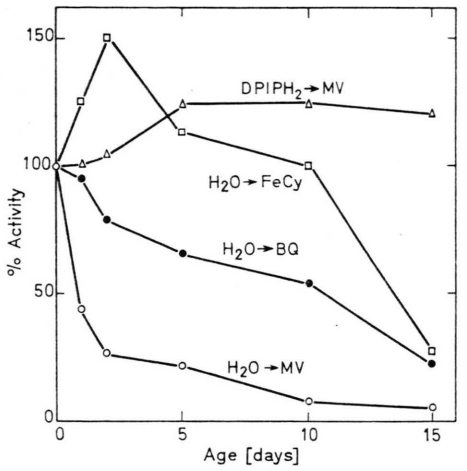

Fig. 1. Changes in the various partial reactions of photosynthesis in *in vitro* ageing spinach chloroplasts, stored at −5 °C in 0.66 M sorbitol. State 2 oxygen evolution and uptake rates were measured as described in Methods. In all the cases, except the PS I reaction, the chlorophyll concentration was 100 μg in the total volume of 2 ml; a five-fold lower concentration was used for the PS I assay. The equivalent 100% value of the state 2 rates were: $H_2O \rightarrow$ FeCy (PS II+I), 32; $H_2O \rightarrow$ BQ (PS II), 120; $H_2O \rightarrow$ MV (PS II+I), 46; ascorbate+DPIP → MV (PS I), 144 μatom O_2/mg Chl·hour.

+I) showed a very rapid loss of activity after 1 day of storage followed by a slower loss. On the other hand, electron transport to FeCy (PS II + I) showed an increase in the rate during the first 2 days of ageing followed by a slow decrease. However, even after 10 days this level was found to be not less than 100%. Further ageing decreased this activity. The PS II reaction mediated by BQ showed a slower inactivation and as much as 65% of the initial activity was observed after 10 days. Electron transport mediated by PS I alone with artificial electron donors to MV was found to be stable over the whole ageing period of 15 days.

Studies were also made of the ADP-induced, state 3 electron transport rate to FeCy – this is a simple method to demonstrate the phosphorylation capacity of the chloroplasts. The terminology state 2, 3 and 4 used here for the various levels of the electron transport is that of Chance and Williams [10] as has been shown for chloroplasts [8, 11]. Changes in the level of state 2 electron transport in chloroplasts stored at −5 °C under different osmotic solutions in the presence and absence of BSA are shown in Table I. A substantial increase (*e.g.* 2.5× with chloroplasts stored in 1.2 M sorbitol without BSA) in the state 2 rate due to uncoupling of phosphorylation was observed in all cases during the first 2 days of ageing, followed then by a decrease. Addition of BSA and/or hypertonic sorbitol substantially decreased the longer term harmful effects.

Changes in the different levels of electron transport, photosynthetic control (state 3/state 4) and ADP/O ratio (μmol ADP added/μatoms O_2 evolved) in chloroplasts stored at different temperatures are shown in Table II. Different extents of uncoupling occur upon storage as is shown by the increased state 2 rate. The smallest increase was found in chloroplasts stored at −5 °C. Gradual decreases in the state 3 and uncoupled rates were found with increasing temperatures. Chloroplasts stored at 15 °C could retain as much at 50 – 65% of the NH_4-uncoupled Hill reaction activity after 2 days. The extent of the loss of state 3 rate was much higher at 15 °C than at 4° and −5 °C. In chloroplasts stored at 77 °C a clear, well-defined state 3 rate was observed even after 10 days of storage.

Discussion

Use of an isolation method with rapid grinding and quick separation of chloroplasts (originally developed by Walker [12, 13]) yielded type A complete chloroplasts which could then be used for ageing studies. At least three major changes in ageing chloroplasts were observed previously [1, 4]: a large increase in the chloroplast volume during the initial

Table I. Effect of different sorbitol concentrations in the chloroplast storage medium kept at −5 °C on the state 2 electron transport rate with ferricyanide as the electron acceptor (expressed as a % of day zero rate). Concentration of BSA was 10 mg/ml. The 100% levels were 25 – 36 μatom O_2/mg Chl·hour.

Age [days]	1.2 M		0.66 M		0.33 M		0.1 M	
	+BSA	−BSA	+BSA	−BSA	+BSA	−BSA	+BSA	−BSA
2	171.4	250.0	150.0	143.8	185.7	127.3	122.2	136.4
5	114.3	162.5	112.5	137.5	100.0	36.4	100.0	18.2
10	114.3	0.0	100.0	0.0	92.9	0.0	66.7	0.0
15	118.6	0.0	25.0	0.0	28.6	0.0	22.2	0.0

Table II. Changes in the different states of electron transport (with ferricyanide as the electron acceptor), photosynthetic control and ADP/O ratio in chloroplasts aged at different temperatures. Chloroplasts were aged in 0.66 M sorbitol.

Age [days]	Storage Temp.	Oxygen evolution: μmol/mg Chl·hour				Photosynthetic control	ADP/O *
		State 2	State 3	State 4	$+NH_4Cl$		
Fresh	—	16	56	18	108	3.1	1.9
1	15 °C	20	28	17	60	1.7	2.8
	4 °C	26	50	16	111	3.1	—
	−5 °C	20	46	22	108	2.9	2.6
2	15 °C	20	20	16	46	1.3	—
	4 °C	24	34	14	88	2.4	5.6
	−5 °C	24	34	22	96	1.6	3.3
5	15 °C	10	10	10	10	1.0	—
	4 °C	28	28	28	40	1.0	—
	−5 °C	18	32	26	60	1.2	—
	77 °C	28	56	28	88	2.0	2.2
10	15 °C	2	2	2	2	1.0	—
	4 °C	20	20	20	20	1.0	—
	−5 °C	16	22	20	24	1.1	—
	77 °C	24	46	26	80	1.8	2.6

* Ratio calculated with subtraction of state 2 rate from state 3 (ref. [7]).

6 – 8 hours of storage at room temperature, a greatly increased electron transport to acceptors like FeCy and DPIP due to uncoupling, and an increase in the level of free fatty acids in chloroplasts. To avoid extensive breakage upon swelling, chloroplasts were stored at relatively high osmotic concentrations (0.66 and 1.2 M sorbitol). Even though this gave increased preservation, complete breakage of all chloroplasts had occurred within 5 days (see ref. 14 for ultrastructural changes).

The difference in ageing effects on $H_2O \rightarrow$ FeCy and $H_2O \rightarrow$ MV (Fig. 1) which both required PS II + I for non-cyclic electron transfer is interesting since it may indicate the ability of FeCy to accept electrons from PS II alone (instead of at PS I) as the PQ pool situated between PS II and PS I is lost with ageing. Thus FeCy changes from a PS I acceptor to a PS II acceptor as the chloroplasts deteriorate after isolation in the type A complete condition. We have measured the fluorescence induction kinetics to provide some indication of the extent of the PQ pool and electron transport between PS II and PS I (see Fig. 2). A drop in the PQ level over the first 2 days probably resulted in a decrease in the $H_2O \rightarrow$ MV reaction but an increase in the $H_2O \rightarrow$ FeCy reaction as the FeCy switched from accepting electrons at PS I to acting at PS II (similar to the $H_2O \rightarrow$ BQ system). No quantitative correlation was attempted but the results are quite indicative of an ageing effect.

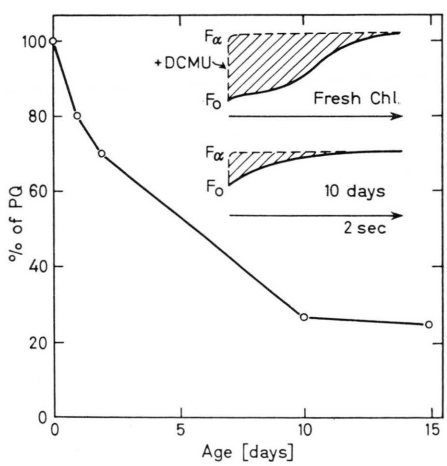

Fig. 2. Changes in the level of plastoquinone (PQ) in chloroplasts ageing at −5 °C in 0.66 M sorbitol. Amount of PQ was calculated by measuring the kinetics of the fluorescence induction in the presence and absence of 1 μM DCMU. The difference (on area basis, shadowed portion in the insert figure) was assumed as a relative unit for the amount of PQ present. Chloroplasts were suspended in 0.33 M sorbitol at a final chlorophyll concentration of 5 μm/ml. Chlorophyll fluorescence was excited by blue light (λ_{max}= 460 nm) and measured at 678 nm. [We are indebted to Dr. J. Barber, Imperial College London for providing measuring facilities.]

Ageing resulted initially in large increases in the state 2 electron transport rate due to uncoupling of the phosphorylation activity (see also refs 1 and 5). However, chloroplasts maintained a good state 3 phosphorylating activity for a least a day. Addition

of BSA decreased the uncoupling effects in all cases. Phosphorylation and electron transport were found to be best preserved by storing chloroplasts in 0.66 M sorbitol in the presence of BSA .

Chloroplasts stored at room temperature in alkaline medium showed an increase in the level of free fatty acids [1]; release of as much as 30% of phospho- and sulpho-lipids from the thylakoids stored under alkaline conditions in a salt medium has also been reported [15]. We attempted to minimize the level of lipid release from the thylakoid membranes by storing them at 77 °K in the presence of BSA. Even though addition of BSA at any temperature decreased the inactivation of the Hill reaction, storing of chloroplasts at 77 °K resulted in a good degree of preservation of the phosphorylation capacity for a maximum period of 10 days. The observed decrease in the photosynthetic reactions was probably due to repeated freezing and thawing of the same sample. Even though an increased state 2 rate was observed in aged chloroplasts, the NH_4-uncoupled electron transport rate showed greater stability towards ageing when stored at -5° and 4 °C.

From a wide range of experimental conditions tried, photosynthetic electron transport reactions were found to be well preserved when type A, complete chloroplasts were stored in relatively high concentrations of sorbitol (0.66 M) in the presence of BSA and at temperatures close to 0 °C. Preservation of phosphorylation for several days could be achieved by storing them at 77 °K, even with freezing and thawing.

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