Photosynthetic Control in Chloroplasts Suspensions Frozen in Liquid Nitrogen in the Presence of Glycerol

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Type A complete spinach chloroplasts (photosynthetic control ratio $\simeq 5$) were frozen in liquid nitrogen, in the presence or absence of 50 percent glycerol (v/v). By considering five parameters related to the non cyclic electron transfer in the light (oxidant : K-ferricyanide) it was shown that the uncoupling due to freezing and thawing was partially prevented by glycerol. However glycerol was responsible for a slight uncoupling in fresh chloroplasts. The clearest uncoupling effect of freezing, and chiefly of glycerol, concerned the inhibition of the electron flow which follows the stimulation caused by a limiting quantity of ADP (state 4).

The relative decrease of the photosynthetic control and of related parameters due to the use of this method of preservation, has to be balanced with the improved homogeneity and convenience resulting from it.

Introduction

The well known lability of chloroplasts suspensions has induced several attempts to preserve these organelles by holding them in subzero temperature [1–5]. It has been reported that chloroplasts suspensions in 50% glycerol (v/v) are entirely protected against the uncoupling effect of liquid nitrogen on cyclic [3] and non cyclic phosphorylation [6]. Photosynthetic control (P.C.) has been shown to be a very sensitive indicator of the biochemical integrity of the chloroplasts, particularly of their coupled state [7, 8]. It is the ratio of two rate parameters, E_3/E_4. E_3 is the rate of the electron flow stimulated by the addition of a limiting quantity of ADP, and E_4 the rate of the inhibited electron flow that follows exhaustion of ADP. Large values of P.C. point to high coupling. However they can be due either to increasing E_3 or to decreasing E_4 or both, so that the meaning of P.C. is relatively ambiguous. A few other kinetic parameters have therefore been measured as well as P.C. in the following experiments, in order to assess more clearly the effect of glycerol on the uncoupling due to freezing.

We have reexamined the protective effect of glycerol on coupled non cyclic electron transport when chloroplasts are frozen. We have used P.C. and related parameters to evaluate the extent of coupling preservation. The cryoprotective effect of glycerol on coupling has been confirmed, but the use of the more sensitive energy parameters (P.C. and related parameters) has revealed that this protection is partial and not complete as was believed formerly [3, 6].

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Materials and Methods

Type A complete chloroplasts [9] were obtained from spinach leaves (var. Géant d'hiver) after a growth of plants for 2 to 3 months in a controlled environment, by the method of Reeves and Hall [10] with the following modifications. A Braun MX 32 blender was used instead of a Polytron. The initial slurry was filtered through cheese-cloth and a layer of cloth with calibrated pores (Blutex 50, Tripette and Renaud, Paris). Ascorbate 10 mM was used instead of isoascorbate.

An aliquot of the final chloroplast suspension (1 to 3 mg total chlorophylls/ml) was immediately frozen as described earlier [11] and the rest of the suspension was used as a fresh control (*cf.* Table I). The first measurement with fresh chloroplasts was made within 30 min of the beginning of the chloroplast extraction. The frozen chloroplasts were generally thawed and used after 3 or 4 hours, but their properties show no visible further modifications after more than two weeks in liquid nitrogen.

Oxygen concentration changes were measured with a Clark type electrode (Rank Bros., Bottisham, U.K.) at 15 °C. The electrode was calibrated in the presence of chloroplasts and K-ferricyanide [8] the concentration of which was measured by its absorbance at 420 nm. The chloroplasts suspension (100 μg of chlorophylls) was added to the incuba-

tion medium[10] in the electrode cuvette so that the total volume was 2 ml. It was equilibrated thermally for about 5 min. A saturating red light was turned on simultaneously as 5 μmol of K-ferricyanide were added to the solution (state E_2). The addition of the ferricyanide simultaneously with the turning on of the light was intended to prevent the damage due to the dark preincubation with this oxidant[12]. After a stable rate was obtained, 250 nmol of ADP were added (state 3). The quantity of O_2 evolved increased linearly with the added quantities of ADP, between 100 nmol and 1000 nmol, as was found by Reeves[13]. In some experiments, 2 μmol of NH_4Cl were added instead of ADP during E_2 (E_2NH_4), or after the addition of ADP, 2 to 3 min after the beginning of E_4 (E_4NH_4). This concentration of NH_4Cl was experimentally found to be optimal in our conditions. Besides P.C., (E_3/E_4), the following ratios were also calculated: 1) $(E_3-E_2)/E_2$; 2) $(E_4-E_2)/E_2$; 3) $(E_4NH_4-E_4)/E_4$. $(E_3-E_2)/E_2$ is a measure of the stimulation of the electron flow by a limiting quantity of ADP. This ratio is positive except when the chloroplasts are fully uncoupled; then it is zero. $(E_4-E_2)/E_2$ is a measure of the inhibition of the electron flow after exhaustion of the added ADP. It is negative unless chloroplasts are fully uncoupled. Then it is zero. Uncouplers inhibit ATP formation without inhibiting electron transport. Even more uncouplers like NH_4Cl accelerate electron transport[14]. The highest accelerations will be found in the more coupled organelles. Thus, the measurement of the acceleration of the electron transport by NH_4Cl during state 2 ($(E_2NH_4-E_2)/E_2$) and state 4 ($(E_4NH_4-E_4)/E_4$) will be two measures of the coupling related respectively to E_2 and E_4. Both these parameters are positive in coupled chloroplasts. All the measurements on one batch of chloroplasts were performed in about two hours. It has been experimentally checked that longer periods of preservation of the chloroplasts suspensions in the same conditions (temperature of zero degree and complete darkness) resulted inevitably in progressive inhibition of the electron flow.

The chemicals were all of the highest purity commercially available except for serumalbumin which is fraction 5 from Sigma.

Results and Discussion

In the absence of glycerol

When chloroplasts are frozen, E_4 is greatly increased (Table I a). P.C., $(E_3-E_2)/E_2$, $(E_4NH_4-E_4)/E_4$ and $(E_4-E_2)/E_2$ are seriously depressed (Table I b). The other parameters are not significantly modified in these conditions. Thus there is a consistent effect of freezing on E_4. In contrast, the effect of freezing on E_2 depends on whether one judges coupling by looking at ADP or NH_4Cl stimulated electron transport.

Table I. Effects of freezing in liquid nitrogen temperature on the coupling of type A complete chloroplasts. For each value the confidence limits for a probability of 95% were calculated. For most data a Kruskal-Wallis analysis was used as a further statistical control. With the exception of P.C. which has no dimension, the parameters are given in μmol O_2/mg chlorophylls · hours (a) and as percentages (b).

Table I a.

	Fresh	Frozen
E_2	63	75
	61	72
E_2NH_4	108	124
	105	139
E_3	89	88
	85	100
E_4	19	64
	30	39
E_4NH_4	86	100
	89	119

Table I b.

	Fresh	Frozen
P.C.	5.2	1.3
	3.0	2.6
$\frac{E_2NH_4-E_2}{E_2}$	72	62
	73	100
$\frac{E_3-E_2}{E_2}$	43	15
	35	38
$\frac{E_4NH_4-E_4}{E_4}$	418	58
	221	210
$\frac{E_4-E_2}{E_2}$	−68	−17
	−52	−44

E_2, state 2; E_3, state 3; E_4, state 4. E_2NH_4, state 2 modified by the addition of NH_4Cl; E_4NH_4, state 4 modified by the addition of NH_4Cl. P.C., photosynthetic control. Fresh, chloroplasts used immediately after processing; Frozen, chloroplasts frozen in liquid nitrogen immediately after extraction, generally thawed and used after 3—4 hours. First line: in the absence of glycerol; second line: in the presence of glycerol.

In the presence of glycerol

In fresh chloroplasts the value of E_4 is increased (Table I a) so that ratios containing E_4, P.C., $(E_4NH_4-E_4)/E_4$ and $(E_4-E_2)/E_2$ are reduced (Table I b). The values of these parameters are intermediate between those of the fresh and those of the frozen chloroplasts in the absence of glycerol. The other parameters are not modified compared with their values in the absence of glycerol. This points to an uncoupling by glycerol itself; this uncoupling is less acute than that due to freezing in the absence of glycerol and it is restricted to E_4. Freezing in the presence of glycerol has a similar effect except that in frozen chloroplasts the value of $(E_2NH_4-E_2)/E_2$ is higher than in fresh ones, even in the absence of glycerol (Table I b). (No direct explanation of this last observation seems available

at present.) Freezing in the presence of glycerol does not seem, therefore to add to the relatively moderate uncoupling concerning E_4, due to glycerol. To distinguish between the uncoupling due to freezing and to glycerol, the effect of washing away the glycerol has been experimented *. Several parameters, including the P.C., were found to exhibit higher values in the washed than in the unwashed chloroplasts. This shows clearly that the uncoupling due to glycerol can be reversed, at least partially, by washing. Thus, at least a part of the uncoupling in the chloroplasts frozen in the presence of glycerol is irreversible and is not due to the cryoprotective.

Conclusion

The analysis of these results points to a partial but not negligible protection by glycerol against the uncoupling due to freezing with liquid nitrogen as preservative. It is clearly E_4 which is specifically affected, in the presence or in the absence of glycerol. An uncoupling affecting E_2 in the absence of glycerol cannot be stressed unambiguously on the basis of these sole experiments. Glycerol by itself uncouples E_4, but the uncoupling is at least partially reversible.

What is the signification of the special sensitivity of E_4 to the uncoupling effects of freezing and in a less measure, by glycerol? The inhibited rate of the electron flow during E_4 could be related to the presence of ATP in certain conditions: indeed ATP has been reported to inhibit the non cyclic electron flow [10, 15]. West and Wiskich relate this inhibition by ATP to a specific proton translocating ATPase, according to the chemiosmotic theory [15]. If this is true, the hypothesis could be proposed that the observed uncoupling affecting E_4 concerns this ATPase.

It is important to note that the chloroplasts frozen in the presence of glycerol in our conditions, retain an appreciable part of their photosynthetic control and related parameters, and thus of their energetic integrity, with the advantage of an appreciable improvement in homogeneity and convenience compared to "fresh" chloroplasts. Moreover, it is not excluded that improvements could still be brought out in the quality of the preservation, for instance, by controlling quantitatively the rate of freezing; this last parameter being recently proved to be crucial in cryobiological experiments with cell suspensions [16, 17].

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