RESTORATION BY SILICO TUNGSTATE OF THE REVERSIBLE LIGHT INDUCED pH RISE IN COUPLING FACTOR (CF_1) DEFICIENT CHLOROPLASTS

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1. Introduction

The coupling factor (CF_1) in chloroplasts has a dual function: this protein plays a catalytic role in the formation and hydrolysis of ATP, and contributes to a large part to the structure and properties of chloroplast membranes [1]. Removal of CF_1 from chloroplast membranes causes a permeability change and the light-induced pH rise as the post-illumination ATP formation is suppressed. The pH change of the chloroplast suspension is interpreted as the result of the acidification of the thylakoid compartment and the generation of a pH difference (ΔpH) across the thylakoid membrane [2].

Assuming that it was possible to modify the chloroplast membrane permeability by attaching charged molecules to the membrane we have tried two polyanions, silicotungstic acid (STA) and phosphotungstic acid; both react with amine groups and are used for negative staining of the membrane in electron microscopy [3].

We found an effect only with STA. The light-induced pH rise in chloroplasts, the coupling factor (CF_1) of which have been removed, can be restored by STA but the post-illumination ATP formation could not. We interpret this action as the result of the STA fixation on the chloroplast membrane. The fixed STA molecules operate by buffering the inner space of thylakoid membrane, as imidazole does [4], following the reaction (1)

$$12\,(SiO_6\,(WO_3)_{11})^{8-} + 52\,H^+ \rightleftharpoons 11\,(SiO_4\,(WO_3)_{12})^{4-} + SiO_2 + 26\,H_2O \qquad (1)$$

On the other hand, we studied the STA action on the different photochemical reactions of the chloroplast: dichlorophenol indophenol (DPIP) mediated photoreactions, NADP photoreduction and pyocyanin mediated cyclic photophosphorylations are very sensitive to STA but ferricyanide mediated O_2 evolution and methyl viologen O_2 uptake are insensitive.

Abbreviations:

ATP : adenosine 5' triphosphate
CF_1 : coupling factor (E. Racker's nomenclature)
DPIP : 2,6-dichlorophenolindophenol
EDTA : ethylenediaminetetraacetic acid
NADP : nicotinamide dinucleotide phosphate
STA : silico tungstic acid

2. Material and methods

Chloroplasts from spinach leaves were prepared in a cold room (5°) by homogenizing 400 g of leaves in a blender with 1,200 ml of extracting medium (4×10^{-1} M sucrose, 2×10^{-2} M Tris pH 7.8). After filtration through cheese cloth, large particles were discarded by centrifugation at 200 *g* for 5 min. The chloroplast fraction was collected by sedimentation at 1,200 *g* for 30 min. Chloroplasts (100 mg of chlorophyll) were broken in 300 ml distilled water for 60 min, then centrifuged at 15,000 *g* for 10 min. The pellet was resuspended in 4×10^{-1} M sucrose.

Uncoupled chloroplasts were prepared by treatment with EDTA. Chloroplasts (1 mg of chlorophyll per 5 ml) were gently stirred in a cold solution (5°) of 10^{-3} M EDTA for 10–25 min at pH 8, then centrifuged at 15,000 *g* for 10 min to remove the coupling factor (CF_1) from the pellet which is resuspended in 4×10^{-1} M sucrose.

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pH measurements were made with a Beckman research pH meter and scaled with a graphispot recorder (Sefram). Proton uptake with chloroplast suspensions is carried out in a water-jacketed cell at 5°. The reacting medium (13 ml) constantly stirred consists of: 10^{-2} M $MgCl_2$; 5×10^{-5} M pyocyanine; 1.5×10^{-3} M Tris-malate, pH 6.2; 50 μg chlorophyll per ml. Light is provided by 650 watt sun gun lamp (Sylvania). The light beam is filtered through 15 cm of water. Calibration is performed in darkness with standard 10^{-2} M NaOH.

Ferricyanide reduction, O_2 evolution and uptake were measured by amperometric techniques. A polarographic apparatus with 3 electrodes (Tacussel potentiostat-PRT 500 L) was used with a vibrating platinum electrode (for ferrocyanide) or a Clark electrode (for oxygen).

NADP and DPIP reduction were measured with a Cary 14 spectrophotometer. NADP reduction, DPIP reduction and ferricyanide reduction were observed with the reaction media of M. Avron [7] and methyl viologen mediated oxygen uptake with the reaction medium of S. Lien and E. Racker [8]. ^{32}P-ATP formation was determined by its specific activity after separating it from the inorganic phosphate by high voltage paper electrophoresis [6].

3. Results

Reversible light-induced pH rise were measured with normal and uncoupled chloroplast suspensions (table 1). In the presence of STA, uncoupled chloroplasts recover the ability to cause a reversible increase of the pH of their suspensions when they are illuminated. The optimal STA concentration varies linearly with the chlorophyll concentration (fig. 1) and is $1-2 \times 10^{-5}$ M for a chloroplast suspension containing 50 μg of chlorophyll per ml. When STA concentration is the limiting factor of the proton pump, the proton uptake is about 4 times the amount of STA added. A higher STA concentration inhibits the light-induced pH increase in normal and uncoupled chloroplasts (table 1). Proton uptake observed with and without STA presents the same variations as a function of the pH (fig. 2). Phosphotungstic acid has no enhancement effect on the light-induced pH change, but only an inhibitory effect at the same concentration as STA does.

Table 1
Dependence of the reversible light induced proton uptake on the STA concentration with normal and uncoupled chloroplast suspensions.

STA concentration ($M \times 10^5$)	Proton uptake (nmole of HCl per mg of chlorophyll)	
	Normal chloroplasts	Uncoupled chloroplasts
0	650	150
0.5	650	550
1	650	700
2	650	700
3	450	450
4	250	150

Initial pH is 6.2, chlorophyll concentration is around 50 μg per ml.

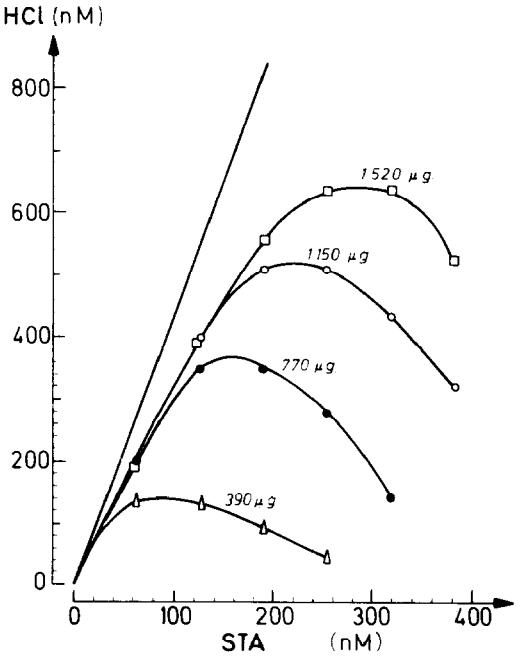

Fig. 1. Extent of the proton uptake as a function of STA amount. Experiments are made as described in the methods except for chlorophyll amounts, which are reported on the top of each curve. All the data, nM of HCl or STA, and μg of chlorophyll, correspond to the quantities present in 13 ml of the reacting medium. The straight line is the theoretical amount of H^+ necessary for the transformation of all the STA molecules, supposed to be in the form $(SiO_6(WO_3)_{11})^{8-}$ to the other form $(SiO_4(WO_3)_{12})^{4-}$ following the reaction (1).

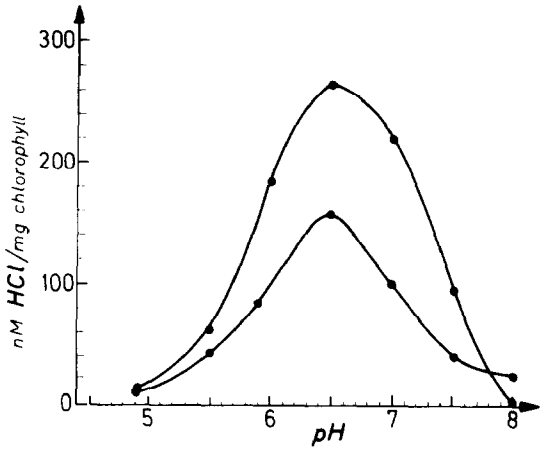

Fig. 2. Extent of proton uptake with and without STA as a function of the pH. Experiments as described in the methods: lyophilized chloroplasts kept for 6 mon at −15° were used. The upper curve is with STA 1 × 10^{-5} M, the lower curve without STA.

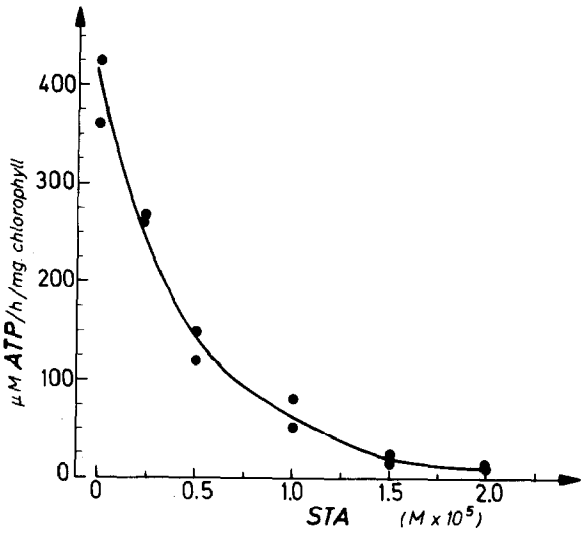

Fig. 3. ATP formation during cyclic photophosphorylation as a function of STA concentration in the light phase. Normal chloroplasts (500 µg of chlorophyll) are incubated at pH 6 in 10 ml of STA solution for 5 min. Then 0.5 ml of the suspension is brought to 1 ml final (4 × 10^{-2} M Tris, pH 8.2; 2.5 × 10^{-3} M Na_2HPO; 2.5 × 10^{-3} M ADP; 5 × 10^{-3} M $MgCl_2$; 5 × 10^{-5} M pyocyanine and 2.5 µCi ^{32}P per ml) and illuminated for 3 min. Reaction is stopped by lowering the pH with 0.2 ml 2 N HCl.

Table 2

% Inhibition of photochemical reactions at different concentrations of STA with normal chloroplast suspensions.

	Chlorophyll concentration (µg per ml)	STA concentration (M × 10^5)	
		0.5	2
NADP reduction H_2O electron donor	20	100	100
NADP reduction with ascorbate and DPIP	20	100	100
DPIP reduction	20	90	90
O_2 evolution with ferricyanide	50	0	0
Ferricyanide reduction	50	0	0
Methyl viologen mediated O_2 uptake	50	0	0

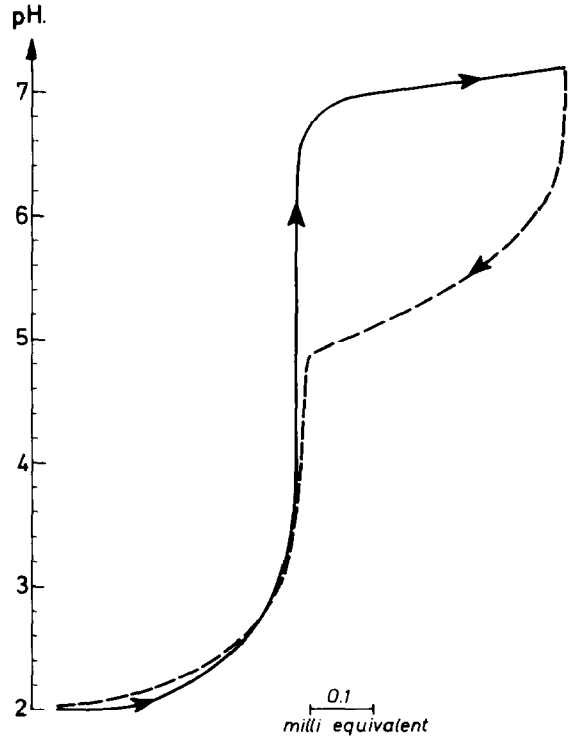

Fig. 4. Acid-base titration of STA. 0.1 mmole of STA is dissolved in 40 ml. Titration is run first with 10^{-1} N NaOH till we get a stable neutralization, then back titration is run with 10^{-1} N HCl.

In the presence of STA, uncoupled chloroplasts do not recover the ability to give a post illumination ATP formation.

ATP formation during pyocyanine mediated cyclic photophosphorylation is strongly inhibited in presence of STA with normal chloroplast suspension (fig. 3).

Ferricyanide mediated O_2 evolution and methyl viologen O_2 uptake are not sensitive to STA but NADP and DPIP reduction are strongly inhibited (table 2).

4. Discussion

Acid-base titration of STA (fig. 4) shows that between pH 7 and 5 a buffering capacity is developed which is very likely the result of the pH dependent interconversion between two different forms of STA.

$$12\,(SiO_6(WO_3)_{11})^{8-} + 52\,H^+ \rightleftarrows 11\,(SiO_4(WO_3)_{12})^{4-} + SiO_2 + 26\,H_2O \quad (1)$$

So we think that light-induced proton influx is buffered by STA molecules which, fixed to the membrane, exist as a form stable [5] at pH 6 and are transformed by fixing about 4 protons per molecule to the other form stable at lower pH according to the reaction (1). The proton uptake extent is increased in the same way as with imidazole [4]. Following this interpretation we can explain the inactivity of phosphotungstic acid which, between pH 2 and 7, exists only in one form [5].

E. Racker has discovered that chloroplast suspensions containing 4 mg of chlorophyll per ml, when treated with STA 3×10^{-3} M at pH 5.5, lose the coupling factor (CF_1) [8]. This concentration of STA is very similar to that we used for optimal H^+ uptake restoration; indeed we have seen that optimal STA concentration varies linearly with the chlorophyll concentration (fig. 1). It is tempting to assume that CF_1 and that STA could play the same role as CF_1 by regulating the buffering properties of the membrane.

STA inhibitory activities on the different photochemical reactions of the chloroplasts cannot be explained by a differential sensitivity of the two photoreactive systems to STA. More likely different enzymatic proteins, necessary for one or more steps of the photochemical reactions, lose their activities in presence of STA.

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