LIGHT-DEPENDENT FIXATION OF POLYAMINES INTO CHLOROPLASTS OF CHINESE CABBAGE

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1. Introduction

We have been studying the infection of Chinese cabbage by turnip yellow mosaic virus (TYMV), an infection which causes vesiculation of chloroplast membranes, aggregation of chloroplasts and accumulation of virus specifically in pockets between the chloroplasts [1,2]. TYMV is rich in spermidine associated with the RNA [3] and the infected plant contains high levels of this triamine [4]. We have observed that a chloroplast fraction isolated from leaf protoplasts can synthesize spermidine from methionine and contains spermidine synthase [5].

The infected plant is also high in putrescine and spermine, and the former increases late in infection, apparently concomitantly with the yellowing and withering of the plant [4]. Since it is known that putrescine accumulates generally in plants in unfavorable soils [6] and is itself toxic to the plant [7], we have asked if the increase of this polyamine in infection is causally related to the developing pathology. Two sets of observations bear on this point:

(1) Putrescine was found to be lethal to a Cyanobacterium, *Anacystis nidulans* in the light, and to bind covalently to various cellular proteins, including ribosomal subunits [8];
(2) Protoplasts of the healthy leaves were found to convert methionine to acid-insoluble form by chloroplasts isolated from disrupted protoplasts.

It has been found that in the presence of light, chloroplasts do bind polyamines in an acid-insoluble form, of which ~1/2 is soluble in 80% acetone or alcohol.

2. Materials and methods

2.1. *Protoplast and chloroplast preparation*

Fresh leaves from either healthy or TYMV-infected Chinese cabbage (*Brassica pekinesis*, var. Pak Choy) (see [5]) were excised when the leaves were <5 cm long at 19–30 days total leaf age. The leaves were rinsed with distilled water and patted dry. The midrib was removed with a razor blade and the undersides of the leaves were cross-hatched with a pair of razor blades. The weighed leaves were placed in a petri dish cross-hatched surface down, in a solution containing 0.5% macerozyme R-10 and 0.5% cellulase Onozuka R-10 (Yakult Biochemicals, Japan) in 0.5 M sorbitol, 1 mM $NaKPO_4$ (pH 5.8) (sterile filter). The material was then incubated overnight in the dark at 17°C. The dish was shaken gently to release protoplasts into the incubation mixture. A sterile wide-bore glass pipet was used to transfer the cells to a sterile glass cylinder containing a cushion of several ml sorbitol solution at pH 5.8. The cells were diluted 3-fold with the same solution and filtered through 8 layers of cheesecloth into a 40 ml glass tube using sterile glassware. The resulting protoplast suspension was centrifuged for 15 min at 20 × *g* at room temperature. The supernatant was discarded and the pellet was washed 2 times in the pH 5.8 buffer and resuspended in 0.33 M sorbitol, 50 mM Tricine, 1 mM Na_2HOP_4 (pH 8.0). The protoplast content of the suspension was determined and the sample was diluted with the pH 8.0 buffer to 3.0–4.7 × 10^5 protoplasts/ml. The protoplast suspension was disrupted by passage through a 21 gauge 5 ml sterile syringe. After 2 cycles of disruption the released chloroplast suspension was found to contain <0.1% of undisrupted protoplasts. The chloroplast suspension was then centrifuged for 15 min at 200 × *g* at room temperature. The supernatant was discarded and the pellet was washed once in the sorbitol buffer at pH 8.5 and finally resuspended at that pH. At this point the resuspended chloroplast fraction, layered thinly (<0.5 cm) in a petri dish, was preincubated for

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15 min under 10 000 lux of white light before addition of radioactive amine. The temperature under the lamp was maintained at 29°C, that of the plant growth chamber.

2.2. *Isotopes*

The following polyamine isotopes were purchased from New England Nuclear (Boston MA): [*tetramethylene*-1,4-^{14}C]spermine · 4 HCl (77 mCi/mmol), [*tetramethylene*-1,4-^{14}C]spermidine · 3 HCl (98.7 mCi/mmol), [1,4-^{14}C]putrescine · 2 HCl (102 mCi/mmol).

3. Results

3.1. *Polyamine fixation and its light dependence*

In initial experiments, mixtures of chloroplasts incubated in the light with radioactive putrescine and spermidine were analysed by sedimenting the chloroplasts through a silicone layer into perchloric acid [9]. The radioactivities of the non-penetrating aqueous fraction revealed disappearance of polyamine into the chloroplast-containing acidic fractions. Isotope was found to increase in the acid-insoluble pellet derived from the aliquots of the incubation mixture. However, the density of chloroplasts changed on incubation, compelling changes in the silicones used at particular time points. Also clumping of the chloroplasts occurred during incubation resulting in difficulties in obtaining reproducible small aliquots. Reproducible quantitative data on polyamine binding was obtained after precipitation of larger aliquots with trichloroacetic acid, followed by centrifugation, transfer of the precipitate to filters and multiple washings with acid and 95% ethanol on the filter. Therefore, this latter method was used in subsequent experiments.

The concentrations of polyamines used initially had been set by the concentration when applied exogenously to this organism which concentrates putrescine intracellularly to >50 mM [8]. Covalent binding of putrescine in *A. nidulans* was observed at intracellular levels of 15 mM and the increased concentration within the cell was demonstrated to occur at pH-values generating significant amounts of non-protonated amine. The rate of putrescine uptake as an acid-soluble component within the bacterium at pH 8.5 is 1–2% of the maximal rate found at pH 10.5 and 10% that at pH 9.5 [11]. However, the fixation of putrescine in an acid-soluble form in the chloroplast preparation at pH 8.5 was essentially linear and ~33–50% that at pH 9.5, i.e., much greater than the rate expected at pH 8.5 if the fixation depended upon an initial penetration of the non-protonated species. The experiments were done at pH 8.5 to minimize unmediated permeation. At this pH all spermidine is protonated [12].

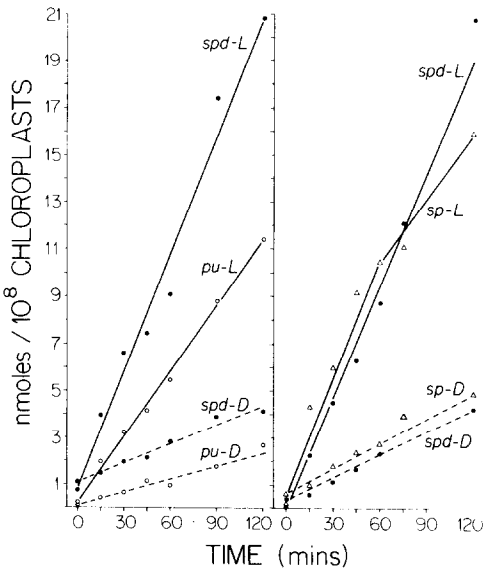

Fig.1. The acid-insoluble association of polyamines with chloroplasts in light (L) and dark (D); left-uptake of putrescine (pu) and spermidine (spd); right-uptake of spermidine and spermine (sp).

The rate of spermidine fixation in the light at pH 8.5 is twice as great as that of putrescine (fig.1). This result has been seen reproducibly, whereas the initial rate of spermine fixation was similar to that of spermidine. Incubation in the dark resulted reproducibly in ~80% reduction in the rates of fixation of these polyamines.

Putrescine was fixed on the chloroplasts in the light for 1 h and the chloroplasts were sedimented and washed twice to remove isotopic diamine. Aliquots were then incubated in the fixation medium in the presence or absence of [^{12}C]putrescine in the presence or absence of light. The fixation was not readily reversible. Light did not lead to an increase or significant fall in the isotope present in the fraction. In the dark there was a slow approximately linear fall in the content of acid-insoluble isotope of 35–45% in 90 min in the presence or absence of cold putrescine.

The rates of fixation at spermidine at 0.033, 0.1,

0.33, and 1.0 mM at pH 8.5 were 2.3, 2.8, 3.7 and 4.8 μmol . h^{-1}. 10^8 chloroplasts^{-1}. At the latter concentration an apparent plateau of bound polyamine was reached at 90 min, amounting to 4×10^7 molecules/chloroplast. Higher plateau values have been observed and in fig.1, a maximum of spermidine fixation had not been achieved at 12×10^7 molecules/chloroplast.

3.2. *Identity of bound polyamine*

Labelled putrescine or spermidine was fixed by chloroplasts in the light for 1 h and precipitated in 5% trichloroacetic acid. The precipitates were washed in acid and in 95% ethanol and, after addition of 0.05 μmol non-radioactive amine, were hydrolysed in 6 N HCl at 110°C in a sealed tube for 18 h. The hydrolysate was adjusted to >pH 13 with NaOH, saturated with Na_2SO_4, and extracted twice with *n*-butanol. The butanol phase was acidified with HCl and taken to dryness. Amines were purified by adsorption and elution on silica [13], dansylated and the dansyl amines were extracted and their radioactivities were determined after separation by thin-layer chromatography [5].

In controls, comparable amounts of labeled polyamines were added to chloroplasts before hydrolysis and recoveries of these amines were estimated after hydrolysis. The yields of added labeled amine were comparable when hydrolysis was effected in 4 N HCl for 4 h or in 6 N HCl for 18 h. However, in similar tests of hydrolysis with the labeled polyamine fixed in the chloroplasts, the final yields of radioactive dansyl amine were sharply reduced (>60%) by the 18 h hydrolysis in 6 N HCl. In all cases studied, i.e., controls and experiments, 79–87% of the radioactivity extractable after hydrolysis was elutable in the polyamine fraction from silica, and 80–100% of the radioactivity applied to the thin-layer plate was recovered as dansyl amine. After putrescine fixation, of the radioactivity liberated as amine by hydrolysis and dansylated 59–75% comigrated with dansyl putrescine. After spermidine fixation, of the radioactivity liberated as amine by hydrolysis and dansylated, 81–86% comigrated with dansyl spermidine.

In the course of identifying fixed spermidine, aliquots of the extracted free amines were also electrophoresed on paper in 0.1 N sodium citrate buffer (pH 3.6). Paper strips of 1 cm were counted and radioactivity was found at the spermidine position in amounts of 79–90% of the total radioactivity on the paper. It appears that prior to fixation there had been little if any conversion of the amine to some other radioactive compound, e.g., an amino acid.

3.3. *Some studies on the mechanism of fixation*

The chloroplast fraction from the disrupted protoplasts was sedimented, carefully drained, resuspended in fresh 0.33 M sorbitol buffer, and tested in putrescine fixation. Each of three such successive washes led to a 30–50% reduction in this activity. The initial wash fluid contained 10–15% of the activity and when this was added back to the sedimented chloroplast fraction there was a slight but incomplete restoration of the activity. It is known from estimation of soluble ribulose biphosphate carboxylase (unpublished) that disruption of the protoplasts produces some leakage of the chloroplasts. Thus, it is not clear if the decrease of activity of the chloroplasts on successive washes is due to damage to the membrane and decreased internal polyamine concentration, or separation of a soluble enzyme from the chloroplast or both.

To test if the activity was similar to the familiar transglutaminase reaction, which generally requires Ca^{2+}, the effects of EDTA and of added Ca^{2+} were determined on a once-washed preparation of chloroplasts [14]: 0.3 mM EDTA produced a 50% stimulation of the activity and 5 mM Ca^{2+} produced a 30% inhibition of the activity at both the 30 min and 1 h points. Neither of these effects support the hypothesis of a typical transglutaminase-catalysed fixation.

In once-washed chloroplast preparations, putrescine was a poor inhibitor of spermidine uptake. At putrescine/spermidine molar ratios of 1, 2 and 10 the rate of fixation of 0.1 mM spermidine was decreased by 16%, 20% and 62%, respectively. By contrast spermidine was a much better inhibitor of putrescine fixation. At molar ratios of spermidine to putrescine of 1 and 2, the rate of fixation of 0.1 mM putrescine was inhibited by 60% and 87%, respectively.

3.4. *Distribution of acid-insoluble polyamine*

In previous experiments, aliquots were precipitated with acid, filtered, and washed on the filter with 95% ethanol, and dried. When acid precipitates were extracted at 4°C with 80% acetone or 95% ethanol, it was found reproducibly that ~50% of the radioactivity was soluble in the organic solvent. A typical experiment is presented in fig.2 in which the total radioactivity rendered acid-insoluble as a func-

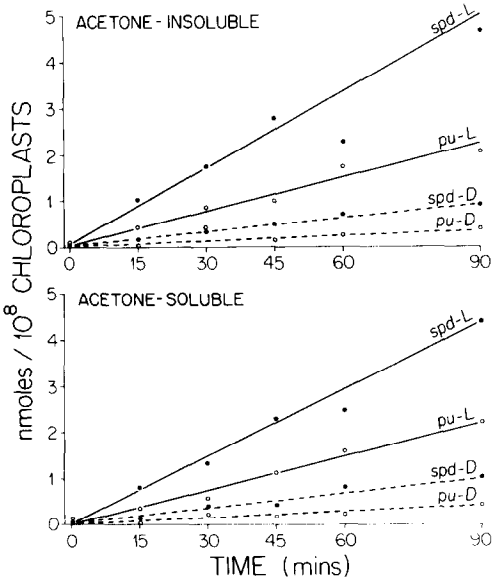

Fig.2. The acid-insoluble association of polyamines with acetone-insoluble and acetone-soluble fractions or chloroplasts in light and dark.

tion of time was subdivided into an acetone-extractable fraction and a residual protein-bound fraction. The acetone-extractable fraction was taken to dryness in vacuo and triturated with 0.03 N HCl. The acidic solution was dried, and the residue was dissolved in water and electrophoresed on paper in 0.1 N citrate buffer, at pH 3.6. The extractable radioactivity migrated as the free original polyamine. It appears that half of the 'fixed' amine had entered a hydrophobic compartment of the chloroplast inaccessible to aqueous acid and after extraction with organic solvents such material could be liberated as the free amine chloride by treatment with aqueous HCl. It is not clear if the apparent and reproducible equivalence of acetone-soluble and acid-insoluble polyamine is fortuitous.

4. Discussion

The time-dependent adventitious binding of thymidine triphosphate [15] to protein-containing fractions has been described and provides a warning concerning the possibly accidental significance of our results. However, 3 circumstances suggest that this present result is physiologically significant:

(i) A comparable acid-precipitated fraction from protoplasts incubated with labeled methionine has, on acid hydrolysis, released isolatable labeled spermidine and a labeled fraction similar to diaminopropane.

(ii) The time-dependent addition of polyamine is light-dependent.

(iii) Apparently fixed polyamine is precipitable with acid even after a thorough disruption of the chloroplast by SDS.

Nevertheless, to begin to understand these results it will be necessary to clarify the nature of the apparent polyamine salt initially unextractable into aqueous acid and soluble in organic solvents and to identify the linkage and component to which polyamine is attached after disruption of the chloroplast by SDS.

The precipitation of acid-insoluble polyamine is not affected by the addition of an excess of non-radioactive polyamine prior to the addition of acid. Also, if after incubation with labeled polyamine, the chloroplasts are lysed by SDS and diluted, the precipitate formed an addition of acid in the presence of an excess of radioactive polyamine still shows a time-dependent fixation of labeled polyamine.

Although the penetration into chloroplasts of monoamines and ammonia has been studied by centrifugation through silicone fluid layers [16] into aqueous acid or sucrose, no effort had been made in those studies to test for the binding of the amine in an acid-insoluble form. Chloroplasts used in uptake studies have most often been 60–80% intact. It is not clear then if the silicone layer technique, even if adapted to permit a simultaneous analysis of both acid-soluble and acid-insoluble amine, would permit a clear interpretation of the relation of the concentration of intrachloroplast acid-soluble amine to the formation of acid-insoluble amine in chloroplasts. In sorting out these relations it seems desirable initially to learn how to produce preparations of a higher percentage of intact organelles.

Acknowledgements

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