Leucyl-Transfer Ribonucleic Acid Synthetase in Senescing Tobacco Leaves

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1. The activity of leucyl-tRNA synthetase obtained from tobacco leaves declined by 50% over a period of 4 days senescence induced by detachment. In addition tRNA^{Leu} from senescing leaves was charged to a lesser extent than tRNA^{Leu} extracted from mature leaves immediately after detachment. 2. tRNA^{Leu} was charged with a synthetase preparation from either mature or senescent leaves and chromatographed on an RPC 3 column. The elution profile showed that the marked decline in specific activity of leucyl-tRNA synthetase in senescent leaves was not associated with a loss of acylation of any isoacceptor of tRNA^{Leu}.

There have been some reports about changes in aminoacyl-tRNA synthetase activity in the course of cell differentiation. Bick & Strehler (1971) showed that the capacity of synthetase to acylate the isoacceptors of tRNA^{Leu} is modified during senescence of soya-bean cotyledons. Investigating the regulation of protein synthesis during the differentiation of *Tenebrio* pupae, Ilan (1969) found that a synthetase preparation from a mature stage increased the extent of acylation of tRNA^{Leu} that had been initially acylated by a synthetase from a younger stage. A summary concerning aminoacyl-tRNA synthetases of plants is presented in the review of Lea & Norris (1972).

The present work relates to the thesis that senescence reflects the final stage of cell differentiation and that the modifications in protein metabolism occurring at this stage may be regulated at the translational stage of protein synthesis (Strehler *et al.*, 1967). There is evidence to indicate that detached leaves maintained in darkness and kept turgid undergo changes comparable with those typically occurring in senescing attached leaves (Wollgiehn, 1967). After research of other investigators (Henshall & Goodwin, 1964; Anderson & Fowden, 1969), we chose to test whether experimentally induced leaf senescence was associated with modifications in aminoacyl-tRNA synthetase activity. This was tested by measuring the activity of leucyl-tRNA synthetase in mature and senescent tobacco leaves and by chromatographic characterization of aminoacylated tRNA^{Leu}.

& Arnon, 1939). After detachment leaves were thoroughly washed as described by Richmond *et al.* (1971). The leaves were then either used immediately (mature, or day-0 leaves) or allowed to senesce for 4 days in dark and humid containers (senescent, or day-4 leaves).

Synthetase preparation

The main veins were removed from 50 g of leaf tissue, which was homogenized in a Waring Blendor with 50 ml of homogenizing buffer, which contained (Anderson & Rowan, 1966a), 30 mM-2-mercaptoethanol and 16 mM-MgCl₂ in 0.1 M-sodium phosphate buffer, pH 7.2. The homogenate was filtered through Miracloth and centrifuged at 12000g for 10 min. The supernatant was collected and centrifuged (105000g, 1 h) and the high-speed supernatant was treated with solid (NH₄)₂SO₄. The fraction precipitated between 40 and 50% saturation of (NH₄)₂SO₄ was collected by centrifugation (20000g for 20 min) as this fraction was initially found to be the most active in acylation of leucine. The pellet was dissolved in a small volume of the homogenizing buffer and the protein was dialysed overnight against half-strength homogenizing solution. Glycerol was added to the dialysed solution to one-third of the initial volume and the preparation was used as the source of synthetase. This procedure yielded a 65-fold increase in the specific activity of the enzyme over the activity in the crude homogenate. Protein content was determined as described by Lowry *et al.* (1951).

Materials and Methods

The first two fully expanded leaves closest to the apex of *Nicotiana rustica* L. plants were used for all the experiments. The plants were grown in a greenhouse on half-strength Hoagland solution (Hoagland

tRNA preparation

tRNA was extracted from tobacco leaves by a procedure adapted from that of Zubay (1962) except that bentonite was added to the homogenizing

buffer to give a final concentration of 0.5%. The nucleic acid solution was passed through a column (1 cm $\times$ 10 cm) of DEAE-cellulose (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and was washed with 0.2 M-NaCl in 0.1 M-Tris-HCl buffer (pH 7.5). tRNA was then eluted with 0.6 M-NaCl in the same buffer. The u.v. absorbance of the fractions collected was measured at 260 and 280 nm, and the fractionated tRNA was precipitated overnight at -15°C in 2 vol. of ethanol containing potassium acetate (final concn. 2%, w/v).

Aminoacylation of tRNA

Aminoacylation was determined as described by Zachau (1970). The final concentrations in the reaction mixture were 10 mM-ATP, 15 mM-NaCl, 15 mM-Mg²⁺, 25 mM-Tris-HCl buffer (pH 7.5), 20 μg of protein, 0.75 E_{260} unit of tRNA and 0.3 μCi of [¹⁴C]leucine (342 mCi/mmol) to a final volume of 110 μl . After 15 min at 25°C the reaction was stopped by pipetting the mixture on to filter-paper discs which were washed successively with cold 5% (w/v) trichloroacetic acid, ethanol and ether, and the radioactivity was measured in toluene containing Liquiflor in a Packard Tri-Carb liquid-scintillation spectro-

meter. For co-chromatography of native and re-natured tRNA^{L^{eu}}, [³H]leucine (500–1000 mCi/mmol) was also used in the aminoacylation assays. All the radioactive material was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Reverse-phase column chromatography

A reaction mixture containing about 10 E_{260} units of tRNA in 2 ml final volume was charged with [¹⁴C]leucine. After incubation the aminoacylated tRNA was precipitated with HCl and purified with phenol and ether as described by Zachau (1970).

A RPC 3 column was used as described by Weiss *et al.* (1968). Chromosorb G obtained from Johns Manville Corp., New York, N.Y., U.S.A. was acid-washed, treated with dimethyldichlorosilane and finally coated with triethylpropylammonium bromide (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.). The column (1.5 cm $\times$ 100 cm) was kept at 37°C . tRNA was eluted with a 2-litre gradient of 0.4–0.7 M-NaCl in the elution buffer. Fractions (8 ml) were collected at a rate of 1.2 ml/min. The radioactive aminoacylated tRNA was precipitated in cold 5% (w/v) trichloroacetic acid, collected on filter-paper discs, and the radioactivity was counted as before.

Fig. 1. Effect of the concentration of day-0 and day-4 enzyme preparation on the aminoacylation of day-0 and day-4 tRNA

A reaction mixture was used as described in the Materials and Methods section. (a) ○, Day-0 tRNA and day-0 enzyme; ●, day-4 tRNA and day-0 enzyme; (b) ○, day-0 tRNA and day-4 enzyme; ●, day-4 tRNA and day-4 enzyme.

Results

A marked modification in aminoacylation capacity occurred in senescing tobacco leaves. This is evident by comparison of the relative aminoacylation of tRNA^{Leu} charged with enzyme preparations from mature (day-0) and senescing (day-4) leaves (Fig. 1). This graph shows maximal acylation of a given quantity of tRNA^{Leu} by increasing quantities of the enzyme preparation. The highest point represents maximal acylation of all the species of tRNA^{Leu} that may be acylated by a given preparation. The aminoacylation potential decreases by about 50% in preparations of leucyl-tRNA synthetase extracted from senescent (day-4) leaves (Fig. 1). In addition, a large decrease took place in the extent of leucine-acylation of tRNA from day-4 leaves as compared with tRNA from day-0 leaves. The enzyme preparation from day-4 leaves was also less active in acylation of tRNA^{Leu} from day-4 leaves than toward tRNA from day-0 leaves. The possibility that the day-4 enzyme preparation contained relatively more factors that affect the extent of aminoacylation *in vitro*, such as ribonuclease or proteinases, was tested. Preparations from either day-0 or day-4 leaves were preincubated with tRNA and the other constituents of the reaction mixture in the absence of amino acid. Preincubation with either preparation caused a slight and similar decrease in the extent of leucine acylation (Table 1).

Table 1. Effect of preincubation of the reaction mixture with the enzyme preparation on the extent of aminoacylation

Preincubation was carried out under conditions identical with those of the aminoacylation assay. Amino acid was absent from the reaction mixture during the preincubation. Change (%) means change in extent of charging after preincubation (b), compared with the value without preincubation (a).

Preincubation	tRNA (day)	Enzyme (day)	Extent of aminoacylation (pmol/E ₂₆₀ unit of tRNA)	Change (%)
(a) -	0	0	34	
(b) +	0	0	31.9	-6.1
(a) -	0	4	18.2	
(b) +	0	4	16.6	-8.3
(a) -	4	0	16.7	
(b) +	4	0	15.5	-7.1
(a) -	4	4	12.4	
(b) +	4	4	11.2	-9.6

There is no evidence to assume that the decline in acylation potential in preparations from senescent leaves stems from an enhanced hydrolytic activity. Likewise, there was no indication that the preparations differed in leucine-degrading (not shown) or adenosine triphosphatase activity.

Fig. 2. Reverse-phase chromatography of [¹⁴C]Leu-tRNA^{Leu} obtained from day-0 leaves and charged with enzyme preparation of either day-0 (a) or day-4 (b) leaves

Details are given in the Materials and Methods section. The vertical axis refers to radioactivity in the 8 ml fractions collected from the column.

Fig. 3. Reverse-phase chromatography of native (—) and renatured (----) $tRNA^{Leu}$ charged with [3H]- and [^{14}C]-leucine respectively

Native refers to the state of tRNA when it was isolated and acylated. Renatured tRNA refers to tRNA that, after isolation, was incubated in 0.01M- $MgCl_2$, 0.001M-EDTA and 0.01M-Tris-HCl buffer (pH8) at 60°C for 5 min and quickly cooled before acylation.

It was queried whether the senescence-induced decline in leucine-acylation activity was associated with a change in acylation of the various isoacceptors of $tRNA^{Leu}$. For this purpose, the isoacceptor profile of $tRNA^{Leu}$ charged by the synthetase from day-0 leaves was compared with that acylated by the enzyme from day-4 leaves. As shown in Fig. 2, the marked decline in total leucyl-tRNA synthetase activity was not associated with a loss of the potential to acylate any specific isoacceptor of $tRNA^{Leu}$.

The chromatographic properties of the fractionated tRNA were not changed after renaturation in the presence of Mg^{2+} at 60°C (Fig. 3). This is consistent with the premise that the six chromatographic species of $tRNA^{Leu}$ obtained in this work consist of intact strands, without fragments or incomplete molecules. That the $tRNA^{Leu}$ species were in monomeric forms was tested by filtration on Sephadex G-100. This procedure yielded a single elution peak, as was also obtained by Barnett *et al.* (1969) and Guderian *et al.* (1972).

Discussion

The decline of some 50% in the activity of leucyl-tRNA synthetase in tobacco leaves found 4 days after leaf detachment is in agreement with the findings of Bick & Strehler (1971), who investigated changes

in leucyl-tRNA synthetase activity in peanut cotyledons. Anderson & Rowan (1966b), however, who investigated total aminoacyl-tRNA synthetase activity in detached tobacco leaves, reported an initial increase in total synthetase activity followed by a decrease. Concomitantly, they obtained an increase in the specific activity of the preparation owing to the more pronounced decline in total leaf protein. Since the results presented here concern a specific synthetase, it may thus be that leaf senescence is involved with different modifications in the various aminoacyl-tRNA synthetases, some species disappearing faster than others.

The data in Fig. 1 demonstrate that the enzyme preparation contained an inhibitory activity for leucine-acylation in both mature and senescent leaves. This was reported by others who worked with preparations from bulbs of *Galega officinalis* L. (Moustafa & Proctor, 1962), tobacco leaves (Anderson & Rowan, 1966a) and pea seeds (Anderson & Fowden, 1969). The latter considered adenosine triphosphatase activity as being a contributing or even a causal factor for the inhibition. In our preparation, the inhibition of leucine-acylation is not due to an indirect effect on the components of the reaction system *in vitro*, i.e. $tRNA^{Leu}$, ATP and [^{14}C]leucine. Preincubation of the enzyme preparation with these reactants did not appreciably decrease the extent of the reaction. Whether this inhibitory activity relates to a significant control mechanism remains to be elucidated. Our finding that the senescence-induced decrease in total leucyl-tRNA synthetase activity was not associated with a significant modification in the relative acylation of the six isoacceptors of leucine is in contrast with the conclusions of Bick & Strehler (1971). They reported that the aging of peanut cotyledons was associated with a modification of the relative acylation of the isoacceptors of leucyl-tRNA, the acylation of $tRNA^{Leu}$ species 5 and 6 increasing in comparison with that of $tRNA^{Leu}$ species 1 and 2. This apparent disagreement with our results is interesting, since like Guderian *et al.* (1972), we also found that $tRNA^{Leu}$ species 5 and 6 are specific to the chloroplasts (not shown). Perhaps in peanut cotyledons, which are not exposed to light, these isoacceptors of $tRNA^{Leu}$ reside in the proplastids and the relative rise in their activity from day 2 to 15 that Bick & Strehler (1971) reported may relate to organelle modifications rather than to senescence.

Our findings support the possibility that the well-established senescence-induced decrease in protein synthesis in leaves is at least partially due to a decrease in aminoacyl-tRNA synthetase activity.

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