IDENTIFICATION OF CHLOROPLAST NADP-LINKED MALATE DEHYDROGENASE AS A PRODUCT OF CHLOROPLAST PROTEIN SYNTHESIS

V. GEETHA*, A. Habib MOHAMED* and A. GNANAM
School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Received 28 January 1981

1. Introduction

Chloroplasts contain transcriptional and translational machineries which closely resemble that of the prokaryotic system and are distinct from that of the nuclear–cytoplasmic systems of higher plants and algae. Perhaps the most direct and effective method of elucidating the coding potential of the chloroplast genome is to follow and analyze the products synthesized by the isolated organelle in vitro. Some of the polypeptides synthesized by the isolated chloroplasts have been identified and characterized [1–4].

We have shown that the mesophyll chloroplasts of *Sorghum vulgare*, a C_4 plant, could be used effectively in the studies of protein synthesis in vitro and as an alternate assay system for exogenous RNAs [5]. *Sorghum vulgare* is an NADP-malic enzyme-type of plant possessing NADP-linked malate dehydrogenase, a key enzyme involved in the reduction of C_4 acids, in the mesophyll chloroplasts. Analyses of the immunoprecipitates of the whole-leaf RNA-translated products with anti NADP-malate dehydrogenase on SDS–urea polyacrylamide gels reveal the syntheses of 2 polypeptides of the enzyme (45 000 and 33 000 M_r), whereas that of the chloroplast RNA translated products only the larger of the 2. Hence, it is suggested that the synthesis of chloroplastic NADP-linked malate dehydrogenase requires the cooperation of both the nuclear and chloroplastic genes.

Abbreviations: MDH, malate dehydrogenase; RuBP carboxylase, ribulose-1,5-bisphosphate carboxylase; SDS, sodium dodecyl sulfate

* Present address: Dept. Biological Sciences, University of Illinois at Chicago Circle, Box 4348, Chicago, IL 60680, USA; to whom correspondence should be directed

2. Materials and methods

Sorghum vulgare var. CO 20 was the source of chloroplasts in the experiments reported here. ^{14}C-Labelled *Chlorella* protein hydrolysate (27–42 Ci/Catom) was purchased from the Isotope Division, Bhabha Atomic Research Centre (Bombay). The chemicals used were of analytical reagent grade obtained from BDH Laboratory Chemicals (Bombay). The finer chemicals were purchased from Sigma Chemicals (St Louis MO).

Isolation of mesophyll chloroplasts and protein synthesis in vitro were performed as in [5]. Reaction mixtures were appropriately scaled up for the analysis of the immunoprecipitates.

Whole leaf RNA from *Sorghum* was extracted by the modified hot phenol method as in [6]; RNA was extracted from chloroplasts as in [7]. NADP-linked malate dehydrogenase (NADP-MDH) was purified from 10-day-old leaves of *Sorghum vulgare* as in [8] with the following modification: the 45–55% $(NH_4)_2SO_4$ precipitate was used for further purification of the enzyme. Assay for NADP-MDH was as in [9].

The purified NADP-MDH (~2 mg) suspended in 1.0 ml phosphate-buffered saline (pH 7.2) (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4 and 0.02% KH_2PO_4) was emulsified with 1.5 ml Freund's complete adjuvant (Difco) and injected into rabbits both intracutaneously (at 10 different places at the back) and intramuscularly in the hind legs. Four weeks later, 3 consecutive boosters of 1.0 mg antigen in 1.0 ml phosphate-buffered saline emulsified with 0.5 ml Freund's incomplete adjuvant were given intravenously. Five days after the last booster injection, the rabbit blood samples were obtained by bleeding the ear veins. The serum was collected and tested for cross-reactivity against the purified NADP-MDH by

Ouchterlony's double diffusion [10] and immunoelectrophoresis [11]. Null serum was taken from the rabbit before immunization. Higher quantity of purified NADP-MDH protein was used for immunization, as we have found the protein to be a poor antigen.

The products synthesized in vitro were analyzed on SDS–polyacrylamide gels as in [12]. The immunoprecipitates of the products synthesized by preincubated chloroplasts primed with *Sorghum* whole leaf or chloroplast RNA with anti-native NADP-MDH, were solubilized by heating in 8.0 M urea and 2.5% 2-mercaptoethanol to which SDS was added to 2% final conc. and the fractionation was done essentially as in [12]. The gels were cut into 1 mm slices in a Mickle gel slicer, solubilized in 30% H_2O_2 and the radioactivity was counted in liquid scintillation counter (LSS 34, Electronic Corporation of India). Chlorophyll and protein were estimated as in [5].

3. Results

The various steps used to purify the NADP-linked malate dehydrogenase and the extent of purification that could be achieved are shown in table 1. Most of the protein fraction having maximal enzyme activity could be precipitated between 45–55% saturation of ammonium sulfate and the resultant enzyme had a specific activity 4-times greater than the crude enzyme. Further purification on DEAE-cellulose column resulted in a greater increase in the specific activity of the enzyme and subsequent efforts to purify it on a Sephadex G-200 column did not bring about any further increase in the specific activity as can be seen from the data. All these steps of purification resulted in an ~50-fold increase in purity and the final yield was 22%. When the eluate from the Sephadex G-200 column was analyzed on 7.5% polyacrylamide gels under non-denaturing conditions, a single homogenous band was obtained. Any possible contamination of other proteins was checked by overloading the gels with the purified protein. A M_r determination of the purified protein on a gel filtration column suggested a value of 76 000 (not shown).

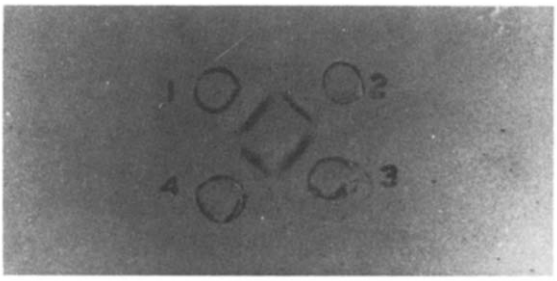

Fig.1. Ouchterlony's immunodiffusion of NADP-malate dehydrogenase at different stages of purification against anti-NADP-MDH in the central well: (1) crude NADP-MDH; (2) 45–55% saturation ammonium sulfate precipitate; (3) DEAE-cellulose eluate; (4) Sephadex G-200 eluate.

The antibody obtained for NADP-MDH was found to be mono-specific as can be seen from immodiffusion. Fig.1 shows the precipitin bands obtained with the enzyme at different stages of purification such as

Table 1
Purification of NADP-linked malate dehydrogenase (EC 1.1.1.82) from the leaves of *Sorghum vulgare*

Fraction	Total protein (mg)	Total units (μmol/min)	Specific activity (μmol . mg protein^{-1} . min^{-1})	Purification (-fold)	Yield (%)
Crude homogenate	696	301.8	0.4	1	100
Ammonium sulfate fractionation 45–55% saturation	62.3	116.5	1.9	4.3	39
DEAE-cellulose chromatography	4.7	89.7	19	43.7	30
Sephadex G-200 filtration	3.3	65.4	20	45.9	22

crude, 45–55% ammonium sulfate precipitate, DEAE-cellulose and Sephadex G-200 eluates against anti-NADPH-MDH placed in the center. No precipitin lines were obtained when the protein fractions were diffused against null serum, confirming the authenticity of the reaction. The specificity of the antiserum was further checked by the near 95% inhibition of the enzyme activity which was unaffected by the pre-immune serum (not shown).

Sorghum mesophyll chloroplasts contain sufficient quantities of NADP-MDH protein endogenously to produce clear precipitin lines; hence the identity of this protein as one of the translational products of protein synthesis in vitro in the preincubated chloroplasts primed with exogenous templates could not be established unambiguously by the commonly used immunological techniques like immunodiffusion and electrophoresis. Consequently, the synthesis of this particular protein was established by the detection of radioactivity in the precipitin bands. Considerable amount of label was present in the precipitin bands formed by the reaction between anti-NADP-MDH and endogenous, *Sorghum* chloroplasts and whole-leaf RNA-translated products, thus indicating the de novo synthesis of MDH. No radioactivity could be found in the precipitin lines formed between preincubated chloroplasts and anti-NADP-MDH, since no protein synthesis could occur in the absence of exogenous templates (table 2). Agar slices on either side of the precipitin lines contained no radioactivity.

Table 2
Radioactivity associated with the precipitation bands formed between different products and anti NADP-malate dehydrogenase

Various products	Precipitation bands of immunodiffusion (cpm/arc)	Precipitation bands of immunoelectrophoresis (cpm/arc)
Endogenous product	844	984
Preincubated chloroplasts (–RNA)	38	61
Chloroplast RNA-translated product	952	1096
Whole-leaf RNA-translated product	1148	1464

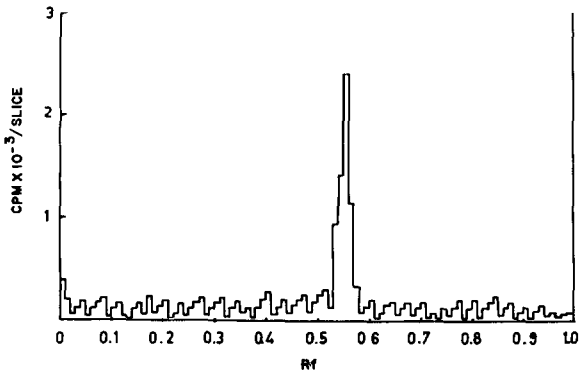

Fig.2. SDS–urea polyacrylamide gel electrophoresis of the immunoprecipitates of the translated products of the preincubated chloroplasts primed with *Sorghum* chloroplast RNA. Similar results were obtained with *Sorghum* endogenous RNA translated products also. The peak corresponds to the polypeptide of 45 000 M_r. In this and in the subsequent figure, controls with the translated products against the pre-immune serum/goat anti-rabbit IgG were co-electrophoresed and the radioactivity (~2%) associated with the corresponding gel slices corrected for the non-specific adsorption of radioactivity, from the experiments.

When the immunoprecipitates of the chloroplast endogenous products or that synthesized by the preincubated chloroplasts primed with *Sorghum* chloroplast RNA with anti-NADP-MDH were analyzed on SDS–urea polyacrylamide gels, a single polypeptide with M_r 45 000 was obtained (fig.2). An additional minor polypeptide of M_r 33 000 was found to be labeled besides the major polypeptide of 45 000 M_r in the case of the translational products made by the preincubated chloroplasts primed with *Sorghum* whole-leaf RNA (fig.3).

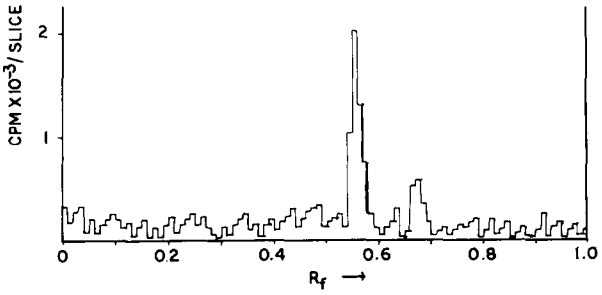

Fig.3. SDS–urea polyacrylamide gel electrophoresis of the translated products of the preincubated chloroplasts primed with *Sorghum* whole leaf RNA. The peaks correspond to the polypeptides of 45 000 and 33 000 M_r.

4. Discussion

Studies on the protein synthesis of isolated chloroplasts of C_3 plants, viz. pea and spinach, have been documented [1,2]. In the light of the known functional differences between the mesophyll chloroplasts of C_3 and C_4 plants, the observations with the isolated chloroplasts of *Sorghum vulgare*, a NADP-malic enzyme type of C_4 plant, are perhaps significant. The *Sorghum* chloroplast protein synthesizing system has several advantages over the other cell-free systems currently in use [5]. It can be easily depleted of the endogenous mRNA by incubating in light at 20 000 lux at 25°C for 1 h without affecting the overall protein synthetic efficiency and the activity can be restored to the original level by adding exogenous *Sorghum* chloroplast or whole leaf RNA. The maximal rate of amino acid incorporation is ~1.5–2.0 nmol leucine . mg chl.$^{-1}$. h^{-1}. The efficiency of translation is also comparable to that of other commonly used systems such as wheat germ [5].

Few of the proteins synthesized in vitro in the isolated chloroplasts are associated with the stroma [13,14]. Excepting the large subunit of RuBP carboxylase and the elongation factors, other soluble proteins synthesized by the chloroplasts have not been conclusively identified. The absence of RuBP carboxylase, a soluble protein which constitutes 50% of the leaf protein, in the C_4 mesophyll chloroplasts of *Sorghum vulgare* prompted us to investigate the synthesis of other soluble proteins. This report presents evidence for the possible synthesis of a subunit of NADP-linked malate dehydrogenase in the C_4 mesophyll chloroplasts. From our data it is likely that NADP-linked malate dehydrogenase of *Sorghum vulgare* possesses two non-identical subunits and that their formation, possibly, the assembly and function apparently require the interaction of the organellar and the nuclear genomes; the larger subunit is made by the chloroplast and the smaller one by the nucleus. However, further experiments which are in progress might unequivocally demonstrate the site of synthesis of the protein. This is perhaps analogous to the synthesis of RuBP carboxylase in chloroplasts [1], the oligomycin-sensitive ATPase and cytochrome oxidase in mitochondria [15], by both the genomes. The multisubunit completion principle [16] which states that complete multimeric proteins are not made solely on organellar ribosomes seems to be true in the case of NADP-linked malate dehydrogenase of *Sorghum vulgare* also.

References

[1] Blair, G. E. and Ellis, R. J. (1973) Biochim. Biophys. Acta 319, 223–243.
[2] Mendiola-Morgenthaler, L. R., Morgenthaler, J. J. and Price, C. A. (1976) FEBS Lett. 62, 96–100.
[3] Geetha, V. and Gnanam, A. (1980) FEBS Lett. 111, 272–276.
[4] Doherty, A. and Gray, J. C. (1979) Eur. J. Biochem. 98, 89–92.
[5] Geetha, V. and Gnanam, A. (1980) J. Biol. Chem. 255, 492–497.
[6] Girard, M. (1967) Methods Enzymol. 12 A, 581–584.
[7] Loening, U. E. and Ingle, J. (1967) Nature 215, 363–367.
[8] Ting, I. P. and Rocha, V. (1971) Arch. Biochem. Biophys. 147, 156–164.
[9] Johnson, H. S. and Hatch, M. D. (1970) Biochem. J. 119, 273–280.
[10] Williams, C. A. and Chase, M. W. (1971) Methods Immunol. Immunochem. 3, 35–56; 146–160; 188–191; 297–298; 435–453.
[11] Campbell, D. H., Garrey, J. S., Cremer, N. E. and Sassdorf, D. H. (1964) Methods Immunol. 143–150.
[12] Laemmli, U. K. (1970) Nature 227, 680–685.
[13] Geetha, V. and Gnanam, A. (1980) Biochim. Biophys. Acta 608, 427–434.
[14] Morgenthaler, J. J. and Mendiola-Morgenthaler, L. R. (1976) Arch. Biochem. Biophys. 172, 51–58.
[15] Poyton, R. O. and Schatz, G. (1975) J. Biol. Chem. 250, 752–761.
[16] Ellis, R. J. (1977) Biochim. Biophys. Acta 463, 185–215.