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PHOTOREGULATION PROCESS OF SORGHUM LEAF PHOSPHOENOLPYRUVATE CARBOXYLASE: STUDY WITH MONOCLONAL ANTIBODIES

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Monoclonal antibodies were produced against the C isozyme subunit of PEP carboxylase (PEPC) from Sorghum leaves by the hybridoma technique. More than 400 antibodies-producing hybridomas to PEPC were produced from the fusion of spleen cells from immunized mice with NS1 myeloma cells. By using an ELISA, three hybridomas (91-G, 83-G, 49-EG) were selected. Monoclonal antibodies were subsequently characterized in a Western experiment; Mabs 83-G and 91-G were found to be highly specific to the G isozyme whereas Mab 49-EG recognized both forms (E and G isozymes) of the enzyme. Addition of Mabs to the enzyme preparation did not modify its catalytic activity nor its activation by glycine. Use of these probes provided direct and definite evidence of the specific enhancing effect of light on the G form and on its corresponding mRNA.

PEPC (EC 4.1.1.31) is involved in the primary step of photosynthetic CO_2 fixation pathway in C_4 type plants (malate former). At least two predominant isozymes (named E and G forms) occur in sorghum leaves; they are oligomeric enzymes composed of four subunits with very similar molecular weight of about 92000 daltons (1). Comparable results showing enzyme polymorphism have also been reported in the case of maïze leaf PEPC (2). Previously, the G form from both plants was purified to homogeneity and specific polyclonal antibodies

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Abbreviations:
BSA, bovine serum albumine; E-PEPC, enzyme present in etiolated leaves; ELISA, enzyme linked immunosorbent assay; G-PEPc, enzyme present in green leaves; HTP, hydroxylapatite; Mab, monoclonal antibody; MW, molecular weight; PEPC, phosphoenopyruvate carboxylase; PBS, phosphate buffer saline pH 7.4; TCA, trichloracetic acid.

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were raised (3-4-5-6-7). The immuneserum was used in several ways: i, to determine PEPC protein amounts during greening of the leaves (1-4-5-7); ii, to isolate antigen in one step purification by using an immunosorbent technique (8); iii, to characterize PEPC peptide among translation products in an *in vitro* translation system programmed by poly A^+ RNAs extracted from leaves (4-5-6-9); iiii, to localize the enzyme in leaf sections (10). These studies led different authors to the conclusion that E form is present in etiolated material and at all steps of the ontogenic process of the plant whereas the appearance of the G form is dependent upon light and strictly restricted to the cytoplasm of mesophyll cells (11); in addition, it has been demonstrated that phytochrome pigment is involved in the photocontrol process (12). However, although sera have been shown, in most cases, to be highly specific for PEPC, they were subsequently found to strongly cross react with other PEPCs from various plant sources, including the E form present in sorghum leaf (13). This property of antibodies has been used in a comparative study on plant PEPC immunochemical relationships (14). As a consequence, each time isozymes are simultaneously present in extracts, they become immunochemically indistinguishable and in such a case, polyclonal antibodies are of a limited value. In order to study the submolecular level of the two isozymes separately, we raised monoclonal antibodies against subunits of the G enzyme from sorghum leaf.

MATERIAL AND METHODS

Antigen extraction and purification.

Seedlings of sorghum vulgare cv. Tamaran FNK 140 were grown as already described (3); PEPC (G form) was extracted and purified from sorghum leaves by ammonium sulfate precipitation followed by DEAE cellulose and HTP chromatography (1-3). Enzyme was immunoprecipitated from extract with polyclonal antibodies according to (1). Before immunization, PEPC subunits were separated from IgG by HPLC (15).

Production of monoclonal antibodies.

Monoclonal antibodies were produced by immunization procedure involving a subcutaneous injection (s.c.) of 50 µg of purified PEPC subunit in Freund's complete adjuvant as the initial dose into Balb/c mice, followed by a s.c. injection of 50 µg in Freund incomplete adjuvant four months later. The animals were boosted by intraperitoneal (50 µg) and intravenous (50 µg) injections, nine and three days respectively before the fusion. The cell fusion was performed by incubating spleen cells with NS1 myeloma cells (5:1) in the presence of 40% polyethylene glycol 1000 (16). Hybridomas were selected by an ELISA, following instructions provided in the Monoclonal Antibody Screening System kit (Goat anti-mouse Ig-HRP Conjugated Screening System; NEN,

Boston, Massachussetts). 96-well plates were coated (1h, 37°C) with 1 µg of PEPC (in 0.1M phosphate buffer, pH 7.4) from crude extract or purified to homogeneity as described above. Hybridomas were cloned twice at limiting dilution and large amount of monoclonal antibodies were obtained from ascitic fluids produced by intraperitoneal inoculation of nude mice with 10^6 hybridoma cells (16). Immunoglobulin isotypes of each clone were determined according to (17).

Characterization of the monoclonal antibodies.

- western blots

PEPC protein or subunits were electrophoretically separated respectively on non-denaturing 7% polyacrylamide gel or 10% denaturing SDS-polyacrylamide gel, and then electrotransferred onto nitrocellulose sheets (0.45 µm pore size, Schleicher and schull) in a Bio-rad transfer apparatus (Bio-rad, Richmond, CA) at 0.17 A for 3h. The sheet was then soaked into blocking buffer (3% BSA in 10mM phosphate buffer pH 7.4, NaCl 0.9%) at 37°C for 1h. The nitrocellulose was incubated overnight at 4°C with the primary antibodies (culture supernatants diluted to 1/50 in blocking buffer supplemented with 0.1% Triton X100). The blot was washed for 1h with 6 changes of PBS containing 0.1% Triton X100. The second antibody, goat anti-mouse IgG [^{125}I] (NEN, Boston, Massachussetts) was diluted to 0.01 µg/ml (2.10^5cpm/ml) with PBS containing 1% BSA and incubated with the blot for 1h at room temperature. After washing, the dried sheet was exposed to an X-Ray film for autoradiography.

- Affinity purification of the antibodies

Mabs were purified by chromatography on anti-mice IgG bound to Affigel (Bio-rad, Richmond, CA) or enzyme-bound Affigel. Goat anti-mice IgG (16 mg) were linked to Affigel (1 ml of deposited gel). Enzyme-bound Affigel was obtained by linking 2 U of G type PEPC (about 60 ug of protein) to 800 µl of deposited gel. PEPC protein used in this experiment was purified to homogeneity by immunochromatography as described elsewhere (1). Mabs were ammonium sulfate precipitated (50% saturation) from ascitic fluids or culture supernatant; protein pellets were redissolved in PBS buffer and deposited onto the column. After rinsing with PBS (5ml), 0.25M Tris/HCl buffer pH 8 containing $(NH_4)_2SO_4$ (5 ml), PBS buffer (5 ml), fixed Mabs were eluted by 4 ml of acetic acid 0.5 M, ammonium sulfate precipitated (40% saturation) and pellets redissolved in PBS buffer.

- Effect of Mabs on enzymatic activity and regulation of the G form

5 µg of purified antibodies were incubated with 0.5 U of PEPC (G form partially purified) in 150 µl of PBS. PEPC activity and its activation by glycine were recorded as a function of time.

RNA extraction and in vitro translation experiment.

They were performed as previously described (9). The immunoprecipitation was carried out as follows: either 8 µl of polyclonal serum or 10 µg of purified Mabs (91 G and 83 G) were added to translation medium (450000 cpm of TCA precipitable material) diluted to 1 ml with TNET buffer (Tris (Cl^-) 20 mM pH 7.5, NaCl 140 mM, EDTA 1 mM, Triton X100 1%, Methionine 2 mM), the mixture was incubated overnight at 4°C. Then, immunecomplexes were selected by addition of protein A-sepharose in the case of polyclonal antibodies and rabbit anti mouse IgG for 83-G and 91-G Mabs. Pellets were washed three times in 1 ml of TNET buffer then dissociated in 25 µl of dissociating buffer and submitted to SDS-gel electrophoresis according to Laemmli (18); gel was dried and fluorographed at -80°C.

RESULTS AND DISCUSSION

Numerous studies have been and are still devoted to the photoregulation process leading to the accumulation of C4 plant PEPC in the course of leaf greening. Previous works had firmly established that this carboxylating activity was in fact catalyzed by several isozymes; two main forms, E and G,

were described in leaves (2,3,4). On the other hand, the hypothesis according which the observed large increase in activity is correlated to synthesis of G enzyme mRNA was put forward and is now widely accepted (5-6-7). However, neither various sera nor complementary DNA raised in several laboratories (1-2-4-5-6-7) to be used in such studies were demonstrated to be specific to the G form or its mRNA. For instance, polyclonal antibodies raised against the G form not only cross-reacted with the E form but also with all plant PEPC tested (13); and, so, only indicated the global fate of isozyme family. Although accumulation of total PEPC protein has become patent during leaf greening, the question of whether the increase in protein amount only concerns the G form or, alternatively, the E form followed by its conversion to G form by post-translational modification, remained opened. Therefore, in order to strictly examine the light dependency of the G isozyme, we raised monoclonal antibodies against the G-PEPC form from sorghum leaf.

200 G-PEPC antibody-producing hybridomas were selected on the basis of highest absorbance readings in the ELISA, and screened separately for their reactivity with E and G forms of sorghum PEPC using a second ELISA test. Three clones, producing IgG1 antibodies, namely 91-G , 83-G and 49-EG were selected for further experiments. The Mabs 83-G and 91-G exhibited a very high specificity to the G form of PEPc, whereas 49-EG recognized both forms. The Mabs were purified from ascitic fluids or from culture supernatant. An immunocolumn was prepared by linking a highly purified G- PEPC to a gel matrix on which Mabs were adsorbed efficiently. The elution was obtained by acid washing of the gel and about 150 µg of Mabs 91-G and 83-G were retained by the affinity column. Mabs were also purified on goat anti-mice IgG Affigel column; 16 mg of goat antibodies, insolubilized on 1 ml of gel, retained about 800 µg of monoclonal antibodies.

The reactivity of monoclonal antibodies with either the subunit or the oligomeric form of the enzyme was proved by a western experiment. Fig. 1 shows that the protein band revealed by antibodies 91-G, 83-G and 49-EG corresponds to the subunit of the G form; a similar result is observed when the G form in

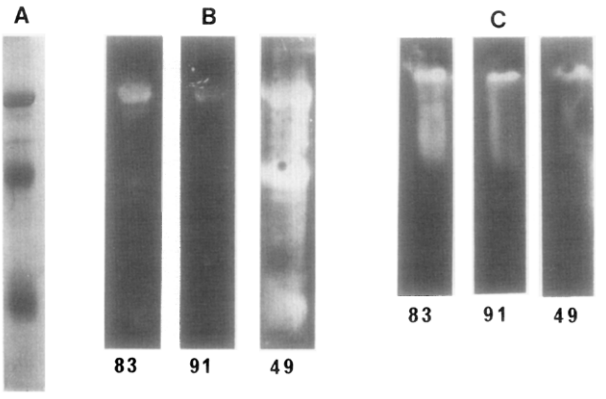

Fig. 1. Binding of monoclonal antibodies to Western blots of PEPC.
1 U (30 µg) of PEPC (essentially of G type) was immunoprecipitated in green leaf extracts by adding rabbit polyclonal antibodies as previously described (1). The precipitated protein was dissociated, then electrophoresed in 10% denaturing SDS-polyacrylamide gel according to Laemmli (18).
1 U (30 µg) of G type PEPc purified by DEAE cellulose and HTP chromatography (3) was electrophoresed in non denaturing 7% polyacrylamide gel according to Laemmli (18).
The blots were successively treated with Mabs solution followed by an anti-mice antibody solution iodine labelled. After rinsing, the blots were autoradiographed for 12h in the case of Mabs 91-G and 83-G or 24h for Mabs 49-EG.
(A): Coomassie blue staining of proteins separated by SDS-gel electrophoresis;
(B): Autoradiography of the corresponding blots;
(C): Autoradiography of blots obtained in a similar experiment except that proteins were not SDS dissociated before gel electrophoresis.

its oligomeric state is used instead of the subunit. Therefore specific epitopes are recognized by antibodies whatever the agregation state of PEPC, monomeric or multimeric, suggesting that they are not masked when subunits are organized in a quaternary structure. The specificity of the Mabs 91-G and 83-G towards the G-PEPC form and common recognition of G and E PEPC forms by the Mab 49-EG was investigated using an ELISA. Fig. 2 shows that at concentrations ranging from 10^{1} to 10^{-2} µg/ml, Mab 91-G is specific to the G-PEPC form. The Mab 49-EG gave a similar response for both forms of the enzyme. No effect was observed on PEPC activity when the enzyme was incubated with Mabs 49-EG or 91-G at saturating concentrations. Moreover the effect of glycine, a potent activator of G-PEPC activity (2-3 fold), was not significantly modified by any of the Mabs. The result suggested that these antibodies did not bind on or in the vicinity of the corresponding activating site of the protein.

After having ascertained the high specificity of Mabs 91-G and 83-G to G PEPC we took advantage of these probes to reexamine the light dependency of the G

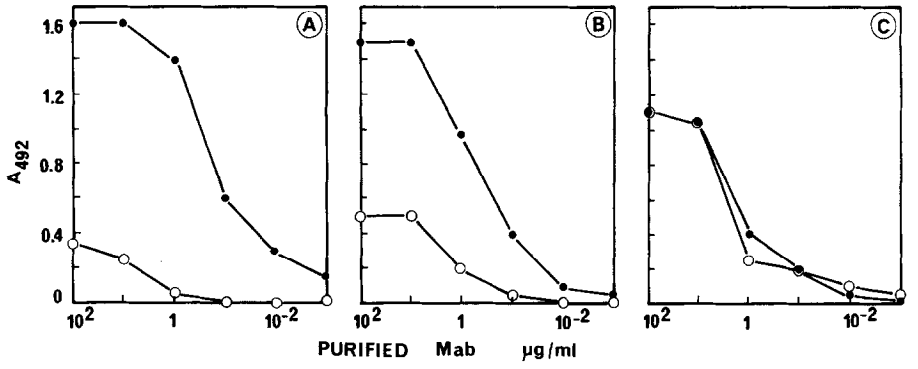

Fig. 2. Relative ELISA activity of the monoclonal antibodies when tested against the two different forms of PEPC.
PEPC protein extracted either from etiolated (O: E type) or green leaves (● :more than 90% of G type) (1 µg/ well) was bound to microtiter plates, blocked, and washed as in screening assays. Six different dilutions of each highly purified monoclonal antibody were added to the wells. The relative amount of antibodies bound to PEPC was assayed by ELISA.
(A)- Dilution curve of Mab 91-G.
(B)- Dilution curve of Mab 83-G.
(C)- Dilution curve of Mab 49-EG.

isozyme by estimating its quantity and the translational capacity of its mRNA as well during leaf greening. Experimental data were as follows: i, an ELISA was carried out at different steps of the greening process; it showed that the G form, virtually absent in etiolated leaf, appears gradually as chlorophylls formation and leaf ontogeny proceed. ii, in vitro translation experiments were performed in reticulocyte lysate programmed by messenger RNA extracted from green or etiolated leaves. In vitro synthesized peptides were immunoselected by adding Mabs to the medium. It was observed that G-PEPC subunit (MW=92,000d) is only produced by messengers from green leaf (fig.3); as expected, use of polyclonal antibodies in a similar experiment revealed both messengers (from green and etiolated leaves) and, so, failed to discriminate G and E messenger products.

Taken together, these results demonstrate that: -the anti G-PEPC Mabs reacted in a very specific manner towards the G isozyme -this protein is undetectable in etiolated material -the G messenger is either absent in etiolated leaf or already present but non-translated; moreover, since Mabs 91 and 83-G were equally capable to react with both in vitro translated peptide and enzyme extracted from leaf, they suggest that these antibodies were not directed to a

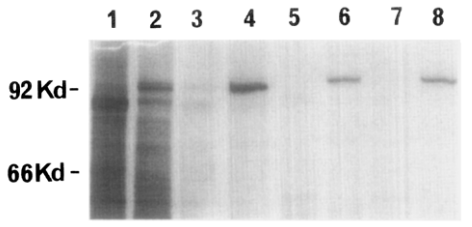

Fig. 3. in vitro translation experiments and immunoprecipitation of the G form of PEPC by using specific Mabs.
Poly(A^+) mRNAs were extracted from etiolated or green leaves and in vitro translated in a reticulocyte lysate (BRL). 45000 cpm of TCA precipitable material were mixed with 8 µl of polyclonal serum or 10 µg of purified Mabs 91-G and 49-EG. The immunecomplexes were dissociated and electrophoresed in a SDS-denaturing 10% polyacrylamide gel according to Laemmli (18). The gel was then fluorographed at -80°C.
Odd numbers correspond to etiolated material, even numbers to green material.
Lanes (1-2): total translation products; lanes (3-4): polyclonal antibodies immunoselected peptides; lanes (5-6): Mabs 91-G immunoselected peptide; lanes (7-8): Mabs 83-G immunoselected peptide.

post-translationnally modified site of the protein. Thus, the hypothesis of PEPC polymorphism resulting of precursor conversion i.e. G form arising from E form, can be completely ruled out. Finally, results also imply that at least two different messengers encoding PEPC subunits do exist in Sorghum leaf and that the one which had been previously postulated to increase following exposure to light is a genuine G messenger encoding G-PEPC form. This is consistent with recent work by Harpster and Taylor (5) who found, by means of a cDNA probe, two prevalent PEPC mRNAs in green maïze leaves.

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