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Phosphorylation of glyoxysomal malate synthase from castor oil seeds *Ricinus communis* L.

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When glyoxysomes isolated from endosperm of 3-day germinated *Ricinus communis* L. seedlings were incubated with [γ-^{32}P]ATP, phosphorylation of a small number of endogenous glyoxysomal proteins occurrred. A 64 kDa glyoxysomal matrix protein was predominately phosphorylated. Based upon results of immunoprecipitation, 2D-polyacrylamide gel electrophoresis and Western blotting, the 64 kDa phosphorylated protein is identified as the subunit of malate synthase (EC 4.1.3.2). Phosphorylation of malate synthase was completed within 5 min and the amino acid residue phosphorylated was serine. Phosphorylation was inhibited by EDTA, EGTA and NaF, but enhanced by Triton X-100.

Malate synthase; Protein phosphorylation; Glyoxysome

1. INTRODUCTION

Protein phosphorylation and dephosphorylation are recognized as important regulatory mechanisms for many cellular processes. The discovery of putative 'second messengers', e.g. cAMP, cGMP, Ca^{2+} and inositol triphosphates [1], in animal systems has considerably expanded our understanding of signal transduction and the role that protein phosphorylation/dephosphorylation plays in the 'signal system'.

Various higher plant proteins undergo post-translational phosphorylation, including nuclear, ribosomal, mitochondrial, chloroplastic, membrane and cytosolic proteins [2–4]. The consequence of the phosphorylation is known for very few, e.g. phosphorylation of the light harvesting chlorophyll *a/b* binding protein regulates the distribution of excitation energy between photosystems I and II [5]. The phosphorylation and dephosphorylation of two enzymes, pyruvate dehydrogenase complex [6] and pyruvate, P_i, dikinase [7], are well characterized and result in inactivation and reactivation, respectively. Phosphorylation of other plant enzymes, e.g. quinate: NAD oxidoreductase [8,9] PEP carboxylase [10] and 6-phosphofructo-2-kinase [11], has also been reported with a resultant effect on activity.

Most reports on protein phosphorylation in higher plants do not identify the phosphorylated protein(s). Substrates for most protein kinase investigations are exogenous proteins such as histones, casein or phosvitin. Consequently, very little is understood about the function of protein kinases and protein phosphorylation/dephosphorylation in plants.

We have recently purified a Ca^{2+}-dependent, calmodulin activated protein kinase from endosperm tissue of castor oil seeds [12] and have been attempting to understand the function of protein phosphorylation in this tissue. This tissue contains specialized peroxisomes termed glyoxysomes which are characterized by very active gluconeogenesis from lipids utilizing the glyoxylate cycle [13]. There is no previous report on protein phos-

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phorylation in glyoxysomes nor is there any published evidence for post-translational modification of glyoxylate cycle enzymes by phosphorylation in higher plants. However, the glyoxylate cycle enzyme, isocitrate lyase is phosphorylated in *E. coli* [14] and a 63 kDa membrane protein in rat liver hepatocyte peroxisomes is phosphorylated in vivo and in vitro [15]. In this paper we report the identification of the 64 kDa matrix protein that is phosphorylated in castor oil seed glyoxysomes as malate synthase (EC 4.1.3.2).

2. MATERIALS AND METHODS

Castor oil seeds (*Ricinus communis* L.) were obtained from Bothwell Enterprise, Plainview, TX. Carrier-free [^{32}P]orthophosphoric acid was from New England Nuclear and was used to prepare [γ-^{32}P]ATP as described [16]. Chemicals for electrophoresis were from Bio-Rad. Other biochemicals were from Sigma.

Castor oil seeds were germinated in the dark at 35°C in moist vermiculite after 24 h soaking in running water. Endosperm tissue was routinely used at 3 days post inhibition and the organelles isolated according to Rapp and Randall [17]. Fumarase and catalase were assayed to evaluate the purity and breakage of organelles [13].

The protein phosphorylation assay mixture contained 20 mM Tris-HCl (pH 8.0), 5 mM DTT, 10 mM $MgCl_2$, 0.1% Triton X-100 and 25 μM [γ-^{32}P]ATP (1000 cpm/pmol) plus 200–250 μg of glyoxysomal protein in a total volume of 1 ml. The assay was performed at 30°C for 5–30 min and the reaction was stopped by precipitating the protein with cold 10% trichloroacetic acid (final concentration). Proteins were collected by centrifugation and dissolved in SDS sample buffer [18] at a final protein concentration of approx. 2 mg/ml.

Matrix and membrane fractions of isolated glyoxysomes were prepared as described [19]. Following the addition of 0.05 M $NaHCO_3$ and 0.25 M NaCl to the glyoxysomes at a 1:1 ratio, the mixture was centrifuged at 110000 × g for 30 min. The supernatant or matrix proteins were precipitated with 10% trichloroacetic acid (final concentration) and collected by centrifugation. The 110000 × g pellet (membrane proteins) was washed twice with the above buffer, then dissolved in SDS sample buffer.

Immunoprecipitation and Western blotting were done using antisera containing antibodies raised against cottonseed malate synthase [20] and isocitrate lyase [21]. The second antibody used for immunostaining of Western blots was anti-rabbit IgG linked to alkaline phosphatase with Fast blue RR and Fast red TR dyes (Sigma) as substrates. After phosphorylation of glyoxysomal proteins, antiserum (20 μl) was added to the 1 ml reaction mixture and further incubated at room temperature for 1 h. Protein-A-Sepharose was then added and the beads collected by centrifugation. All pellets were washed twice by resuspending in 0.1 M Tris-HCl (pH 8) containing 0.1 M NaCl and 1 mM $MgCl_2$.

Denaturing slab gel electrophoresis was performed using 12.5% polyacrylamide gels and a discontinuous buffer system [18]. Two dimensional gel electrophoresis was according to O'Farrell et al. [22]. Western blotting of 2D gels followed the procedure that Towbin et al. [23] described for 1D gels. The dried gels or nitrocellulose membranes were exposed to Kodak X-Omat AR2 films in the presence of Cronex Lightning-Plus intensifying screens (DuPont) at −70°C for autoradiography.

Phosphorylated malate synthase was electrophoresed on a 10% SDS polyacrylamide gel. The gel was stained with Coomassie brilliant G-250 and the phosphorylated malate synthase (^{32}P labeled) located by autoradiography was excised from the gel. The protein was electroeluted using an ISCO electroconcentrator set at 3 W for 6–7 h. The protein was hydrolyzed using 6 N HCl for 2 h and phosphoamino acids were analyzed by two dimensional TLC as described [24].

3. RESULTS

The glyoxysomes were isolated by techniques commonly used for castor oil seed endosperm and

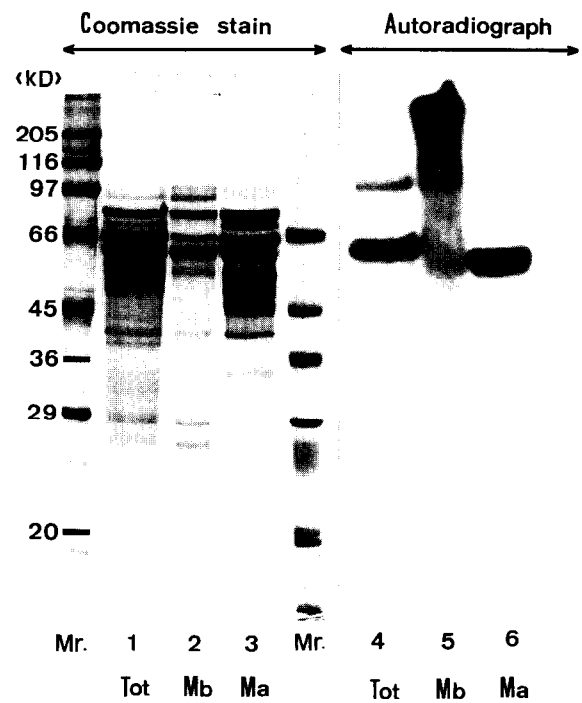

Fig.1. Phosphorylation of glyoxysomal proteins. Glyoxysomal proteins were phosphorylated in vitro using [γ-^{32}P]ATP (1000 cpm/pmol) as described in section 2. Lanes 1,4 show the total glyoxysomal protein (Tot) and lanes 2,5 and 3,6 are glyoxysomal membrane (Mb) and matrix (Ma) proteins, respectively, separated by one-dimension SDS-PAGE with 12.5% separation and 6% stacking gels. Lanes 1–3: Coomassie brilliant blue stained gel. Lanes 4–6: autoradiograph of lanes 1–3 (24 h exposure).

yielded glyoxysomes with very little contamination by mitochondria as judged by marker enzymes (e.g. 1.2% of fumarase activity was present in glyoxysome fraction). Incubation of the isolated glyoxysomes with [γ-^{32}P]ATP resulted in phosphorylation of a small number of proteins (fig.1). Separation of the matrix proteins from the glyoxysomal membrane indicated that a 64 kDa matrix protein was predominantly phosphorylated (fig.1, lanes 4,6). The phosphorylation was completely inhibited by EDTA (fig.2, lane 3) indicating that Mg-ATP is the substrate for the glyoxysomal protein kinase. Phosphorylation of the 64 kDa protein was inhibited by NaF (fig.2, lane 5) but not by NaCl (fig.2, lane 6). EGTA also inhibited the phosphorylation suggesting that Ca^{2+} might be involved in the regulation of the phosphorylation of the 64 kDa protein (lane 9). The phosphorylation was maximal between pH 6 to 7 and was completed within 5 min (not shown). The 64 kDa phosphoprotein exhibited an isoelectric point about pH 5.0 (not shown). In the absence of Triton X-100 the phosphorylation was greatly decreased (fig.2, lane 2).

Considering the molecular mass and subcellular location [19] in the glyoxysome, two enzymes, malate synthase (64 kDa/subunits [25]) and

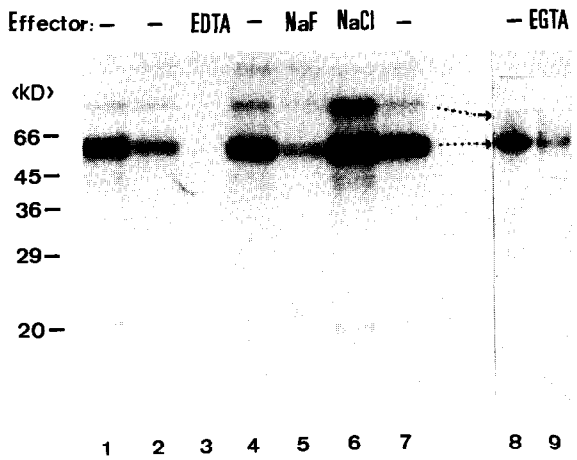

Fig.2. Effect of EDTA, EGTA, NaF and NaCl on 64 kDa polypeptide phosphorylation. Phosphorylation of glyoxysomal proteins with [γ-^{32}P]ATP was carried out in the presence of 20 mM EDTA (lane 3), 20 mM NaF (lane 5), 20 mM NaCl (lane 6), or 2 mM EGTA (lane 9). All samples contained 0.1% Triton X-100 except that in lane 2. Samples were electrophoresed on SDS-PAGE and an autoradiograph prepared (24 h exposure).

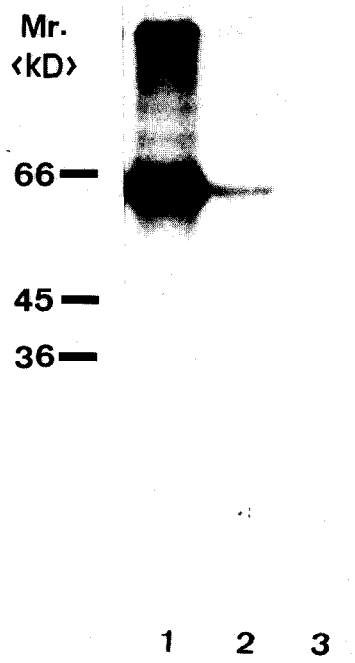

Fig.3. Identification of 64 kDa phosphoprotein as malate synthase by immunoprecipitation. The phosphorylated glyoxysomal proteins (used for lanes 1,4,7 in fig.2) were subjected to immunoprecipitation with malate synthase antiserum (lane 1) or preimmune serum (lane 2). The resulting precipitates and the protein left in the supernatant after immunoprecipitation with malate synthase antiserum (lane 3) were analyzed by SDS-PAGE and an autoradiograph prepared (24 h exposure).

isocitrate lyase (66 kDa [26]), were chosen as potential candidates for phosphorylation. As shown in fig.3, the phosphorylated 64 kDa protein was immunoprecipitable by anti-malate synthase antiserum (lane 1), but not by the preimmune serum (lane 2). After immunoprecipitation of malate synthase, the phosphorylated band was greatly reduced in the supernatant (lane 3).

After 2D gel electrophoresis of the ^{32}P labeled glyoxysomal proteins and Western blotting of the gel, immunostaining of the Western blot using isocitrate lyase antiserum (ICL) and malate synthase antiserum (MS) was performed (fig.4A). Only malate synthase showed the incorporation of ^{32}P as indicated by the corresponding autoradiogram (fig.4B). Immunostaining of Western blots of immunoprecipitated phosphorylated glyoxysomal proteins subjected to 2D gel electrophoresis also revealed a radioactive spot coincident with the spot

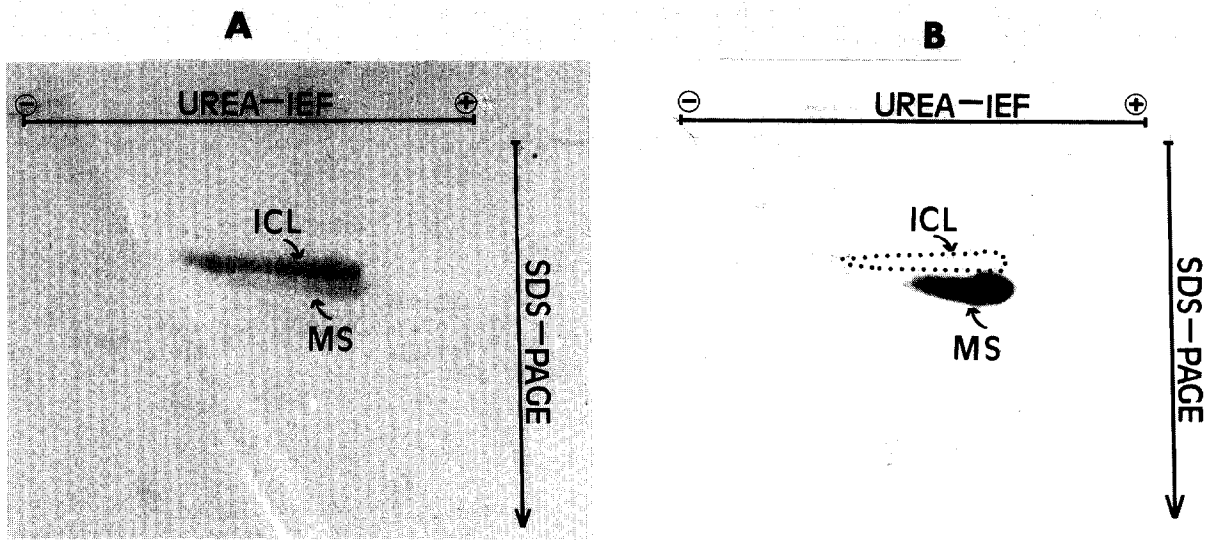

Fig.4. Western blot analysis showing specific recognition between 64 kDa phosphopolypeptide and malate synthase antiserum. After in vitro phosphorylation, glyoxysomal proteins were separated by 2D gel electrophoresis, electro-transferred to nitrocellulose, probed with isocitrate lyase (ICL) antiserum and malate synthase antiserum (MS), separately (A). An autoradiograph of the immunostained Western blot is shown in B (24 h exposure).

detectable by anti-malate synthase antiserum (not shown).

Chemical hydrolysis of the 64 kDa phosphoprotein followed by two-dimensional thin-layer electrophoresis indicated that the phosphorylation of malate synthase occurred exclusively on a serine residue (not shown).

4. DISCUSSION

Experimental evidence is presented demonstrating that malate synthase, an enzyme of the glyoxylate cycle, undergoes in vitro phosphorylation. To our knowledge this is the first report describing the post-translational phosphorylation of malate synthase. The function of this phosphorylation remains obscure and is now under active investigation. The phosphorylation of malate synthase is inhibited by EGTA (fig.2, lane 9) and is stimulated by Ca^{2+} (not shown) suggesting that calcium is potentially involved, either directly or indirectly through protein phosphorylation, in the regulation of triacylglycerol catabolism in endosperm tissue during post germinative growth of *Ricinus* seeds.

It is unusual that NaF inhibits phosphorylation of malate synthase as shown in this study, since fluoride is typically an inhibitor for most phosphoprotein phosphatases. However in previous work we also found that protein kinase isolated from castor oil seed endosperms was inhibited by NaF (unpublished). We do not know the significance of this inhibition or if there is any relationship between the protein kinase activity previously demonstrated from castor oil seed with histone H1 as substrate [12] and malate synthase phosphorylation; however, this is under active study.

The 63 kDa membrane protein that is phosphorylated in rat hepatocyte peroxisomes [15] is distinctly different from the phosphorylated malate synthase reported here. Our separation of membrane and matrix fractions is comparable to that used for the rat peroxisomes, thus eliminating malate synthase as the membrane protein. Malate synthase is unique to the glyoxylate cycle and not present in mammalian peroxisomes. Skorin et al. [15] do not report phosphorylation of any matrix proteins in their rat peroxisomes. The difference in migration of the 64 kDa phosphoprotein on SDS-PAGE between total glyoxysomal fraction and the matrix fraction was observed repeatedly for some unknown reason(s) (fig.1). Both bands, however, reacted positively with malate synthase antiserum (not shown).

The function of malate synthase phosphorylation has not been established and is under active investigation in our laboratories. We are also trying to purify the malate synthase kinase or glyoxysomal protein kinase in order to characterize its regulation.

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REFERENCES

[1] Cohen, P. (1982) Nature 296, 613–620.
[2] Budde, R.J.A. and Chollet, R. (1988) Physiologia Plantarum 72, 435–439.
[3] Ranjeva, R. and Boudet, A.M. (1987) Annu. Rev. Plant Physiol. 38, 73–93.
[4] Rubin, C.S. and Rosen, O.M. (1975) Annu. Rev. Biochem. 44, 831–877.
[5] Bennett, J. (1977) Nature 269, 344–346.
[6] Randall, D.D. and Rubin, P.M. (1977) Plant Physiol. 59, 1–3.
[7] Edwards, G.E., Nakamoto, H., Burnell, J.N. and Hatch, M.D. (1985) Annu. Rev. Plant Physiol. 36, 255–286.
[8] Refeno, G., Ranjeva, R. and Boudet, A.M. (1982) Planta 154, 193–198.
[9] Refeno, G., Ramjeva, R., Fontaine-Delvare, S. and Boudet, A.M. (1982) Plant Cell Physiol. 23, 1137–1144.
[10] Budde, R.J.A. and Chollet, R. (1986) Plant Physiol. 82, 1107–1114.
[11] Walker, G.H. and Huber, S.C. (1987) FEBS Lett. 213, 375–380.
[12] Yang, Y.P. and Randall, D.D. (1987) Curr. Top. Plant Biochem. Physiol. 6, 179.
[13] Cooper, T.G. and Beevers, H. (1969) J. Biol. Chem. 244, 3507–3513.
[14] Robertson, E.F., Hoyt, J.C. and Reeves, H.C. (1988) J. Biol. Chem. 263, 2477–2482.
[15] Skorin, C., Soto, U., Necochea, C. and Leighton, F. (1986) Biochem. Biophys. Res. Commun. 140, 188–194.
[16] Schendel, P. and Wells, R.D. (1973) J. Biol. Chem. 248, 8319–8331.
[17] Rapp, B.J. and Randall, D.D. (1980) Plant Physiol. 65, 314–318.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Fang, T.K., Donaldson, R.P. and Vigil, E.L. (1987) Planta 172, 1–13.
[20] Trelease, R.N., Hermerath, C.A., Turley, R.B. and Kunce, C.M. (1987) Plant Physiol. 84, 1343–1349.
[21] Doman, D.C. and Trelease, R.N. (1985) Protoplasma 124, 157–167.
[22] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell 12, 1133–1142.
[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[24] Huganir, R.L., Miles, K. and Greengard, P. (1984) Proc. Natl. Acad. Sci. USA 81, 6968–6972.
[25] Bowden, L. and Lord, J.M. (1978) Plant Physiol. 61, 259–265.
[26] Martin, C. and Northcote, D.H. (1982) Planta 154, 174–183.