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DARK-LIGHT REGULATION OF PYRUVATE,P_i DIKINASE IN C_4 PLANTS: EVIDENCE THAT THE SAME PROTEIN CATALYSES ACTIVATION AND INACTIVATION

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SUMMARY: Evidence is provided that the ADP plus ATP-dependent inactivation and the P_i-dependent activation of pyruvate,P_i dikinase is catalysed by the same protein. Essentially identical behaviour of these two activities was observed during partial purification by $(NH_4)_2SO_4$ fractionation, affinity chromatography on Sepharose 4B-Blue dextran and filtration on Sepharose 4B. This protein factor, which is sensitive to heat treatment and tryptic digestion, has a molecular weight of approximately 90,000 at pH 7.5 and 180,000 at pH 8.3.

Pyruvate,P_i dikinase (EC 2.7.9.1) catalyses the conversion of pyruvate to phosphoenolpyruvate, the primary CO_2 acceptor in C_4 photosynthesis. This enzyme undergoes rapid light-mediated activation and dark-mediated inactivation in leaves (1,2) and this regulation is closely linked with the photosynthetic electron transport chain (3). Dark-inactivated pyruvate,P_i dikinase from maize leaves can be activated by P_i (4,5) and this process is catalysed by a heat-labile protein (6,7). Other studies with leaf and chloroplast extracts from maize demonstrated that the active pyruvate,P_i dikinase is inactivated by a process requiring ADP, a trace amount of ATP and one or more heat-labile proteins (4,5,8).

In the present study we report on the isolation and partial purification of a protein capable of catalysing the inactivation of pyruvate,P_i dikinase. We present evidence that this protein is identical with the one that catalyses the activation of dark-inactivated pyruvate,P_i dikinase in the presence of P_i. The protein will be referred to as the pyruvate,P_i dikinase regulatory protein (PDRP).

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METHODS

For the partial purification of PDRP 50g of maize leaves were sliced finely with a razor blade and blended at 0°C in 200 ml of buffer containing 100 mM Tris-Cl, pH 8.3, 10 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT. The homogenate was filtered through two layers of Miracloth and centrifuged at 25,000 g for 10 min. The supernatant was fractionated with solid $(NH_4)_2SO_4$ and the precipitate obtained between 20 and 45% saturation was dissolved in 15 ml of 50 mM Tris-Cl, pH 8.3, containing 5 mM $MgCl_2$, 0.1 mM EDTA and 2 mM DTT, referred to below as the buffer mixture. The solution was desalted on a Sephadex G-25 column equilibrated with the above buffer mixture. Twenty ml of the dark green eluate was collected and applied to a column (20 ml) of Sepharose 4B-Blue dextran equilibrated with the buffer mixture. The column was washed with approximately 100 ml 0.2 M KCl dissolved in the buffer mixture. The column was then washed with 50 ml 0.5 M KCl dissolved in the same buffer. Four ml fractions were collected and assayed for inactivating factor activity. Those fractions containing the highest PDRP activity were pooled and the protein precipitated by the addition of solid $(NH_4)_2SO_4$ to 70% saturation. The protein recovered by centrifugation was dissolved in 2 ml of buffer mixture and applied to a column (2.5 x 40 cm) of Sepharose 4B and eluted with the buffer mixture. Two ml fractions were collected and assayed for both inactivation and activation activity. The active fractions were frozen at -80°C until required.

For following the inactivation of pyruvate,P_i dikinase, reactions contained 50 mM Tris-Cl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and varying amounts of pyruvate,P_i dikinase and PDRP in a total volume of 0.24 ml. Inactivation was initiated by the addition of 10 µl 25 mM ADP and 5 mM ATP. Samples (25 µl) were withdrawn at various times and the pyruvate,P_i dikinase activity determined as described previously (5).

For following the activation of pyruvate,P_i dikinase, reaction mixtures contained 50 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT and varying amounts of PDRP and dark inactivated pyruvate,P_i dikinase in a total volume of 0.24 ml. Activation was initiated by the addition of 10 µl 25 mM P_i. Samples (25 µl) were taken at varying time intervals and the pyruvate,P_i dikinase activity determined.

Active and dark-inactivated pyruvate,P_i dikinase were purified from maize leaves as described by Hatch (9) and Nakamoto and Sugiyama (7), respectively.

RESULTS AND DISCUSSION

More than ninety percent of the inactivation activity of PDRP in leaf extracts was recovered in the 20 to 45% $(NH_4)_2SO_4$ fraction. However, this fraction also contained some pyruvate,P_i dikinase. Pyruvate,P_i dikinase and PDRP could be separated by polyethylene glycol fractionation; the PDRP precipitated between 10 and 20% (w/v) polyethylene glycol and pyruvate,P_i dikinase remained in solution above 30% (w/v) polyethylene glycol. However, polyethylene glycol fractionation was not routinely used for the purification of PDRP as the yields were low.

The PDRP was separated from pyruvate,P_i dikinase on a column of Sepharose 4B-Blue dextran. Both this factor and pyruvate,P_i dikinase bound to the

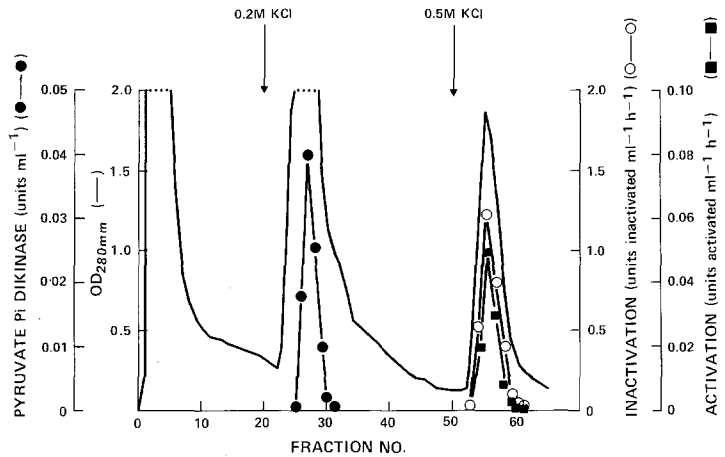

Figure 1. Affinity chromatography of PDRP on Sepharose 4B-Blue dextran. Protein was extracted from 50 g leaf tissue from maize plants, and the extract, following $(NH_4)_2SO_4$ fractionation and Sepharose G-25 column chromatography, was applied to a column (20 ml) of Sepharose 4B-Blue dextran. For other details see METHODS. Activating and inactivating activity of PDRP is expressed as the initial rate of a change of pyruvate,P_i dikinase activity expressed as units ($\mu mol.min^{-1}$) per ml of the eluted fraction per hour.

column but pyruvate,P_i dikinase was completely eluted with 0.2 M KCl whilst PDRP required 0.5 M KCl for elution (Fig. 1). Since the inactivating activity of PDRP was strongly inhibited by KCl, the fractions eluted from Sepharose 4B-Blue dextran by 0.5 M KCl were desalted using Sephadex G-25 prior to assaying. The inactivating and the activating activities of PDRP coeluted from the Sepharose 4B-Blue dextran column (Fig. 1).

Column chromatography of the inactivating activity of PDRP on Sepharose 4B at pH 8.3 indicated a molecular weight for this protein of approximately 180,000 (Fig. 2). When the pH of the Sepharose 4B column was adjusted to pH 7.5 the inactivating activity of PDRP eluted with a molecular weight of approximately 90,000. Under both pH conditions the protein catalysing P_i-dependent activation of pyruvate,P_i dikinase coeluted with the protein catalyzing inactivation of the enzyme (Fig. 2). Further evidence that the proteins catalyzing activation and inactivation were identical was provided by comparing the elution of activities from Sephadex G-200. When a mixture of PDRP (2 mg) and blue dextran (0.05%, w/v) was applied to a column of Sephadex G-200 at pH 8.3 the inactivating and activating activities coeluted

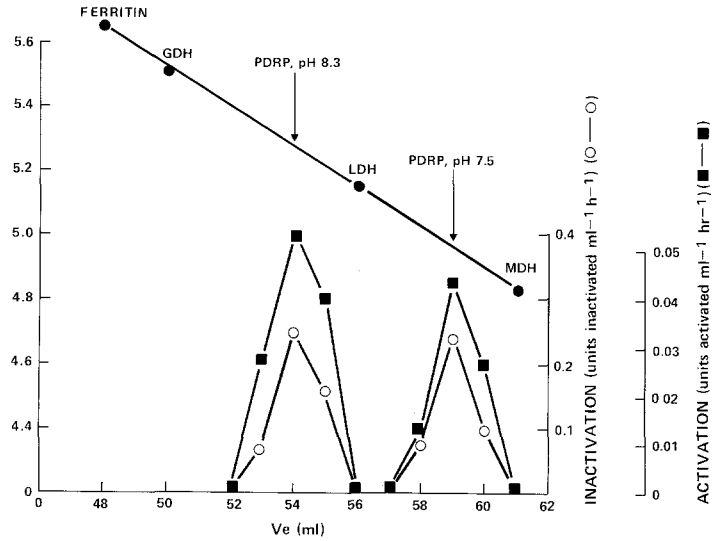

Figure 2. Estimation of the molecular weight of the PDRP. Column chromatography on Sepharose 4B (1.5 x 40 cm) was conducted at pH 7.5 and pH 8.3 with the following standards (mol wt): Ferritin, 440,000; glutamate dehydrogenase, 320,000; lactate dehydrogenase, 140,000; malate dehydrogenase, 67,000. One ml fractions were collected at a flow rate of 20 ml/hr.

with blue dextran in the void volume. When blue dextran was replaced by Cibacron blue, the dye moiety of blue dextran (1 μM), both activities of PDRP coeluted after the excluded protein peak. These results agree with the observation of Nakamoto and Sugiyama (7) that the activating protein binds to blue dextran.

With PDRP purified by sequential treatment on Sepharose 4B-Blue dextran and then Sepharose 4B, inactivation was absolutely dependent on ADP plus ATP and the enzyme was reactivated by P_i after processing on Sephadex G-25 to remove ADP (Fig. 3). Separate experiments showed that the rates of activation and inactivation were proportional to the amount of PDRP and the initial level of either inactive or active pyruvate,P_i dikinase, respectively. The dependence of the rates of both activation and inactivation upon the concentration of both PDRP and either inactive or active pyruvate,P_i dikinase at present preclude an accurate determination of the specific activities during the purification procedure. However the inactivation activity of PDRP was purified approximately 80-fold with a recovery of 16%

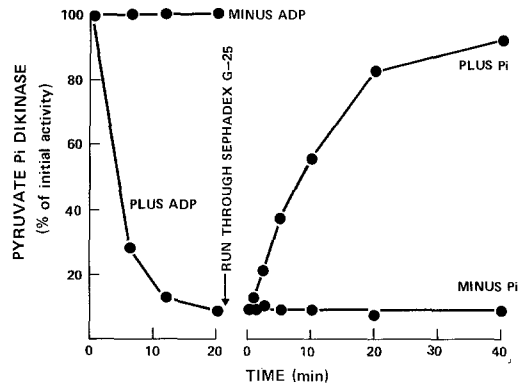

Figure 3. Inactivation and activation activity of purified PDRP chromatographed on Sepharose 4B.

An inactivation reaction was run containing PDRP purified by $(NH_4)_2SO_4$ fractionation and Sepharose 4B-Blue dextran and Sepharose 4B chromatography (see METHODS). When the pyruvate,P_i dikinase was 95% inactivated the reaction mixture was processed on a column of Sephadex G-25 to remove the nucleotides. Activating factor activity of the nucleotide-free column eluate was then followed after the addition of 1 mM P_i as described in the METHODS.

during the purification procedure described in the METHODS. In the experiment described in Fig. 3 where the amounts of PDRP and concentrations of pyruvate,P_i dikinase were essentially the same the relative initial rates of activation and inactivation were approximately 0.27 to 1. Boiling and trypsin treatment (see ref. 8) of the purified PDRP resulted in complete loss of the capacity for inactivating and activating pyruvate,P_i dikinase (results not shown).

CONCLUDING REMARKS

The present studies confirm earlier indirect evidence (8) that ADP plus ATP mediated inactivation of pyruvate,P_i dikinase is a protein-catalysed reaction. The fact that both the inactivation and activation activities coprecipitate between 20 and 45% $(NH_4)_2SO_4$, bind and coelute from Sepharose 4B-Blue dextran, and exhibit identical changes in molecular weight between pH 7.5 and 8.3 as judged by their mobility on Sepharose 4B, provides evidence that these two activities are catalysed by the same protein. This is presumably identical with the partially purified protein factor previously shown to catalyse P_i-dependent activation of pyruvate,P_i dikinase (7).

Sugiyama and Hatch (8) suggested that the pyruvate,P_i dikinase activity prevailing in leaves may be modulated by light intensity through changes in the levels of chloroplast stromal ATP, ADP, AMP and P_i. With the evidence from the current study that the same protein factor mediates both activation and inactivation of pyruvate,P_i dikinase, various possibilities for explaining light-dark regulation of this enzyme become apparent. The most obvious is that changes in levels of these or other metabolites may modulate either the activating or inactivating capacity of the PDRP. Alternatively, the protein may be reversibly convertible between forms that catalyse activation and inactivation. We will be investigating these possibilities.

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