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GTP-BINDING PROTEINS IN ETIOLATED EPICOTYLS OF *Pisum sativum* (Alaska) SEEDLINGS

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Seven fractions of GTP-binding proteins separated by gel filtration of an extract of epicotyls of *Pisum sativum* seedlings were partially characterized. Seven fractions of GTP-binding proteins tentatively designated GP1 to GP7 had the capacity to be ADP-ribosylated by pertussis toxin. Pooled fractions of GP2 to GP7 showed Km values 2, 20, 50, 10, 3 and 1 nM, respectively. The binding of [^{35}S]GTPγS to GTP-binding proteins was prevented competitively in the presence of 0.1 mM GTP and also prevented in the presence of 0.1 mM ATP. Binding of [^{35}S]GTPγS to the proteins produced a decrease in their molecular weights.

The epicotyls of etiolated *Pisum sativum* seedlings have been well analyzed for their response to plant growth regulators, such as gibberellic acid (1), indole acetic acid (2) and ethylene (3) and to light (4,5). The effects of plant growth regulators and of light on the growth responses of several portions of the epicotyl interact with each other (3,6). In spite of these analyses the mechanism of signal transduction of plant growth regulators and transduction of light signals are not known, although the signals of red and far red light are known to be perceived by phytochrome (7).

In *Lemna paucicostata* 441 we detected GTP-binding proteins (8) and demonstrated that red or far red light can modulate the rate of the binding of [^{35}S]GTPγS to GTP-binding proteins (9). Far red

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light caused a change in the intracellular concentrations of cyclic 3',5'-AMP and -GMP in plants subjected to 4 hr of darkness (10). The stimulation of spore germination in ferns by red light is also associated with the reduction in the concentrations of cyclic 3',5'-AMP and -GMP (11). Thus light signals perceived by these plants were suggested to be transduced by phytochrome, GTP-binding protein and cyclic phosphodiesterase as has been established in the retinal rod outer segment of the frog eye (12). In animals adenylate cyclase and polyphosphoinositide phosphodiesterase can be regulated by GTP-binding proteins through the action of a hormone-hormone receptor complex (13). Plant growth regulators may also control the binding rate of [^{35}S]GTPγS to the GTP-binding protein. In the present paper we report GTP-binding proteins in the epicotyls of Pisum sativum (Alaska) seedlings.

EXPERIMENTAL

Growth of pea seeds: About 800 pea seeds (Pisum sativum L. var Alaska) were soaked in 500 ml of 5 % antiformin for 20 min, and then washed 10 times with 1 l of distilled water. They were divided into two lots and each was soaked in 1 l of distilled water for 5 hr. About 100 seeds thus treated were placed in a sterilized container on four sheets of filter paper moistened with sterilized water and incubated at 23 °C for 3 days in darkness. The epicotyls including the plumular hook and the 3rd internode (1) were cut off under dim green safelight and stored at -80 °C in darkness.

Preparation of crude extract: Frozen epicotyls (15 g) were thoroughly macerated in liquid nitrogen in a mortar and mixed with 30 ml of extraction buffer (8). The thoroughly macerated frozen powder was then allowed to melt. After thawing, the extract was centrifuged at 15,000 xg for 20 min at 2 °C. The supernatant (39 ml) was divided into 1 ml aliquots and stored at -80 °C. The crude extract contained 2.19 mg protein/ml.

Assay for GTP-binding activity: The assay for the binding of [^{35}S]GTPγS (Guanosine 5'[γ-thio]triphosphate, [^{35}S]-; 1013 Ci/mmol, NEG-030H) to GTP-binding protein was performed according to the method described (8) using a Norit solution. Protein was measured using a Bio-Rad protein assay kit.

ADP-ribosylation of GTP-binding protein: ADP-ribosylation of GTP-binding proteins by pertussis toxin (Kaken Pharmaceutical Co.) was performed according to the method described (9) using [^{32}P]NAD (NEG-023X:0.504 nmol 0.5 mCi^{-1}).

RESULTS AND DISCUSSION

Detection of GTP-binding proteins: The crude extract containing membrane components was treated with 1 % Lubrol PX and then subjected to gel filtration through a Sephadex G-100 column. The results presented in Fig. 1a show the binding activity of GTP-binding proteins to [^{35}S]GTPγS and ADP-ribosylation of proteins by pertussis toxin with [^{32}P]NAD. Fractions 9 to 11 showed ADP-ribosylation activity, although the capacity to bind [^{35}S]GTPγS was very low. Fractions 13-15, 16-18, 19-21, 22-24, 26-28, 30-32 were active in binding [^{35}S]GTPγS to proteins and also contained proteins that were ADP-ribosylated by pertussis toxin. These GTP-binding proteins, indicated by bars in Fig. 1a, were tentatively designated GP1, GP2, GP3, GP4, GP5, GP6 and GP7. Since the ADP-ribosylation of GP7 was so low, further work will be needed to determine its capacity.

To assay the binding of [^{35}S]GTPγS to GTP-binding protein 2.5 nM (0.5 μCi) of [^{35}S]GTPγS was included for each 200 μl reaction mixture. To the reaction mixture 0.1 mM GTP or 0.1 mM ATP was added to observe competitive prevention of the binding of [^{35}S]GTPγS to the GTP-binding protein. The results shown in Fig. 1b indicate that the binding of [^{35}S]GTPγS in the presence of 0.1 mM GTP to GP1, GP2, GP3, GP4, GP5, GP6 and GP7 was 0, 0, 5.6, 40.3, 50.3, 18.6 and 0.3 % of that in the absence of GTP, and that the binding of [^{35}S]GTPγS in the presence of 0.1 mM ATP to these GTP-binding proteins was 23.5, 16.9, 14.2, 6.0, 9.5, 30.2 and 61.5 % of that in the control experiment. Except for GP7 all the other GTP-binding proteins showed strong affinities to ATP. Especially GP4 and GP5 showed stronger affinities to ATP than to GTP. Thus GP4 and GP5 may represent previously unknown ATP-binding proteins. Further characterization is needed to determine their functional properties.

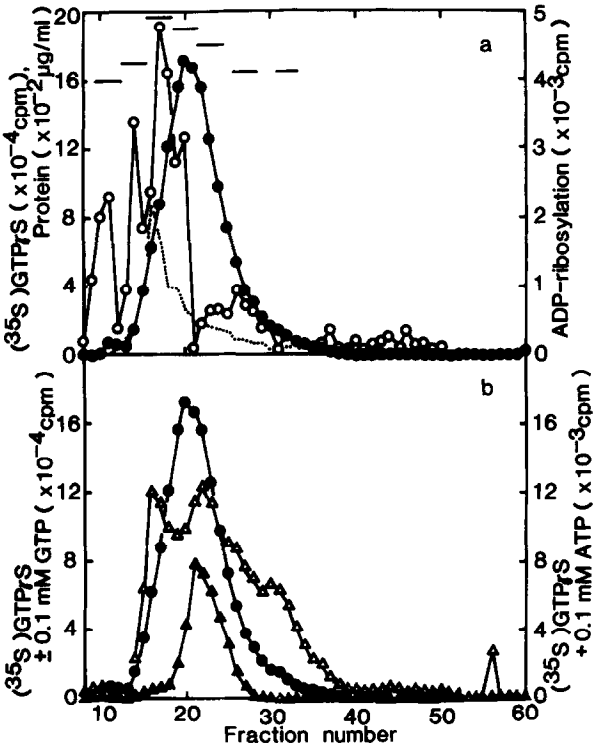

Fig. 1. Gel filtration through a Sephadex G-100 column of a crude extract treated with 1 % Lubrol PX. The crude extract containing membrane components (4.38 mg protein) was made 1 % with Lubrol PX and incubated at 25 °C for 10 min. After chilling in ice-water it was loaded onto a Sephadex G-100 column (2×32 cm) equilibrated with 20 mM Tris-HCl, pH 7.2, 20 mM NaCl, 25 mM $MgCl_2$, 0.1 % Lubrol PX and 1 mM phenyl methylsulfonyl fluoride (buffer A), and eluted with the same buffer into 2.7 ml fractions. (a) Binding of [^{35}S]GTPγS to GTP-binding protein (—●—), ADP-ribosylation of proteins (—○—) and protein concentraion (·····). (b) Binding of [^{35}S]GTPγS to GTP-binding protein in control experiment (—●—), in the presence of 0.1 mM GTP (—▲—) and in the presence of 0.1 mM ATP (—△—).

Km values of these GTP-binding proteins were also determined using the pooled fractions as indicated by the bars in Fig. 1a. The linearity of the assays by the binding of [^{35}S]GTPγS was presented in Fig. 2. Km's for GP2, GP3, GP4, GP5, GP6 and GP7 were 2, 20, 50, 10, 3 and 1 nM, respectively. These values are very close to those of Ns and Ni of adenylate cyclase and of transducin (14).

Gel filtration analysis of reaction products: The crude extract containing membrane components (1.6 ml) was mixed with 0.4 ml of a 5-fold concentrated reaction mixture containing 5 μCi of [^{35}S]GTPγS and incubated at 25 °C for 30 min. The reaction was

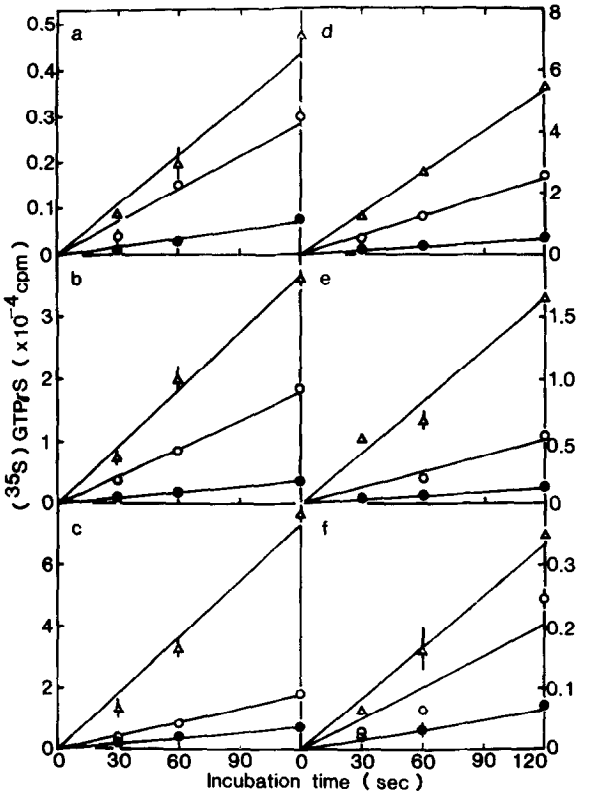

Fig. 2. Rate of the binding of [^{35}S]GTPγS to GTP-binding protein in each pooled fraction. Pooled fractions indicated by bars corresponding to GP2 to GP7 in Fig. 1a were used. GP2(a), GP3(b), GP4(c), GP5(d), GP6(e) and GP7(f). The substrate concentrations in the reaction mixtures were 0.5 nM (0.1 μCi) (—●—), 2.5 nM (0.5 μCi) (—○—) and 5nM (1.0 μCi) (—△—) with error bars.

stopped by adding 2 ml of a 2-fold concentrated quenching mixture containing 40 mM Pipes, pH 6.4 , 0.2 % Lubrol PX, 0.2 M NaCl, 20 mM $MgCl_2$, 2 mM β-mercaptoethanol and 0.2 mM GTP. After chilling in ice-water, it was loaded onto a Sephadex G-100 column (Fig. 3). Fractions 10-12 corresponded to GP1, 13-15 to GP2, 16-18 to GP3, 19-21 to GP4, 22-24 to GP5, 26-28 to GP6 and 31-33 to GP7. The activity counted in each fraction with or without treatment with a Norit solution indicated an absence of radioactivity in the GP4, GP5 and GP6 peaks, and apparent radioactivity in fractions 38 to 47. Thus the proteins in fractions GP4, GP5 and GP6 apparently have reduced molecular weights after the binding of [^{35}S]GTPγS. Similar results were obtained in the GTP-binding proteins of Lemna

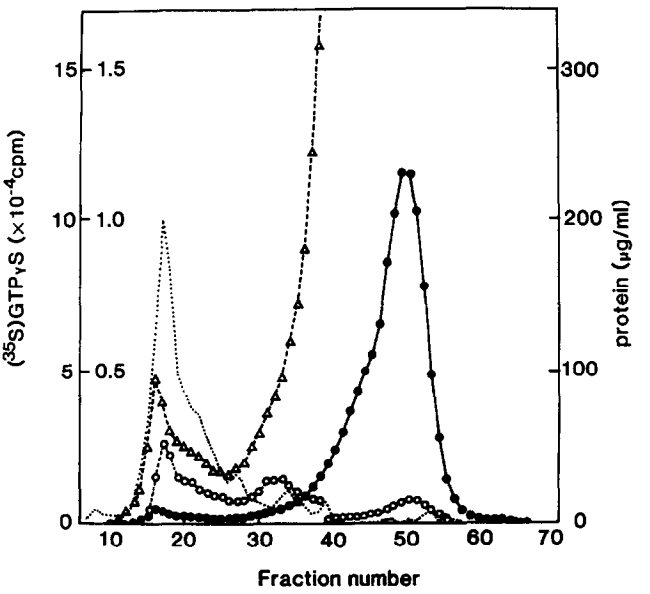

Fig. 3. Sephadex G-100 gel filtration of the reaction product of [^{35}S]GTPγS and the crude extract. The reaction product obtained as described in the text was loaded onto a Sephadex G-100 gel column (2×32 cm) equilibrated with buffer A and eluted with the same buffer into 2.7 ml fractions. Radioactivity counted in each 0.2 ml fraction:(——●——) outside scale, (---Δ---)inside scale . To the 0.2 ml of each fraction, 0.8 ml of a Norit solution was mixed. After centrifugation 0.7 ml of supernatant was taken for radioactivity counting. The radioactivity was calculated as the value which was expected when all the supernatant was recovered for counting. Left side plotting should be read with the inside scale and right side plotting with the outside scale (---O---). Protein concentration (·····).

paucicostata 441 (9) and also in transducin (12). These GTP-binding proteins thus seem to have the general characteristics of GTP-binding proteins found in animals as they show specific affinities to ATP. The characterization of the biological significance of these proteins is now under investigation.

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