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Are guanine nucleotide-binding proteins involved in regulation of thylakoid protein kinase activity?

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The slow ATP-induced decrease in chlorophyll fluorescence associated with the phosphorylation of LHC II seen in spinach thylakoids was inhibited by GTP and its non-hydrolysable analogues β, γ-imidoguanosine 5′-triphosphate and guanosine 5′-*O*-(3-thiotriphosphate), but not by other nucleotide triphosphates or diphosphates. Inhibition by guanosine 5′-*O*-(3-thiotriphosphate) appeared to require prior exposure of the thylakoid membranes to the guanine nucleotide under conditions where the protein kinase was active but unable to turnover. Binding studies with thylakoid membranes using [5′,8-^{3}H]GTP or [^{35}S]guanosine 5′-*O*-(3-thiotriphosphate) show that under similar conditions an increase in specific binding of these radiolabelled nucleotides occurs. The data presented provide evidence for the existence of a guanine nucleotide-binding regulatory protein that is able to interact with the thylakoid protein kinase.

Thylakoid protein kinase; Regulation; Guanine nucleotide; G-protein

1. INTRODUCTION

The discovery of thylakoid protein phosphorylation by Bennett [1,2] has led to a large body of work principally concerned with the physiological significance of this covalent modification. In particular, the reversible phosphorylation of LHC II which is modulated by the redox state of plastoquinone within the thylakoid membrane, is known to underpin an important regulatory system responsible for balancing the quantal input to each photosystem under a range of environmental light conditions ([3,4] and review [5]). Thylakoid protein phosphorylation has also been reported to have a number of more direct effects on the organization [6] and operation of photosystem II [7] and recently the non-LHC II phosphoproteins have been confirmed to be components of photosystem II [8,9]. Although the consequences of thylakoid protein phosphorylation are fairly well understood, somewhat less is known about the molecular identity and operation of the enzymes responsible for catalysing thylakoid protein phosphorylation. Whilst the isolation of thylakoid protein kinases has been reported [10–13] only the 64 kDa preparation of Coughlan and Hind [12,13] was able to phosphorylate isolated LHC II. Moreover, antibodies raised to this isolated enzyme were able to prevent phosphorylation of LHC II and photosystem II components within the native thylakoid membrane and to abolish ATP-induced fluorescence quenching [13]. This preparation, therefore, probably represents the physiological LHC II kinase. In spite of this recent

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Abbreviations: GppNHp, β,γ-imidoguanosine 5′-triphosphate; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Fd, ferredoxin; LHC II, photosystem II light harvesting complex; G-protein, guanine nucleotide-binding regulatory protein; Chl, chlorophyll

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advance the mechanism whereby the kinase perceives the redox state of the thylakoid membrane, and the factors that may regulate its activity in vivo remain unknown.

In certain other membrane bound receptors that respond to external stimuli, notaby the 'photon receptor' rhodopsin and the β-adrenergic and muscarinic hormone receptors, signal transduction is affected by guanine nucleotide-binding regulatory proteins (G-proteins) that are only activated on binding to the stimulated receptor [14,15]. In these systems, desensitization of the receptor to continued stimulation occurs by phosphorylation of the receptor which subsequently leads to a decreased interaction with its G-protein [16,17]. Since phosphorylation of LHC II can be considered to bring about the desensitization of photosystem II to excessive quantal input, I decided to investigate whether guanine nucleotides were able to affect thylakoid protein kinase mediated events. The work reported concerns the effect of guanine nucleotides on the ATP-induced fluorescence decrease catalysed by the thylakoid protein kinase and the specific binding of guanine nucleotides by thylakoid membranes.

2. MATERIALS AND METHODS

2.1. *Materials*

[5',8-^{3}H]GTP and [^{35}S]GTPγS were purchased from New England Nuclear. All other chemicals were of 'Analar' or equivalent quality and nucleotides were prepared freshly in 5 mM tricine-NaOH, pH 7.5, prior to use.

2.2. *Preparation of thylakoid membranes*

Spinach plants (*Spinacea oleracea* var. Bloomingdale) were cultivated in a growth chamber of 6–8 weeks prior to harvesting the leaves. Chloroplasts were prepared according to [18]. Immediately prior to use, the chloroplasts were osmotically shocked by dilution into 10 vols of 5 mM tricine, pH 7.5, and 5 mM $MgCl_2$ for 2 min, prior to the addition of an equal volume of ice-cold reaction medium (50 mM tricine, pH 7.5; 330 mM sorbitol; 5 mM $MgCl_2$; 15 mM KCl) and centrifugation at 5000 × *g* for 5 min. The resulting thylakoid pellet was resuspended in a small volume of reaction medium.

2.3. *Measurement of fluorescence changes*

The ATP-induced decrease in chlorophyll fluorescence was monitored at 20°C essentially as in [19] but using a commercially available (Hansatech, England) modulated fluorescence apparatus. Thylakoids, at a chlorophyll concentration of 10 $\mu g \cdot ml^{-1}$ were suspended in the reaction medium described with 1 μM gramicidin and 5 μM nucleotide present. After turning on the actinic light for 10 s, ATP was added to give a final concentration of 0.5 mM and the fluorescence decrease followed for 5–6 min.

2.4. *Binding assay for [^{35}S]GTPγS and [5',8-^{3}H]GTP*

Assays for the specific binding of [^{35}S]GTPγS and [5',8-^{3}H]GTP were carried out as in [20]. The binding assay was started by resuspending thylakoids equivalent to 27.5 μg Chl in 110 μl of a medium which consisted of 33 mM tricine, pH 7.5, 220 mM sorbitol, 3.3 mM $MgCl_2$, 10 mM KCl, 100 nM GTP or GTPγS, and approx. 7.3 kBq of radiolabelled ligand. In some cases 2.5 μM ferredoxin and 1 mM NADPH (or NADP) were also present. For determination of non-specific binding 2.5 mM GTP was included in the reaction mixture. After incubating the reaction mixture for 10 min in white light at an intensity of approx. 200 $W \cdot m^{-2}$ or in the dark, at 20°C, 100 μl of the reaction mixture was rapidly loaded into a 500 μl microcentrifuge tube containing 100 μl of 1 M sucrose overlayered with 200 μl of a dinonylphthalate/dibutylphthalate mixture (80:20, v/v). Following immediate centrifugation for 6 min at 10000 × *g* (MSE microfuge) the tubes were frozen in liquid N_2 and the bottom 50 μl, containing the thylakoid pellet, was clipped off into 400 μl of 1% SDS (w/v). The pellets were then left overnight to solubilize prior to addition of 4 ml scintillation fluid and determination of the radiolabel present.

3. RESULTS

In preliminary experiments, it became apparent that somewhat variable results were obtained, for the effect of GTP and its analogues on the slow thylakoid protein kinase-mediated fluorescence decrease, when ATP was added promptly after initiating actinic illumination. However, when the

thylakoid membranes were preilluminated for 10–20 s in the presence of the guanine nucleotide triphosphates prior to addition of ATP, this variability was removed. To probe this effect more fully the experiment detailed in fig.1 was carried out. Here, thylakoids were preincubated with 10 μM GTPγS, harvested and resuspended in fresh medium. The ATP-induced fluorescence quenching was then monitored using these membranes. It is clearly apparent that inhibition was observed only when thylakoids had been preincubated in the light with GTPγS. Since the thylakoids had been resuspended in fresh medium, this inhibition must have resulted from GTPγS bound during the preincubation step. The effects of a series of non-hydrolysable GTP and GDP analogues on the ATP-induced fluorescence quenching are shown in fig.2. These analogues were used rather than GTP or GDP, since their non-hydrolysable nature precluded effects which could have arisen with GTP due to hydrolysis of the terminal phosphoester bond. In the control traces, addition of ATP to 0.5 mM caused a steady decrease in variable fluorescence which remained approximately linear for the duration of the experiment (fig.2a), whereas omission of the ATP

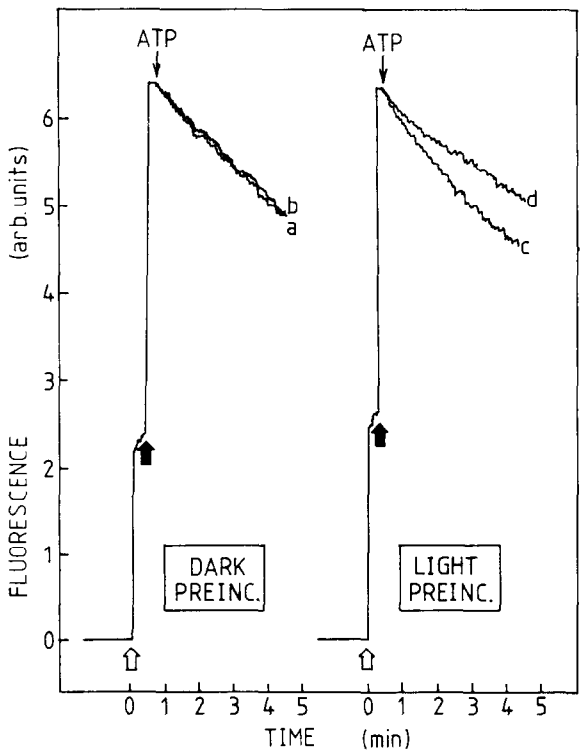

Fig.1. Effect of preincubation with GTPγS on ATP-induced fluorescence quenching in spinach thylakoids. Thylakoids (30 μg Chl), suspended in 1.0 ml of reaction medium, pH 7.5, were preincubated for 5 min in the presence or absence of 10 μM GTPγS under the conditions indicated. After harvesting the thylakoids by centrifugation for 40 s at 12000 × g, the thylakoid pellet was rapidly resuspended in 3 ml of fresh reaction medium (pH 7.5) plus 1 μM gramicidin and the ATP-induced fluorescence decrease was measured as described in section 2. The modulated light (⬆) and actinic light (⇧) were switched on as indicated and ATP added to a concentration of 0.5 mM after 10 s of actinic illumination. Controls (a,c), preincubation with GTPγS (b,d).

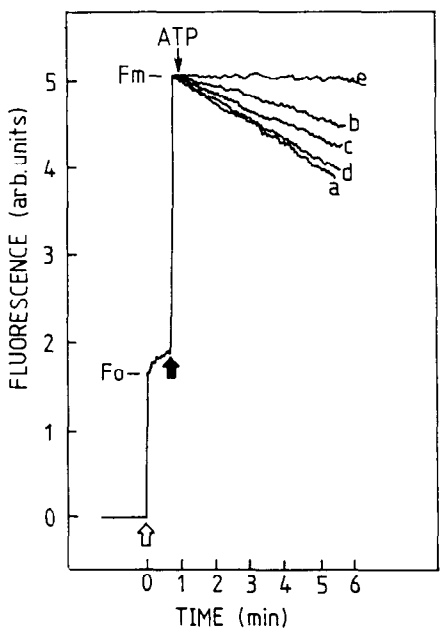

Fig.2. Effect of nucleotides on ATP-induced fluorescence quenching in spinach thylakoids. Modulated fluorescence was measured as described in section 2 using thylakoids equal to 10 μg (Chl) ml^{-1} in reaction medium (pH 7.5) plus 1 μM gramicidin. The modulated light (⬆) and actinic light (⇧) were switched on as indicated and ATP was added to 0.5 mM following 10 s of actinic illumination. Nucleotides were also present in, or absent from the reaction mixture as follows: Control, no guanine nucleotide (trace a); 5 μM GTPγS (trace b); 5 μM GppNHp (trace c); 5 μM GDPβS (trace d); control, no guanine nucleotide or ATP added (trace e).

resulted in a constant level of fluorescence (fig.2e). This indicates that the fluorescence quenching was due to operation of the thylakoid protein kinase. Usually, 1 μM gramicidin was present in the reaction mixture. However, including greater concentrations of gramicidin or utilizing 1 to 2 μM nigericin as uncoupler had no effect on the fluorescence signal indicating that high-energy quenching, q_E [21], did not contribute significantly to the fluorescence decrease. Under these conditions GTPγS gave the maximum inhibition of fluorescence quenching (fig.2b) usually by some 50–60% at 5 μM. GppNHp at the same concentration also inhibited the fluorescence quenching (fig.2c) whilst GDPβS was without effect (fig.2d). Typical rates of ATP-induced fluorescence quenching observed under these conditions are given in table 1 (expt 1). Again it is evident that only GTP analogues were effective in causing inhibition. In experiments where GTP itself, and other nucleotides were present, only the former was effective in inhibiting the fluorescence quenching (table 1, expt 2) and 5 μM GDP or other nucleotide triphosphates did not alter the rate of fluorescence quenching.

Data concerning the binding of [^{35}S]GTPγS and [5′,8-^{3}H]GTP by thylakoid membranes are presented in table 2; in these experiments 100 nM radiolabelled ligand was employed since at the

Table 1

Effects of nucleotide diphosphates and triphosphates on the rate of ATP-induced fluorescence quenching in spinach thylakoids

	Nucleotide present	$\Delta F/F_v$ min^{-1}	% of control
Expt 1	control	8.39	100
	GppNHp	4.99	60
	GTPγS	4.40	52
	dGTP	5.31	63
	GDPβS	8.15	97
Expt 2	control	10.40	100
	GTP	7.71	74
	GDP	10.51	101
	TTP	10.62	102
	CTP	10.86	104
	UTP	10.31	99

Conditions here are as described in legend to fig.1. The nucleotides listed were present at 5 μM except for dGTP and TTP which were 6.25 μM. The control rates in expts 1 and 2 represent that found in the absence of nucleotides other than 0.5 mM ATP which was added in all cases to initiate the fluorescence quenching. The rate of fluorescence decrease, $\Delta F/F_v$ min^{-1} (where $F_v = F_m - F_o$) is in arbitrary units and values presented are the means of 2–4 measurements

Table 2

Binding of [^{35}S]GTPγS and [5′,8-^{3}H]GTP to spinach thylakoid membranes

Binding conditions	Total binding (dpm)	Nonspecific binding (dpm)	Specific binding [pmol·mg^{-1} (Chl)]
(a) [^{35}S]GTPγS			
Light	33 869	6 951	26.9
Dark	8 481	2 981	5.5
Dark, 2 μM Fd, 1 mM NADPH	6 089	2 015	4.1
Dark, 2 μM Fd, 1 mM NADP	7 628	2 505	5.1
(b) [^{35}S]GTPγS			
Light, 2 mM TTP, GTP absent	20 803	16 086	–
Light, 2 mM UTP	20 092	19 045	–
(c) [5′,8-^{3}H]GTP			
Light	18 968	610	18.4
Dark	6 972	529	6.4
Dark, 2 μM Fd, 1 mM NADPH	4 058	425	3.6
Dark, 2 μM Fd, 1 mM NADP	5 185	527	4.7

Binding assays were carried out by addition of thylakoids, equal to 27.5 μg Chl to 100 μl (total volume) of a reaction medium consisting of 33 mM tricine, pH 7.5, 220 mM sorbitol, 10 mM KCl, 3.3 mM $MgCl_2$, 100 nM GTPγS or GTP and 7.3 kBq of the corresponding radiolabelled nucleotide. In some cases 2 μM Fd and 1 mM NADPH (or NADP) were also present. Non-specific binding was determined by including 2.5 mM GTP in the incubation mixture, whereas in b, 2 mM TTP or UTP was included instead. Binding was allowed to proceed for 10 min prior to determination of thylakoid-bound radiolabel as described in section 2. The values presented are the means of triplicate determinations, with a typical standard error of 2 to 4%

higher concentration used in fluorescence experiments (i.e., 5 μM) the non-specific binding component was appreciable. It is apparent from these data that a large increase in the binding of radiolabelled GTPγS and GTP to thylakoids occurred when binding was carried out in the light as compared with that in the dark. The displacement of a large proportion of the radiolabel by unlabelled GTP but not by other nucleotide triphosphates such as TTP and UTP (table 2b, 'Non-specific binding') or CTP (not shown) indicate that this represents specific binding of GTP and its analogue GTPγS. The binding assay was also performed in the dark using ferredoxin and NADPH to reduce the plastoquinone pool and thus activate the kinase. Under these conditions, no large increase in binding of the type seen in the light was observed. In fact, binding was reduced with NADPH/Fd present (reducing conditions) compared with NADP/Fd (oxidizing conditions) and in both cases the specific binding of ligand was below that seen in the dark without additions. Interestingly under all conditions the non-hydrolysable [^{35}S]GTPγS always gave much higher amounts of non-specific binding than [5′,8-^{3}H]-GTP. Finally, preliminary experiments have indicated that ATP was able, under some conditions to displace bound GTP or GTPγS (not shown). However, the relationship between ATP and GTP binding is complex and will be the subject of further communication.

4. DISCUSSION

The work presented shows that spinach thylakoid membranes were able to bind GTP and its non-hydrolysable analogue GTPγS in a specific and light-dependent fashion. The bound guanine nucleotide triphosphate subsequently causes inhibition of the ATP-induced decrease in chlorophyll fluorescence that results from operation of the thylakoid LHC II kinase. In addition to the data presented similar inhibition and binding data were also obtained with pea thylakoids.

At present, the mechanism of interaction of guanine nucleoside triphosphates with the thylakoid membrane is unclear. The data may indicate a binding site for GTP on the LHC II kinase itself or GTP binding by a regulatory protein (G-protein) that is then able to interact with the kinase. In animal cell membrane-bound receptor systems that involve G-proteins, including visual rhodopsin and β-adrenergic hormone receptors [14,15], binding of GTP by the relevant G-protein only occurs when the latter is able to interact with an activated receptor [14,15,22–24]. The active G-protein-GTP complex then undergoes a complex kinetic cycle in which deactivation is partly caused by hydrolysis of the bound GTP to GDP [22–24]. In such systems, non-hydrolysable GTP analogues such as GTPγS and GppNHp thus lead to an enhanced receptor-mediated effect due to permanent activation of its G-protein, e.g. elevation of cellular cAMP level. Whilst the above systems have been found in eukaryotic cells and the chloroplast is prokaryotic in nature, it should be noted that the elongation factor Tu in prokaryotic protein synthesis is also a G-protein [22,24] and is considered to be ancestral to the eukaryotic G-proteins [24]. The observation presented here, that binding of GTP and GTPγS was a strongly light-stimulated event and that the non-hydrolysable guanine nucleotides GTPγS and GppNHp were more effective inhibitors of ATP-induced fluorescence quenching than GTP strongly supports the idea that a G-protein or proteins may interact in some way with the LHC II protein kinase. Moreover the much lower levels of bound [5′,8-^{3}H]GTP than bound [^{35}S]GTPγS in the non-specific binding samples (table 2), could be explained by the accelerated turnover, and thus replacement of [5′,8-^{3}H]GTP with unlabelled GTP in the former case. At present, the binding data obtained in the presence of Fd and NADPH (or NADP) that is shown in table 2a,c cannot be easily explained. However, these data could indicate that reduced Fd is in some way able to prevent GTP binding to the thylakoid membranes, perhaps by physically obscuring the GTP binding site.

The present report, therefore, provides strong initial evidence for the existence of a guanine nucleotide-binding regulatory protein, or proteins, associated with chloroplast thylakoid membranes. In addition to GTPase activity, G-proteins possess a range of specific molecular attributes [22–24]. These include, in many cases, the ability to serve as substrates for cholera toxin and other toxins (which cause a covalent ADP-ribosylation and impairment of GTP binding), non-physiological ac-

tivation by AlF_4^- and a characteristic subunit organization and molecular mass. In a continuing investigation these characteristics will be sought in order to enable further biochemical evidence for the putative thylakoid G-protein.

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