m-Acetylanilido-GTP, a novel photoaffinity label for GTP-binding proteins: synthesis and application

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A novel photoaffinity label, *m*-acetylanilido-GTP (*m*-AcAGTP), was synthesized and used to identify GTP-binding proteins (G-proteins). This GTP analogue is easily prepared and can be used for photoaffinity labelling of G-proteins without chromatographic purification. In the presence of the β -adrenergic agonist isoprenaline, it activates turkey erythrocyte adenylate cyclase. This activation persists even when the β -adrenergic receptor is subsequently blocked by antagonist, indicating that the GTP analogue is resistant to hydrolysis. The apparent K_a for activation of turkey erythrocyte adenylate cyclase by *m*-AcAGTP was found to be 0.21 μ M, a value similar to that for guanosine 5'-[β , γ -imido]triphosphate. *m*-AcAGTP also effectively inhibited the

light-dependent GTPase of *Musca* fly eye membranes. Photoaffinity labelling of fly eye membranes with [α - 32 P]*m*-AcAGTP, followed by immunoprecipitation of G-protein G_q , identified a labelled protein band with the mobility of a 41.5 kDa protein on SDS/PAGE. Labelling of this protein was enhanced 9-fold in blue over red illuminated membranes, containing metarhodopsin and rhodopsin respectively. Labelling of α -subunits of heterotrimeric G-proteins was also demonstrated in turkey erythrocyte membranes. The ease of preparation of *m*-AcAGTP and the chemical properties of the photoreactive acetophenone make this affinity label an important new tool in studies of cellular phenomena mediated by guanine nucleotide-binding proteins.

INTRODUCTION

Photoaffinity labelling is one of the methods used for identification of distinct molecular components in biochemical processes [1]. The growing complexity of the superfamily of GTP-binding proteins (G-proteins) requires the use of photoaffinity labelling, among other methods, for identification of distinct members of this family. The simplest procedure for labelling G-proteins is by u.v. irradiation of the labelled physiological ligand, [α - 32 P]GTP [2]. This method, however, gives a low level of covalent incorporation and hence has low sensitivity. Chemical affinity labelling was used by Low et al. [3] for labelling of G-proteins. Photoaffinity labelling is usually preferred over chemical affinity labelling because the photoaffinity label is generally more stable in solution, photoactivation is time controlled and chemical affinity labels usually react only with certain amino acids. The most widely used photoaffinity label for G-proteins is azidoanilido-GTP, first synthesized and used by Pfeuffer [4]. However, this compound suffers from several shortcomings such as chemical instability and a tendency to react with water or to rearrange after photoactivation. Furthermore, because of the photochemical reactivity of azidoanilido-GTP, its synthesis, purification and application must be performed in the dark.

To overcome some of these disadvantages, we have developed a new photoaffinity label for G-proteins, *m*-acetylanilido-GTP (*m*-AcAGTP) (Figure 1). Acetophenone, the photoreactive component of this compound, like other aryl ketones, is excited by u.v. light to a triplet biradical state, which is highly reactive towards C–H bonds but nearly inert towards water [5]. Particularly reactive are tertiary C–H bonds (as in valine, leucine and isoleucine), and C–H bonds adjacent to heteroatoms, as in methionine or C_α -H bonds of all amino acids [6]. Photolysis of *m*-AcAGTP bound to the G-protein results in formation of a

covalent bond between the carbonyl of acetophenone and an amino acid in the GTP-binding site of the G-protein.

A photoaffinity label based on benzophenone attached to the ribose ring of ATP (BzATP) has been previously used for photoaffinity labelling of ATP-binding proteins [7]. On the other hand, a similar GTP derivative was unable to label the visual G-protein transducin [8]. In the present work, *m*-aminoacetophenone was attached to GTP via the γ -phosphate, as previous work [4] suggested that modification at this position does not disturb binding to G-proteins, the coupling of the anilide to the

Figure 1 Structure and u.v. spectrum of *m*-AcAGTP

Abbreviations used: *m*-AcAGTP, *m*-acetylanilido-GTP; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate; EDC, *N*-ethyl-*N'*-(3-dimethylamino-propyl)carbodi-imide hydrochloride; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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GTP γ -phosphate is readily performed and the phosphoramidate renders the GTP analogue resistant to hydrolysis. Acetophenone was used rather than benzophenone because of its smaller size, thereby decreasing the steric interference with specific binding to G-proteins. A photoaffinity label for G-proteins based on the coupling of a benzophenone derivative to the γ -phosphate of GTP was recently described [9]. However, the preparation of this label, γ -*N*-[4-benzoylphenylmethyl]-GTP phosphoramidate, requires several steps and this probe has not been shown to react with transducing G-proteins.

We report the synthesis and characterization of m-AcAGTP, describe its application for identification of different G-proteins in several biological systems and suggest an easy and rapid 'one-pot reaction' that yields a product that can be used in labelling experiments without chromatographic purification.

EXPERIMENTAL

Materials

[α - 32 P]GTP and [γ - 32 P]GTP were from Amersham, *m*-aminoacetophenone, alkaline phosphatase (*Escherichia coli*) and snake venom phosphodiesterase were from Sigma, Protein A-agarose was from Affinity Chromatography Ltd. (Isle of Man, UK), *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC) was from Fluka, Na₂H₂GTP was from Parma-Waldhof and 1,4-dioxane was from Mallinckrodt. The G_q-directed antiserum (QL), prepared against the C-terminal decapeptide of the α -subunit, was a generous gift from Thresa L. Z. Jones (NIH, Bethesda, MD, U.S.A.).

Preparation of m-AcAGTP

Na₂H₂GTP (20 μ mol; 1 equiv.) and EDC (100 μ mol; 5 equiv.) were dissolved in 0.5 ml of 0.125 M Mes buffer, pH 6.8. *m*-Aminoacetophenone (200 μ mol; 10 equiv.), dissolved in 0.2 ml of 1,4-dioxane, was added and the mixture was kept at room temperature. Quantitative conversion of GTP into m-AcAGTP was achieved overnight. The solution was applied to an anion-exchange column (Partisil 10 SAX; 250 mm $\times$ 4.6 mm) in an h.p.l.c. apparatus. Gradient salt elution was performed and fractions eluted at 0.75 M NaCl were pooled and lyophilized. The elution programme started with 0.4 M NaCl for 10 min, increased in linear steps to 0.56 M and 0.75 M after 30 and 35 min respectively and continued isocratically with 0.75 M NaCl until 50 min. Flow rate was 1 ml/min. For final purification, the lyophilized preparation was dissolved in water, applied to a preparative RP-8 h.p.l.c. column (250 mm $\times$ 10 mm), gradient methanol elution was performed and the product-containing fractions eluted at 12% (v/v) methanol were pooled. Methanol concentration was increased linearly from 5 to 12% over 19 min, followed by isocratic elution with 12% methanol for 21 min. The flow rate was 2 ml/min and the solutions contained 30 mM triethylammonium bicarbonate, pH 7.5. The purified m-AcAGTP was subsequently lyophilized, dissolved in 0.1 M Mops buffer (pH 7.0) and stored at -18°C . Under these conditions no decomposition was detected by h.p.l.c. within 6 months.

Preparation of radioactive m-AcAGTP

[α - 32 P]GTP (400 Ci/mmol) or [γ - 32 P]GTP (> 5000 Ci/mmol) was freeze-dried and redissolved in 50 μ l of 0.125 M Mes buffer, pH 6.5. EDC (10 μ mol) was then dissolved in this solution and *m*-aminoacetophenone (20 μ mol) in 20 μ l of 1,4-dioxane was added. After 5 h at room temperature the mixture was freeze-

dried and redissolved in 10 mM Mops buffer (pH 7.0). Residual insoluble *m*-aminoacetophenone was removed by centrifugation and the product [32 P]m-AcAGTP was ready for use in labelling experiments. The radioactive photoaffinity label, kept at -18°C , was stable for at least a month.

Assay of adenylate cyclase activity

Turkey erythrocytes [10] and rat parotid [11] membranes were prepared as previously described. The ability of m-AcAGTP to persistently activate the adenylate cyclase was determined as described by Cassel and Selinger [10]. The GTP analogue was preincubated with the membranes (50 μ g of protein) for 10 min at 37°C in the presence of 10 μ M D,L-isoprenaline. The preincubation medium contained (in mM) Mops buffer (pH 7.4) (40), MgCl₂ (6), EGTA (0.2), β_2 -mercaptoethanol (4) and 0.2 mg/ml BSA. Subsequently, adenylate cyclase activity was assayed in the presence of propranolol. The adenylate cyclase reaction was initiated by the addition of 0.3 mM ATP and [α - 32 P]ATP (about 1 μ Ci) in a reaction medium containing (in mM) propranolol (0.05), cyclic AMP (1), phosphocreatine (15), 5 units of creatine kinase and other components as in the preincubation medium. The final volume was 0.1 ml and reaction time was 30 min at 37°C . The reaction was terminated by the addition of 0.2% SDS, and cyclic AMP was measured by the method of Salomon et al. [12].

Assay of GTPase activity

White-eyed *Musca domestica* photoreceptor membranes were prepared as previously described [13]. The photoreceptor membranes (10 μ g of protein) were illuminated for 15 s with either blue ($\lambda < 480$ nm) or red ($\lambda > 570$ nm) light for activation or deactivation of rhodopsin respectively. The assay was initiated by the addition of [γ - 32 P]GTP (1.84 Ci/mmol; 0.25–0.75 μ M) in an incubation solution containing (in mM) Mops buffer (pH 6.7) (40), MgCl₂ (3), KCl (48), phenylmethanesulphonyl fluoride (0.4), dithioerythritol (1), ATP (0.6), 0.2 mg/ml BSA, 4 μ g/ml leupeptin and 0.4 μ g/ml pepstatin A. When present, concentrations of m-AcAGTP or guanosine 5'-[β , γ -imidio]triphosphate (p[NH]ppG), which are both inhibitors of the GTPase reaction, were 2 μ M. The total volume was 100 μ l and reaction time was 5 min at $0-4^{\circ}\text{C}$. The reaction was terminated by the addition of 500 μ l of 5% charcoal (Norit A) suspension in sodium phosphate buffer (20 mM, pH 2.5). The suspension was centrifuged (1000 *g* for 5 min) and radioactivity, representing liberated [32 P]P_i, was determined on portions of the supernatant. The assay was performed in triplicate.

Photoaffinity labelling of fly photoreceptor membranes

White-eyed *Musca domestica* photoreceptor membranes were prepared as previously described [13]. The photoreceptor membranes (175 μ g of protein) were illuminated for 30 s with either blue ($\lambda < 480$ nm) or red ($\lambda > 570$ nm) light for activation or deactivation of rhodopsin respectively. The membranes were then incubated with [γ - 32 P]m-AcAGTP (about 500 Ci/mmol; 96 nM; 3 μ Ci) in an incubation solution containing (in mM) Mops buffer (pH 6.7) (40), MgCl₂ (3), KCl (48), phenylmethanesulphonyl fluoride (0.4), dithioerythritol (1), ATP (0.6), phosphocreatine (15), 5 units of creatine kinase, 0.2 mg/ml BSA, 4 μ g/ml leupeptin and 0.4 μ g/ml pepstatin A. The total volume was 62.5 μ l and incubation time was 10 min at $0-4^{\circ}\text{C}$. The reaction was terminated by dilution with 400 μ l of ice-cold incubation solution. Excess of photoaffinity label was removed by centri-

fugation at 4 °C (12000 *g* for 10 min) and the pellet was resuspended in 60 μ l of the incubation solution, not containing m-AcAGTP and ATP.

Membrane samples were placed for irradiation in dimples made in an aluminium foil pressed (reflecting side up) against an empty rack of large pipette tips. The aluminium foil was wrapped over and beneath a glass Petri dish cover which was kept on ice. The shallow level of the sample, the reflectance of the aluminium foil and its good thermal conductivity give rise to effective photoactivation than can be applied for an extended period with only a slight decrease in sample volume. U.v. irradiation ($\lambda > 320$ nm) was applied for 30 min by an Hg(Ar) pencil lamp (Oriol, no. 6035) equipped with a long-wave filter (Oriol, no. 6042) and set at a distance of 0.5 cm from the samples. The long-wave filter was used in order to reduce the photodamage to proteins [14].

The membranes were then subjected to immunoprecipitation as follows: an equal volume of 2% (w/v) Lubrol in 20 mM Tris/HCl (pH 7.4) containing 150 mM NaCl and 10 mM MgCl₂ was added and the sample was incubated on ice for 60 min. Insoluble material was removed by centrifugation and the supernatant representing the detergent extract was recovered. Affinity-purified QL antibody [15] was added to a final concentration of 60 μ g/ml and the samples were incubated for 2 h at 4 °C. Then 50 μ l of 10% (v/v) Protein A-agarose beads was added and samples were further incubated overnight under constant rotation at 4 °C. The beads were pelleted (12000 *g* for 5 min at 4 °C) and washed twice with 180 μ l of washing buffer A [50 mM Tris/HCl (pH 7.4), 600 mM NaCl, 0.5% (w/v) SDS, 1% (v/v) Nonidet P-40] and once with washing buffer B [100 mM Tris/HCl (pH 7.4), 300 mM NaCl, 10 mM EDTA]. Sample buffer (100 μ l) was added, the mixture was boiled for 10 min and centrifuged as above. Half the supernatant was then subjected to SDS/PAGE, performed on 10% polyacrylamide gel by the method of Laemmli [16]. Radioactive molecular-mass markers (Amersham) were used while fixation and staining were avoided as the P-N bond in the probe is acid-labile. The gels were visualized by phosphorimaging and X-ray autoradiography.

Photoaffinity labelling of turkey erythrocytes

Membranes of turkey erythrocytes (25 μ g of protein) were incubated for 10 min at 37 °C with [α -³²P]m-AcAGTP (400 Ci/mmol; 42 nM; 0.5 μ Ci) in 30 μ l of medium containing (in mM) Mops buffer (pH 7.1) (20), MgCl₂ (2), EGTA (1), KCl (10), 2-mercapthoethanol (3), ATP (0.3), 0.2 mg/ml BSA and 10 μ g/ml leupeptin. The reaction was terminated by transfer to 0 °C and 10-fold dilution with ice-cold medium. Unbound photoaffinity label was removed by centrifugation (12000 *g* for 5 min at 4 °C) and pellets were resuspended in 20 μ l of the incubation medium, not containing m-AcAGTP and ATP. U.v. irradiation, SDS/PAGE and autoradiography were carried out as described above, except that 7.5–15% polyacrylamide gradient was used for the SDS/PAGE.

Protein determination

Protein was determined by the Bradford procedure [17] with BSA as standard.

RESULTS

Synthesis and purification of m-AcAGTP

Figure 1 shows the structure of the photoaffinity label, m-AcAGTP. The photoreactive acetophenone moiety was coupled

Figure 2 Photoreaction of m-AcAGTP on polyethyleneimine-cellulose t.l.c. plate

[α -³²P]m-AcAGTP (lanes 1 and 3) and [α -³²P]GTP (lanes 2 and 4) were spotted on polyethyleneimine-cellulose plates. Lanes 3 and 4 were exposed to u.v. light for 10 min. Analysis was performed by autoradiography after development in 1 M LiCl.

to the γ -phosphate of GTP using the water-soluble EDC. The coupling reaction proceeds readily, reaching 93% yield in 2 h and 99% if left for about 5 h. The unlabelled m-AcAGTP can be purified to more than 95% by either one of the two h.p.l.c. steps described in the Experimental section. However, in order to obtain complete purification both steps were carried out. Both the coupling reaction and purification of the product can be performed in full light. The excellent yield and lack of side reactions make it possible to prepare radioactive m-AcAGTP and use it for photoaffinity labelling experiments without chromatographic purification (see below and Figure 6).

Chemical characterization of m-AcAGTP

Determination of m-AcAGTP concentration by an assay of total phosphate content [18] and spectrophotometric analysis yielded a molar absorption coefficient for m-AcAGTP of 28 800 M⁻¹·cm⁻¹ at 232 nm. The u.v. spectrum of the purified product, shown in Figure 1, is a composite of the spectra of GTP and m-aminoacetophenone: λ_{max} of GTP is at 250 nm, whereas m-aminoacetophenone has two absorbance peaks, at 232 nm and a small broad peak above 300 nm corresponding to the $n \rightarrow \pi^*$ absorption transition of the carbonyl group of acetophenone [6]. All three peaks are observed in the u.v. spectrum of the product. The photoreactivity of m-AcAGTP was tested by u.v. irradiation of the affinity label spotted on polyethyleneimine-cellulose plates followed by chromatography with 1 M LiCl; 35% of the irradiated photoaffinity label did not migrate from the origin. In contrast, non-illuminated m-AcAGTP gave an R_f value of 0.71 and GTP had an R_f value of 0.39 whether it was illuminated or not (Figure 2). The structure of m-AcAGTP (Figure 1) was further analysed by enzymic hydrolysis with alkaline phosphatase and snake venom phosphodiesterase. The analogue m-AcAGTP was found to be resistant to treatment with alkaline phosphatase but was rapidly converted into 5'-GMP by phosphodiesterase (not shown). These results are consistent with the structure shown in Figure 1. Finally, fast atom bombardment mass spectra gave a molecular peak ($M-H$) at m/z 639 and three higher molecular peaks corresponding to the molecular mass of m-AcAGTP and m-AcAGTP with one, two and three Na⁺ ions.

Biochemical characterization of m-AcAGTP

The affinity of m-AcAGTP for the G-protein G_s was determined by activation of turkey erythrocyte adenylate cyclase with different concentrations of m-AcAGTP in the presence of iso-

Figure 3 Lineweaver-Burk plot of adenylate cyclase activation by m-AcAGTP and p[NH]ppG

Turkey erythrocyte membranes were preincubated for 10 min at 37 °C with 10 μM D,L-isoprenaline and either m-AcAGTP, 8.3–500 nM (○), or p[NH]ppG, 8.3–100 nM (●). Adenylate cyclase activity was subsequently assayed at 37 °C for 30 min in the presence of 50 μM propranolol as described in the Experimental section. Each value is the mean of three independent determinations of adenylate cyclase activity (S.E.M. < 3%).

Table 1 Persistent activation of adenylate cyclase by GTP analogues

Adenylate cyclase activity of turkey erythrocyte membranes (50 μg) was determined in the presence of the β-adrenergic agonist isoprenaline (10 μM), GTP analogues and the substrate [α -³²P]ATP for 30 min at 37 °C. Persistent adenylate cyclase activation was determined by preincubation (10 min, 37 °C) of turkey erythrocyte membranes (50 μg) with isoprenaline (10 μM) and GTP analogues, followed by assay (30 min, 37 °C) of enzyme activity in the presence of the β-adrenergic antagonist propranolol (50 μM) and the substrate [α -³²P]ATP. The concentrations of GTP and analogues (in μM) were: GTP (50), m-AcAGTP (50), p[NH]ppG (50) and GTP[S] (10). Conditions for the assay are described in the Experimental section. The results are means ± S.E.M. of three independent determinations of adenylate cyclase activity, expressed as pmol of cyclic AMP/min per mg of protein.

GTP analogue	Adenylate cyclase activity	Persistent adenylate cyclase activity
GTP	30 ± 1	< 1
m-AcAGTP	27.7 ± 0.1	29.6 ± 0.3
p[NH]ppG	94 ± 15	83 ± 1
GTP[S]	122 ± 1	103 ± 2

prelaine. Figure 3 shows the Lineweaver-Burk plot of adenylate cyclase activity which yields apparent K_a values of 0.21 μM and 0.30 μM for m-AcAGTP and p[NH]ppG respectively. Persistent activation of the adenylate cyclase by m-AcAGTP was tested by preincubation of the label with turkey erythrocyte membranes in the presence of the β-adrenergic agonist isoprenaline followed by assay of adenylate cyclase activity in the presence of the β-adrenergic blocker propranolol. During the preincubation period, the activated β-adrenergic receptor charges the G-protein with the GTP analogue, and the subsequent assay of adenylate cyclase activity in the presence of propranolol measures whether the G-protein remains persistently active, as was indeed found

Figure 4 Lineweaver-Burk plot of inhibition of GTPase activity by m-AcAGTP and p[NH]ppG

Fly photoreceptor membranes (10 μg of protein) were illuminated for 15 s with blue light for the conversion of rhodopsin into the activated metarhodopsin. GTPase activity was assayed in the dark at 0–4 °C for 5 min, as described in the Experimental section. The reaction was initiated by the addition of [γ -³²P]GTP (1.84 Ci/mmol; 0.25–0.75 μM) in the absence (■) or presence of 2 μM m-AcAGTP (●) or 2 μM p[NH]ppG (▲). The reaction was terminated by the addition of 0.5 ml of 5% charcoal (Norit A) suspension in sodium phosphate buffer (20 mM, pH 2.5). The values represent the light-induced GTPase activity, calculated by subtracting the basal GTPase activity (measured in the same procedure after a 15 s illumination with red light) from the blue light-induced GTPase activity. Each value is a mean of three independent determinations (S.E.M. < 5%).

for G-protein charged with the hydrolysis-resistant GTP analogues p[NH]ppG and guanosine [γ -thio]triphosphate (GTP[S]) [10]. This protocol gave persistent adenylate cyclase activity when m-AcAGTP, p[NH]ppG or GTP[S] were present during the preincubation period, whereas GTP did not persistently activate the enzyme (Table 1). m-AcAGTP only partially activated the adenylate cyclase indicating that it serves as a partial agonist in this system. The partial activation by m-AcAGTP cannot be easily explained by the co-activation of a G_i -protein by the high concentration of m-AcAGTP used in this experiment, as the same values of V_{max} are obtained by extrapolation of the linear Lineweaver-Burk plots for both m-AcAGTP and p[NH]ppG (Figure 3). Furthermore, activation of the G_i -protein by p[NH]ppG or GTP[S] in the absence of a specific agonist is almost undetectable [19,20]. Results similar to those shown in Figure 3 and Table 1 were obtained with the rat parotid adenylate cyclase.

The affinity of m-AcAGTP for the G-protein G_q was determined by inhibition of the light-induced GTPase activity of fly eye membranes with m-AcAGTP. The Lineweaver-Burk plot shows that both m-AcAGTP and p[NH]ppG competitively inhibited the light-induced GTPase activity with K_i values of 1.8 μM and 1.2 μM respectively. The K_m of GTP was found to be 0.7 μM in the same experiment (Figure 4).

Taken together, these results show that m-AcAGTP has a high affinity for several G-proteins and its activity is characterized by resistance of the bound analogue to enzymic hydrolysis.

Light-dependent labelling of the fly photoreceptor G-protein

Fly eye membranes were illuminated with either red ($\lambda > 570$ nm) or blue ($\lambda < 480$ nm) light to generate rhodopsin and meta-rhodopsin respectively. Incubation of these membranes with

Figure 5 Light-dependent photoaffinity labelling of G-protein in fly photoreceptor membranes

[α - 32 P]m-AcAGTP (500 Ci/mmol; 96 nM; 3 μ Ci) was incubated for 10 min at 0–4 °C with fly photoreceptor membranes (175 μ g of protein) pre-illuminated with either red (lane 1) or blue (lane 2) light and processed as described in the Experimental section. Photoactivation was by u.v. illumination ($\lambda > 320$ nm) for 30 min at 0–4 °C, using a mercury/argon lamp set at a distance of 0.5 cm from the sample. The G_q -protein was immunoprecipitated by an affinity-purified QL antibody directed against the C-terminal decapeptide of the protein. Labelling was visualized by SDS/PAGE followed by X-ray film autoradiography and quantification by phosphorimaging. Competition of the photoaffinity label with p[NH]ppG or p[NH]ppA (10 μ M) was performed on blue-illuminated membranes, using the same procedure (lanes 3 and 4, respectively).

[α - 32 P]m-AcAGTP followed by photoactivation with u.v. light, immunoprecipitation of the G_q -protein and analysis by SDS/PAGE resulted in labelling of one protein band with mobility of a 41.5 kDa protein (Figure 5). Labelling of the 41.5 kDa band by [α - 32 P]m-AcAGTP was enhanced 9-fold by blue light over that by red light (Figure 5, lanes 2 and 1 respectively). This result is consistent with the following previously published data: the fly eye is specialized in signal transduction of light stimuli; the light-activated G-protein, which is an abundant protein in this tissue, is known to activate phospholipase C [13]; the magnitude of the enhanced labelling by blue light is similar to the magnitude of light-dependent increase in GTPase activity in the fly eye membranes [21]. The labelling was completely abolished by addition of p[NH]ppG together with m-AcAGTP (Figure 5, lane 3) but was only slightly reduced (20 %) by addition of p[NH]ppA together with m-AcAGTP (Figure 5, lane 4). Photolabelling by m-AcAGTP was completely dependent on photolysis as no incorporation was observed if u.v. illumination was omitted. No labelling was observed when [α - 32 P]m-AcAGTP was replaced by [α - 32 P]GTP (results not shown). Taken together, these results suggest that G_q , the labelled 41.5 kDa protein in fly eye membranes, is a G-protein that mediates the transduction of the light stimulus.

Photoaffinity labelling of G-proteins in turkey erythrocytes

In order to establish that m-AcAGTP can be used for labelling of G-proteins in signalling systems other than the fly visual system, we have tested the photoaffinity label on turkey erythrocyte membranes. In the course of these studies we have found that in practice there is no need to purify the label by chromatography. Both m-AcAGTP extracted from the lyophilized reaction mixture and the h.p.l.c.-purified m-AcAGTP

Figure 6 Photoaffinity labelling of G-proteins in turkey erythrocyte membranes

[α - 32 P]m-AcAGTP (400 Ci/mmol; 42 nM; 0.5 μ Ci) was incubated for 10 min at 37 °C with 25 μ g of turkey erythrocyte membranes as described in the Experimental section. H.p.l.c.-purified [α - 32 P]m-AcAGTP (lane 1) or unpurified [α - 32 P]m-AcAGTP (lane 2) was used as photoaffinity label. Excess probe was removed by centrifugation and the pellet was resuspended and illuminated for 30 min at 0–4 °C by u.v. light ($\lambda > 320$ nm) of a mercury/argon lamp set 0.5 cm from the sample. Labelling was visualized by SDS/PAGE followed by X-ray autoradiography.

gave similar labelling patterns of 40–45 kDa proteins which correspond to α -subunits of heterotrimeric G-proteins (Figure 6).

Other experiments in several membrane preparations revealed that [γ - 32 P]m-AcAGTP of high specific radioactivity (> 5000 Ci/mmol) greatly increased the sensitivity of the photoaffinity labelling.

DISCUSSION

The GTP analogue, m-AcAGTP, is a unique photoaffinity label, as it can be synthesized and applied to biological preparations without the need to protect it from light. This unusual but convenient behaviour can be explained in the following way. Aryl ketones are excited by u.v. light to a triplet biradical intermediate which is incorporated into a nearby C–H bond but is inert towards water. In the absence of a C–H bond in close proximity, the excited intermediate quenches back to the ground state and the aryl ketone remains excitable if hit again by another photon [5,6]. In the case of m-aminoacetophenone, the low absorption at the wavelength that causes excitation further contributes to the relative insensitivity of m-AcAGTP to light. These properties, however, dictate the need for prolonged photoactivation of the G-protein-bound m-AcAGTP by intense

long-wave u.v. illumination ($\lambda > 320$ nm). m-AcAGTP also has several other desirable properties: (1) Synthesis of the probe is very simple, only requiring mixing of three commercially available compounds, labelled GTP, m-aminoacetophenone and the water-soluble carbodi-imide EDC, for the coupling reaction. (2) Quantitative conversion of GTP into m-AcAGTP is achieved unattended within a few hours. (3) Chromatographic purification of the product is not needed for labelling experiments. (4) The product has excellent chemical stability in solution. (5) It has high affinity for G_s and G_q and probably also for other G-proteins. The finding that m-AcAGTP is an apparent partial agonist of the adenylate cyclase does not influence the labelling experiments which depend on the high affinity for the G-protein rather than on the extent of activation of the effector. (6) The G-protein-bound m-AcAGTP is resistant to hydrolysis and m-AcAGTP remains bound to the G-protein during centrifugal washing. Taken together, these properties make photoaffinity labelling of G-proteins with m-AcAGTP a simple procedure with good sensitivity and specificity.

This procedure can be used to assign labelling of a particular G-protein to activation of a specific receptor, as we have demonstrated for the fly photoreceptor system. By combination of m-AcAGTP-mediated photoaffinity labelling and immunoprecipitation techniques, we have shown that G_q is a G-protein responsible for transduction of the light stimulus in fly eye membranes. In other membrane systems, demonstration of a connection between activation of a specific receptor and labelling of a particular G-protein is not as easy as in the fly membrane preparation. It requires detailed kinetic analysis and titration of optimal concentrations of Mg^{2+} and GDP which we considered to be outside the scope of the present work. It should be noted, however, that such an assignment has been achieved in the elegant experiments of Laugwitz et al. [22] using azidoanilido-GTP and immunoprecipitation with G_{12} -specific antisera. The proteins labelled by m-AcAGTP in this work were definitely G-proteins, as p[NH]ppG abolished the labelling whereas p[NH]ppA did not. In all the experiments, specific binding to G-proteins was ensured by the presence of unlabelled ATP at a concentration four orders of magnitude higher than that of the photoaffinity label.

It can be expected that the novel photoaffinity label, m-AcAGTP, will be a useful tool in studies of cellular phenomena

mediated by guanine nucleotide-binding proteins, such as signal transduction, secretion, endocytosis and cellular growth control. The methodology described here can be easily extended to preparation of photoaffinity labels for other nucleotide-binding proteins.

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