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# Heterotrimeric G proteins in synaptoneurosome membranes are crosslinked by *p*-phenylenedimaleimide, yielding structures comparable in size to crosslinked tubulin and F-actin

**(signal transduction/biopolymers/guanine nucleotide-binding protein)**

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ABSTRACT **We have treated rat brain synaptoneurosomes with the crosslinking agent *N,N'*-1,4-phenylenedimaleimide under conditions that cause extensive crosslinking of tubulin, F-actin, and the $\alpha$ and $\beta$ subunits of three major types of heterotrimeric GTP-binding regulatory proteins ($G_o$, $G_s$, $G_i$) present in brain membranes. The major crosslinked products are coeluted from Bio-Gel sizing columns as very large structures that do not penetrate stacking gels during SDS/PAGE. The $\alpha$ subunits but not the $\beta$ subunits of $G_s$, $G_o$, and $G_i$ also yield crosslinked products of intermediate sizes. None of the products are as small as the heterotrimeric G proteins extracted from brain by cholate or Lubrol. However, the large and intermediate crosslinked structures are strikingly similar to the large, polydisperse structures of the $\alpha$ subunits of $G_s$, $G_i$, and $G_o$ extracted from synaptoneurosomes by the detergent octyl glucoside, which have sedimentation properties of multimeric proteins. Several ways in which multimeric forms of G proteins can explain the dynamic and pleiotropic actions of hormones and GTP on signal-transducing systems are discussed.**

Guanine nucleotide-binding proteins, commonly termed G proteins, are a family of proteins that serve as the transduction elements between hormone receptors bound to the cell surface and signal-producing systems (adenylyl cyclases, phospholipases, ion channels) that are responsible for initiating the regulation of a variety of processes within hormone-targeted cells. During the past decade many investigations have established that receptor-coupled G proteins minimally consist of heterotrimers of which the $\alpha$ subunits bind and hydrolyze GTP and the $\beta$ and $\gamma$ subunits form a strongly bonded $\beta\gamma$ complex that is necessary for functional interaction of the $\alpha$ subunit with receptors (1). Great strides have been made in ascertaining the number and types of the three subunits (2). Most of the current hypotheses (see ref. 3 for detailed descriptions) of how G proteins serve as transducers between activated receptors and effectors rely on information gained from their behavior in reconstituted systems with purified components. While such knowledge is unquestionably useful, left uncertain are the structures and topological organization of receptors, G proteins, and effectors as they exist in their native membrane environment.

One approach to this problem has been to use irradiation or target size analysis as a means of determining the functional size of the transduction systems in native membranes. In the case of the glucagon-sensitive system in rat liver membranes, it was ascertained (4) that the functional size of the system prior to activation by ligands (hormones, guanine nucleotides, fluoride ion) was $\approx$1500 kDa. When activated the functional mass was reduced by a factor of 4. The 1500-kDa mass could not be accommodated by the now established combined masses of the glucagon receptor [65 kDa (5)], $G\alpha_s$ [the G-protein subunit (45–54 kDa) that stimulates adenylyl cyclase], and adenylyl cyclase [$\approx$210 kDa (6)]. Based on this apparent discrepancy, it was postulated (7) that the activation process may involve the breakdown of a putative multimeric structure containing receptors and $G\alpha_s$ to smaller structures minimally composed of a monomeric, activated form of $G\alpha_s$ linked to adenylyl cyclase. Since similar target sizes were found for the $G\alpha_i$-inhibited process in rat adipocytes (8), it seemed possible that this hypothesis might apply generally to transduction systems involving G proteins.

It was subsequently found (9) that octyl glucoside extracts G proteins that, on hydrodynamic evaluation, are polydisperse structures having sedimentation values far larger than those exhibited by heterotrimeric G proteins extracted with sodium cholate or Lubrol, the usual detergents employed for extraction and purification (10). These larger structures were devoid of the $\beta$ subunits (and presumably $\beta\gamma$ complexes) yet were susceptible to the same disaggregating or dissociative effects of activating ligands such as guanosine 5'-[$\gamma$-thio]triphosphate (GTP[$\gamma$S]), guanosine 5'-[$\beta,\gamma$-imido]triphosphate, and aluminum fluoride observed with purified heterotrimeric G proteins in cholate or Lubrol detergents (1). It became apparent that G proteins do not exist only as heterotrimers; depending on the type of detergent used in their extraction, they can also exist as multimers of varying size and sensitivity to the activating effects of guanine nucleotides.

Pretreatment of rat liver membranes with glucagon and GTP[$\gamma$S] followed by extraction with octyl glucoside led to the formation of immunologically detected structures of $G\alpha_s$ having much smaller sizes than the parent $G\alpha_s$ from control membranes (11). These findings are strikingly similar to the changes observed with target size analysis. Moreover, only about 10% of the total $G\alpha_s$ in the liver membranes was activated by hormonal treatment. This small fraction was derived from material large enough to sediment through a sucrose gradient. One inference from these findings is that the glucagon receptor interacts preferentially with a multimeric form of $G\alpha_s$ in native liver membranes, again consistent with target size analysis of the glucagon-sensitive cyclase system.

In addition to target size analysis, another means of assessing the structure of G proteins in their native membrane environment is to examine the nearest-neighbor relationships of the components with the use of crosslinking agents. If multimeric rather than heterotrimeric structures of G proteins exist in cell membranes, theoretically they should be susceptible to cross-linking, yielding structures having much higher molecular weights than heterotrimeric G pro-

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Abbreviations: PDM, *N,N'*-1,4-phenylenedimaleimide; GTP[$\gamma$S], guanosine 5'-[$\gamma$-thio]triphosphate.

teins. We have employed the crosslinking reagent *N,N'*-1,4-phenylenedimaleimide (PDM) to test this possibility, using rat brain synaptoneurosomes as the source of various G proteins ($G_s$, $G_i$, $G_o$).

## MATERIALS AND METHODS

**Reagents.** Sources of the antisera that react selectively with $G\alpha_s$, $G\alpha_i$, $G\alpha_o$, and the $\beta$ subunits of G proteins were as described (9). Antisera against tubulin and actin were purchased from Biomedical Technologies (Stoughton, MA). PDM was from Aldrich; dithiobis(succinimidyl propionate) and ethylene glycolbis(succinimidyl succinate) were from Pierce. All other chemicals were of reagent grade.

**Incubation Procedures.** Rat brain synaptoneurosomes (9) were stored at −70°C. Prior to each experiment, an aliquot was rapidly thawed and diluted to 1 mg of protein per ml in phosphate-buffered saline (PBS: 150 mM NaCl/10 mM potassium phosphate, pH 7.4). Crosslinking reagents were dissolved in dimethylformamide; in all cases (controls and crosslinked) the final concentration of dimethylformamide was 10% (vol/vol). In preliminary experiments, each crosslinking agent was tested, over a concentration range of 5–500 $\mu$M, for efficacy of crosslinking by treating the membranes (1 mg/ml in PBS) at either 4°C or room temperature. Efficacy of crosslinking of G proteins was evaluated by the extent to which the electrophoretic bands (Western blots) of a given $\alpha$ or $\beta$ subunit decreased in density from its control value. Dithiobis(succinimidyl propionate) proved of little value since it caused general and extensive crosslinking of membrane proteins as determined from the smeared Coomassie blue stains of electrophoresed protein; ethylene glycolbis(succinimidyl succinate), also a cleavable crosslinking reagent, caused crosslinking only at very high concentrations (>1 mM). By contrast, PDM crosslinked <10% of the total membrane protein (Fig. 1). Most of this material probably represented crosslinked tubulin and F-actin. These multimeric proteins, particularly tubulin, displayed extensive crosslinking and were present at relatively high concentrations in the synaptoneurosomes (Fig. 2). In the case of $G\alpha_i$ and $G\alpha_o$, incubation of the membranes at 4°C for 25 min with 75 $\mu$M PDM was sufficient to cause essentially complete crosslinking, as judged by the disappearance of the 40/41-kDa bands typical of these proteins (see Fig. 5). The rate of crosslinking of $G\alpha_s$ and $G_\beta$ was much slower at 4°C (data not shown). Optimal incubation conditions chosen for all of the G-protein subunits and for tubulin and actin were 75 $\mu$M PDM in PBS, pH 7.4, for 1 hr at room temperature. After 1 hr of incubation, 2-mercaptoethanol was added (7 mM) to terminate the crosslinking reaction. The suspension was centrifuged at 8000 rpm for 15 min in a Sorvall RC-5B, SM-24 rotor. The supernatant was discarded and the pellet was suspended in PBS. A solution of SDS was added to give a final concentration of 5%. The mixture was allowed to stand at room temperature until complete solubilization occurred (generally 30 min). Bromphenol blue was then added as a tracking dye.

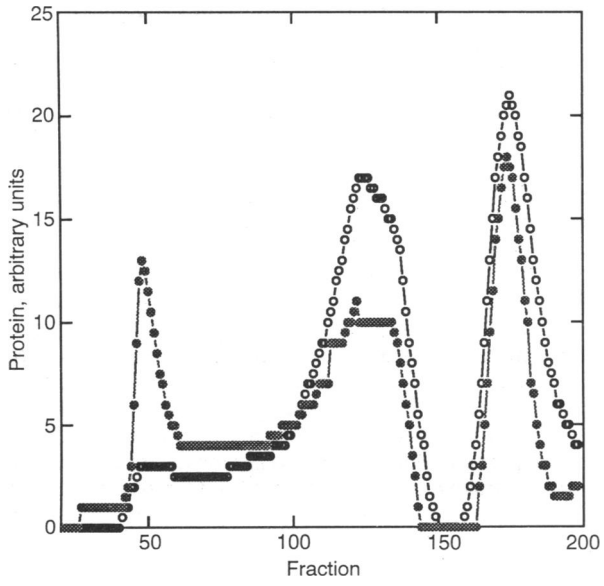


FIG. 1. Crosslinking of proteins in rat brain synaptoneurosomes. Membranes (5 mg) were incubated at room temperature for 1 hr in 5 ml of PBS with (○) or without (●) 75 $\mu$M PDM. The reaction was stopped with 7 mM 2-mercaptoethanol. The membranes were washed in PBS and dissolved in 5% SDS, and the clear solution was injected onto tandemly arrayed Bio-Gel A-50m and A-5m columns. Elution was at room temperature with 10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% SDS at 0.1 ml/min. Protein was monitored continuously at 275 nm.

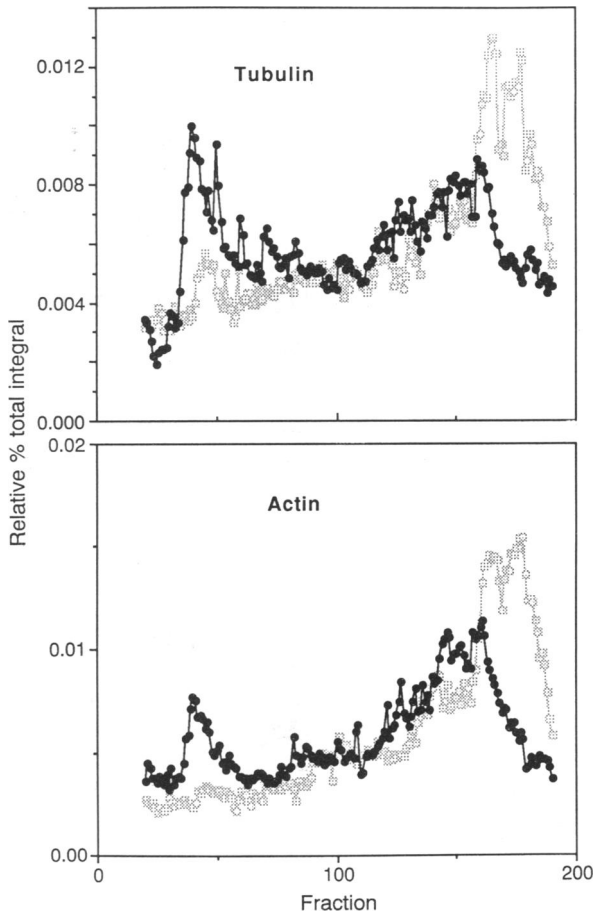


FIG. 2. Crosslinking of tubulin and actin. Synaptoneurosome membranes and procedures were the same as for Fig. 1. Tubulin and actin were detected by dot blotting each fraction with specific antisera. Light symbols, control; ●, crosslinked.

**Chromatography.** The solubilized material was injected onto tandemly arrayed Bio-Gel A-50m/A-5m (Bio-Rad) columns (1.5 × 40 cm each) kept at room temperature. Elution was at room temperature with 10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% SDS at 0.1 ml/min. Eluted protein was monitored at 275 nm, $OD_{max}$ = 0.05. One-milliliter fractions were collected.

**Immunodetection.** Aliquots of each fraction were screened with each of the antisera by dot blotting using a Minifold (Schleicher & Schuell) filtration apparatus. Dot blots were quantitated with a Chromoscan 3 (Joyce–Loebl) densitometer in its reflectance mode. The data were normalized by summing the values for each type of G-protein subunit (control or crosslinked) and plotting the results in terms of the relative

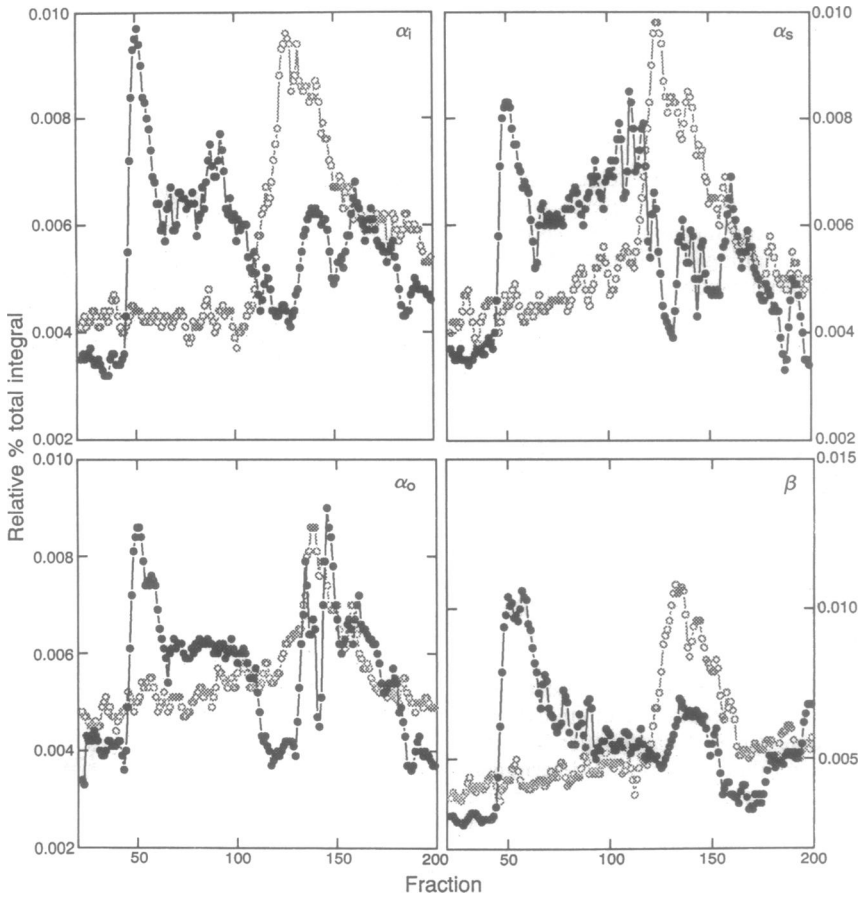


FIG. 3. Crosslinking of $\alpha$ and $\beta$ subunits of $G_s$, $G_i$, and $G_o$ in synaptoneurosomes. Methods were the same as for Figs. 1 and 2. Subunits were detected by dot blotting each fraction with antisera specific for the indicated subunits. ○, Control; ●, crosslinked.

percent of the total integral. The results represent at least two experiments with different synaptosome preparations.

**Immunoblotting.** The peak fractions from the sizing columns were also monitored for the types and sizes of the control and crosslinked G-protein subunits. Generally 10 pooled fractions (see legend to Fig. 4) were electrophoresed in SDS/polyacrylamide gels at 40 mA per gel. Transfers (12) were carried out on nitrocellulose overnight at 30 V, with the last hour at 60 V. Immunostaining was accomplished by a standard peroxidase method (13).

## RESULTS

Typical patterns of crosslinking by PDM of three types of G$\alpha$ proteins and the $\beta$-subunits present in brain synaptoneurosomes are shown in Fig. 3. In all cases, the most striking changes from control membranes were the large structures that appeared in the void volume of Bio-Gel A-50m. Their precise sizes could not be deciphered because, unlike globular proteins, crosslinked proteins in general do not show a normal relationship between their gel elution patterns and molecular size. However, because the crosslinked G-protein subunits were eluted in fractions similar to that of crosslinked tubulin and actin (Fig. 2), it can be reasonably assumed that their native membrane structures are more like the polymeric forms of tubulin and F-actin than heterotrimeric G proteins. Indeed, the elution patterns are in marked contrast to the behavior of purified crosslinked heterotrimeric G proteins; crosslinked $G_o$, for example, elutes from Ultrogel AcA columns in a manner identical to that of uncrosslinked $G_o$ (14).

In addition to the very large structures, each of the G$\alpha$ proteins ($G\alpha_s$, $G\alpha_i$, $G\alpha_o$) displayed a range of sizes intermediate between the largest and the smallest structures. The crosslinked G$\beta$ proteins also were eluted as large structures (Fig. 3) but, unlike the G$\alpha$ proteins, no intermediate-size structures were detected by the dot-blot procedure.

As a means of judging the relative sizes of the crosslinked proteins, fractions encompassing the major peaks from the columns were combined and subjected to SDS/PAGE followed by Western blotting with specific antisera. The crosslinked material in the fractions containing the largest sizes did not enter the stacking gels and hence could not be detected on the gels. The intermediate-size fractions entered the gels (Fig. 4). Each successive fraction contained multiple bands that displayed decreasing sizes in accordance with their elution from the Bio-Gel columns. These findings are markedly different from the reported behavior of purified heterotrimeric G proteins (14); when crosslinked, $G_o$ yielded only two major bands of crosslinked material. It is likely that the intermediate-size crosslinked structures are derived from multiple forms of the G$\alpha$ proteins in synaptoneurosomes, consistent with previous findings that G$\alpha$ proteins extracted from synaptoneurosomes by octyl glucoside are polydisperse structures ranging in *s* values from about 5 S to over 12 S (9).

We were surprised to find that the G$\beta$ subunits also formed large crosslinked structures. Previous studies (9) indicated that the octyl glucoside-extracted G$\beta$ (presumably the $\beta\gamma$ complexes), in contrast to G$\alpha$, behaved hydrodynamically on sucrose gradients as a sharp peak of about 4.5 S, approximating that given by purified heterotrimeric G proteins (15). The extracted $\beta$ subunits were not crosslinked by PDM; i.e., their electrophoretic mobility did not change after incubation. By contrast, the $\alpha$ subunits extracted with octyl glucoside formed large crosslinked structures that did not penetrate the stacking gels (data not shown). These data further support the suggestion (9) that octyl glucoside may disrupt

the bonding between multimeric $\alpha$ proteins and the $\beta\gamma$ complexes.

Another possibility that may explain the apparent crosslinking of the $\beta$ subunits in membranes is that the dot-blotting procedure detected other proteins eluted from the Bio-Gel columns. The antiserum used for detecting $\beta$ subunits was derived from the $\beta\gamma$ complex prepared from transducin ($G_t$) and contains contaminating polyclonal antibodies against $G\alpha_t$, which crossreact with $G\alpha_i$ (16). This is shown by the upper band (41 kDa) observed in control membranes with antibodies against the $\beta$ subunits (35/36-kDa bands) (Fig. 5). To examine which of the bands became crosslinked with PDM, this antiserum was used to compare the degree and rates of crosslinking by PDM of $G\alpha_i$ and $G\beta$ in synaptosomal membranes (Fig. 5). The $\alpha_i$ band disappeared (no longer entered the gel) within 2 min of incubation with PDM whereas cross-linking of the $\beta$ subunits required minimally 10 min to detect any loss due to crosslinking. Moreover, only about 50% of the $\beta$ subunits were crosslinked after 1 hr of incubation at room temperature, in contrast to nearly complete crosslinking of all of the $\alpha$ subunits examined. We conclude that the large crosslinked material detected by dot blotting with the antiserum to the $\beta$ subunits represents both $\alpha_i$ and $\beta$ subunits. The $\beta$ subunits form crosslinked structures comparable in size to crosslinked $\alpha$ subunits. These structures cannot be derived from crosslinking heterotrimeric G proteins. If the latter exist in native synaptoneurosome membranes, they are present in very low abundance compared with the large and intermediate-size structures of the G protein. Possibly crosslinked heterotrimers are present in the fractions (nos. 60–180) containing the smallest proteins (Fig. 3), which were too dilute to detect by immunostaining.

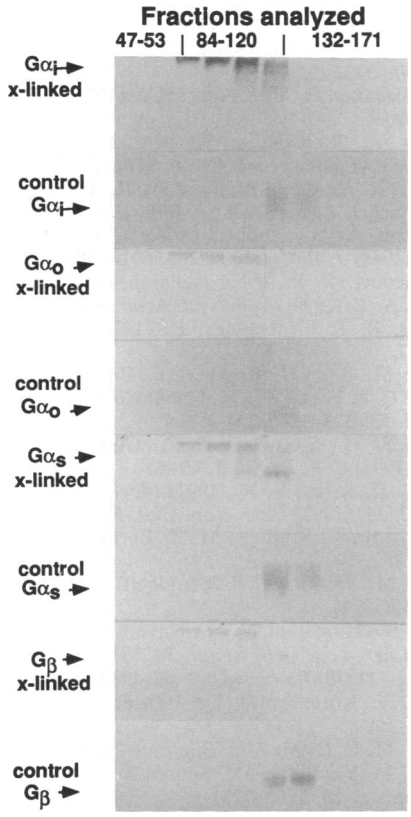


FIG. 4. Electrophoretic behavior of crosslinked $G\alpha_i$, $G\alpha_o$, $G\alpha_s$, and $\beta$. Aliquots of the peak fractions (crosslinked and controls) from the Bio-Gel columns, as detected by dot blotting (see Fig. 3), were analyzed by SDS/10% PAGE followed by Western blotting with specific antisera to the indicated G-protein subunits. This is a composite photograph showing both control and crosslinked products. The very large crosslinked structures (fractions 47–53) failed to enter the stacking gels and were not detected on the gels.

## DISCUSSION

Previous studies of G-protein crosslinking have concentrated on the nature of the interactions between the subunits of purified heterotrimeric G proteins ($G_t$ and $G_o$) in solution (14, 17, 18). In one of these studies (14), crosslinking of purified heterotrimeric $G_o$ by 1–3 mM 1,6-bismaleimidohexane, a sulfhydryl-reactive homobifunctional crosslinking reagent, was compared with crosslinking of $G_o$ in brain membranes. The same crosslinked products of $G_o$ were formed in both cases, from which it was concluded that heterotrimeric $G_o$ is the native structure in brain membranes. However, it appears that crosslinking of both purified and membrane-associated $G_o$ was carried out in the presence of 0.4% Lubrol. Both sodium cholate and Lubrol extract heterotrimeric G proteins but destroy the large multimeric structures extracted from brain membranes with octyl glucoside (ref. 9 and unpublished observations); this is the likely explanation for Yi *et al.* (14) obtaining identical crosslinked products. Indeed, because detergents affect the structure of G proteins in different ways, their presence during the crosslinking reaction was studiously avoided in the present study. Under these conditions, we obtained crosslinked products that were considerably larger than crosslinked heterotrimers and that bore striking resemblance to the polydisperse large structures extracted from synaptoneurosomes with octyl glucoside (9). The latter structures were devoid of $\beta$ (presumably $\beta\gamma$) subunits and were sensitive to the disaggregating actions of GTP[$\gamma$S]. In the present study we found that the $\beta$ subunits were crosslinked to form structures in the same size range as the crosslinked $\alpha$ subunits, indicating that $\beta$ (likely $\beta\gamma$) subunits are part of a large structure associated with synaptoneurosome membranes. The precise composition of the crosslinked structures remains unknown but it is reasonable to suggest that these structures are multimers of heterotrimeric G proteins. Vaillancourt *et al.* (18), using a photoactivatable probe covalently linked to $Cys^{347}$ of $G\alpha_t$, obtained evidence suggesting that $G_t$ is constructed of multimers (dimers, trimers) of the $\alpha$ subunits stabilized by close association with the $\gamma$ subunits. No evidence for linkage between

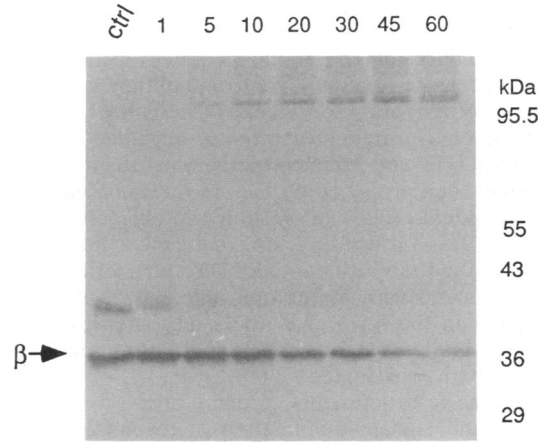


FIG. 5. Comparative electrophoretic behavior of $G\alpha_i$ and $\beta$ in synaptoneurosome membranes after exposure to PDM for various times. Membranes were treated without (ctrl) or with 75 $\mu$M PDM for 1–60 min; reactions were stopped with 7 mM 2-mercaptoethanol. Membranes were extracted and electrophoresed (SDS/PAGE). Electrophoretic bands were detected by blotting using antiserum against $\beta$ subunits that also reacts weakly with $G\alpha_i$ (band at 40 kDa). Increase in intensity of staining of crosslinked material at about 100 kDa is coincident with loss of immunoreactive material representing the $\beta$ subunits.

$\alpha$ and $\beta$ subunits was obtained. Multimer formation and disruption appeared to be $G_t$ concentration-dependent. Accordingly, they proposed that positive cooperative activation of bleached rhodopsin could regulate $G_t$ in intact rods by controlling the latter's functional concentration, presumably leading to breakdown of the multimers to smaller heterotrimeric units. In many respects, the multimeric structure and cooperative behavior of $G_t$ are consistent with the original "disaggregation" theory that was based on target analysis (7). Coupled with the evidence presented here, it would appear that G proteins associated with native biological membranes interact to form multimers of varying sizes, as suggested by the range of intermediate-size crosslinked structures of $\alpha_s$, $\alpha_i$, and $\alpha_o$. Further study is necessary to determine whether the intermediate and large structures are identical in subunit composition and whether there is a precursor–product relationship between these structures. Conceivably, the use of cleavable crosslinking agents that are otherwise structurally similar to PDM can satisfactorily answer these questions.

How does a multimeric structure of G proteins contribute to the signal transduction process? Current models (1, 19) assume that receptors are coupled to "monomeric" G proteins and that activation of the G proteins by hormones and GTP converts the heterotrimeric structure to free $\alpha$ subunits and the $\beta\gamma$ complexes. Since $\alpha$ subunits are generally hydrophilic, it has been suggested that interaction with effector units in the plasma membrane presumably requires some type of shuttling mechanism, possibly involving release to the cytosol ("hopping") and then association with effector units (20). A different view emerges if it is assumed that receptors are coupled to multimeric structures and that activation by hormones and GTP leads not to disruption of the individual heterotrimers (monomers) but rather to release of GTP-bound monomers from the multimeric complex. Monomers remain associated with the membrane, possibly through the isoprenylated $\gamma$ subunits (21), and form associations with effectors. Conversion of GTP to GDP and $P_i$ during or as a result of this interaction produces a conformational change that induces both dissociation of the $\alpha$ subunits from $\beta\gamma$ complexes and changes in the activities of the effector molecules. Modeling of the kinetics of phototransduction via $G_t$ (22) and adenylyl cyclase stimulation via $G_s$ (23, 24) fit best with this concept. Overall, hormonally induced exchange of GTP for GDP results in two independent reactions (25), one leading to release of monomers from multimers and changes in affinity of receptors for agonists (see below), the other in disruption of G-protein structure accompanied by increased turnover of GTP and enhanced activity of effectors. A similar model has been proposed by Ho *et al.* (26) based in part on GTP-dependent release of $G_t$ from photobleached rod outer segments (27), a procedure, not requiring splitting of GTP, that led to the first purification of heterotrimeric G proteins. $G_t$ is associated with the cytoplasmic aspect of rhodopsin-filled disks in the form of 8- to 12-nm particles (28), large enough to imply the presence of the multimeric structures observed with crosslinked $G_t$ (18).

A multimeric structure of G proteins allows for cooperative behavior and signal amplification in response to external signals. It also can serve as a "template" that ensures a high-affinity, tightly linked form of receptors with G proteins and as a reservoir of monomers for "quantal" release and excitation of effectors. Release of monomeric G proteins from multimers by the actions of sensory agents and GTP can readily explain the ubiquitous GTP-dependent reduction in the affinity of receptors for agonists (7). Finally, in this model, release of $\alpha$ subunits from membranes does not occur during the entire process of signal transduction.

The existence of multimeric G proteins brings into focus the possibility that they are similar in their structural and dynamic properties to tubulin and actin, multimeric proteins that utilize the binding and splitting of GTP or ATP in their dynamic behavior (23, 29, 30). Also, given recent evidence that luteinizing hormone and parathyroid hormone activate, each through a single receptor molecule, two or more G protein-mediated signal pathways (31, 32), an interesting speculation is that multimers are composed of more than one type of G protein; i.e., each receptor can activate simultaneously two or more types of G proteins in their multimeric state.


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