Volume 279, number 1, 98–100 FEBS 09381 February 1991

ADONIS 001457939100051M

G_o associates with another 40 kDa brain protein

Bradley M. Denker and Eva J. Neer

Harvard Medical School and Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

Received 6 November 1990

Guanine nucleotide binding proteins (G proteins) mediate a variety of cellular responses to external stimuli. Pure G protein, receptor, and effector are sufficient to reconstitute hormonal activation of an effector in phospholipid vesicles, but other components may be important for specificity or localization in vivo. If another protein associates with G_o, the molecular weight of G_o solubilized from membranes would be larger than the molecular weight of G_o after purification. We find that G_o solubilized from bovine brain membranes by Triton X-100 behaves as a single population of molecules on sucrose density gradients and gel filtration columns. Its molecular mass is about 40 kDa larger than pure G_o. Association of G_o with the other protein is fragile as the proteins dissociate on further purification. There was no difference in ADP-ribosylation or tryptic cleavage of G_o in larger and smaller form. These studies provide a basis for future experiments to stabilize the interaction and identify the protein.

G protein; Signal transduction; Sucrose gradient; Stokes radii; Molecular weight

1. INTRODUCTION

A variety of cellular responses to external stimuli are mediated by a group of guanine nucleotide binding proteins (G proteins) made up of α, β, and γ subunits. The basic components of this signal transduction system are a receptor, an effector, and the G protein that couples the two. Incorporation of pure receptor, G protein and effector into lipid vesicles is sufficient for agonist stimulation of the effector. For example, isoproterenol can stimulate adenylyl cyclase when pure β-adrenergic receptor, G_s, and adenylyl cyclase are incorporated into phospholipid vesicles [1]. While such reconstitution studies reveal the minimum number of components, they cannot reveal whether other proteins interact with the signalling system. Such interactions may modulate the strength or specificity of responses, or localize the signal transducing molecule to membrane domains. For example, in some neural cells, α_o is localized to growth cones where it interacts with GAP 43, an intracellular protein that can modulate GTPγS binding to G_o [2]. Similarly, calmodulin interacts with G proteins and the catalytic unit of adenylyl cyclase [3,4]. Cytoskeletal proteins can also interact with G proteins and other parts of the transduction system [5–11].

Although purification of the components is essential for precise analysis of their structure and function, there is a danger that during purification loosely bound regulatory or localizing proteins may be lost. As an initial step in determining whether other proteins associate with G proteins, we asked whether the molecular weight of the G_o as first solubilized from the membrane is larger than the molecular weight of the pure protein. A larger molecular weight would suggest that G_o is associating with another protein. We measured the molecular weight of G_o in unfractionated solubilized membranes by the hydrodynamic techniques of sucrose density centrifugation and gel filtration. As long as a particular protein complex in a mixture of proteins behaves as a single hydrodynamic particle, its molecular weight can be explicitly determined. To identify G_o, we analyzed fractions from sucrose density gradients and gel filtration by Western blots probed with antibody to α_o and β. These studies show that the whole population of G_o molecules is initially solubilized from brain membranes as a complex with another protein.

Correspondence address: B.M. Denker, Renal Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA

Abbreviations: GDPβs, guanosine 5'-*O*-(2-thiodiphosphate)

2. MATERIALS AND METHODS

2.1. *Triton X-100 solubilized bovine brain membrane preparation*

Calf brains (obtained from a local slaughter house) were dissected on ice, and the cortices (80 g) were homogenized in 400 ml 50 mM Tris (pH 8.0), 75 mM sucrose, 1 mM EDTA, 6 mM $MgCl_2$, 1 μg/ml soybean and lima bean trypsin inhibitors, 3 mM benzamidine, 1 mM dithiothreitol (buffer A). All reagents in buffer A were from Sigma. The homogenate was centrifuged at 10000 × *g* for 20 min at 4°C, the pellet resuspended in 350 ml buffer A and recentrifuged at 10000 × *g* for 20 min at 4°C. The pellet was resuspended in 100 ml of buffer A and frozen at −70°C. Crude membranes (1 ml) were mixed with 1 ml buffer A containing 2 μM GDPβS and 2% Triton X-100 (Fisher) and gently shaken at 4°C for 90 min, then centrifuged at 32000 × *g* for 70 min at 4°C. The supernatant (5.6 mg/ml) was collected for subsequent analysis.

2.2. *Physical properties of Triton-solubilized G_o and purified G_o*

The sedimentation coefficient and $\bar{\nu}$ were determined as previously described [12]. The 5–20% sucrose gradients were made up in buffer A with 0.3% Triton X-100 and were internally calibrated with BSA $s_{20,w}$, 4.31 S, $\bar{\nu}$, 0.732 cm^3/g, carbonic anhydrase, $s_{20,w}$, 2.85 S, $\bar{\nu}$,

Published by Elsevier Science Publishers B.V.

0.735 cm^3/g, and catalase $s_{20,w}$ 11.3 S, $\bar{\nu}$, 0.730 cm^3/g. The Stokes radius was determined by gel filtration on Ultrogel ACA-34 (LKB) columns equilibrated with buffer A, 0.1% Triton X-100 and internally calibrated with β-galactosidase (69 Å), BSA (37 Å), ovalbumin (27 Å), and catalase (52 Å).

2.3. *Miscellaneous methods*

Purified G_o was prepared as previously described [13]. Protein was determined by the method of Lowry [14] and modified by Bailey [15]. SDS-PAGE was carried out as described by Laemmli [16], and Western blots performed by the method of Towbin [17] as described previously [12]. The anti-α_o and anti-β antibodies have been previously described from this laboratory [12]. ADP-ribosylation by pertussis toxin (List Biological) was carried out as previously described [12] for 10–30 min in the presence of 0–10 μM $GTP\gamma S$. Tryptic digestion was carried out as previously described [18] for 10–30 min in the presence of 0–10 μM $GTP\gamma S$.

3. RESULTS

3.1. *G_o is monodisperse in Triton X-100-solubilized brain membranes*

Fig. 1 shows that all of the Triton-soluble G_o applied to a sucrose density gradient sediments as a single peak. On sucrose gradients the peak width at half-height for Triton-soluble G_o is the same as for the marker proteins. The ratio of peak width for Triton-soluble G_o to the peak width for marker proteins was 1.05 ± 0.24 ($n = 13$). By gel filtration, Triton-soluble G_o also elutes as a single peak. The ratio of peak width for Triton-soluble G_o to peak width for marker proteins was 0.85 ± 0.19 ($n = 6$). The observation that there is only one peak of G_o that is no wider than a peak of pure protein suggests that G_o makes up a single population of molecules and is not randomly aggregating with other proteins.

3.2. *The molecular weight of Triton X-100-solubilized G_o is larger than purified G_o*

To compare the molecular weight of unfractionated solubilized G_o to purified G_o, we measured the sedimentation coefficient ($s_{20,w}$), Stokes radius, and partial specific volume ($\bar{\nu}$) of both preparations in parallel. The partial specific volume was determined by the position of G_o relative to markers in H_2O and D_2O sucrose density gradients. Table I shows the measured parameters and calculated molecular weight of Triton-solubilized G_o and purified G_o. There was no significant difference in the partial specific volume for Triton X-100 solubilized G_o and purified G_o. Both $s_{20,w}$ and Stokes radius were larger for Triton-solubilized G_o than for purified G_o. The values obtained for pure G_o are in agreement with those previously reported [12]. The molecular weight of Triton-solubilized G_o (detergent plus protein) is 212000. However, 32% of this mass is detergent so that the molecular weight of the protein is 144000. The molecular weight of pure G_o detergent protein complex is 124000 with 17% Triton bound giving a molecular weight of 101000 for the protein portion. The molecular weight predicted from the sum of the components for α,β, and γ is 85000. The difference in molecular weight between Triton-soluble G_o and pure G_o is 43000 and represents the total mass of material associating with G_o in Triton-solubilized membranes.

The additional mass most likely represents a protein component*. If it were carbohydrate only, the $\bar{\nu}$ would be smaller than that of pure G_o while this mass of lipid would greatly increase the $\bar{\nu}$. The larger form of G_o is

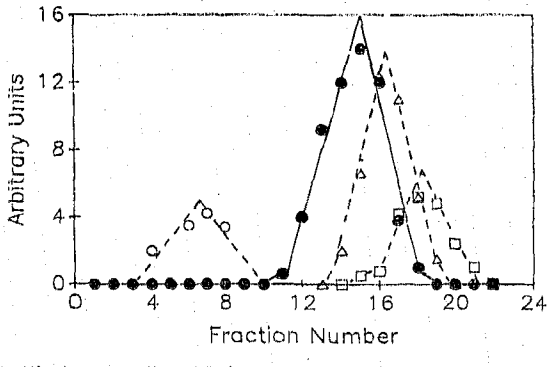

Fig. 1. Elution profile of Triton-solubilized G_o on 5–20% sucrose gradient. Bovine brain membranes were prepared and analyzed as described in Materials and Methods. Coomassie-stained gels and Western blots were scanned with an LKB Laser Densitometer and arbitrary units plotted versus fraction number. (○) catalase; (△) BSA; (□) carbonic anhydrase; and (●) G_o.

Table I

Physical properties of Triton X-100 solubilized and purified G_o in the presence of 1 μM $GDP\beta S$

Parameter[a]	Triton X-100 sol.	Purified G_o
Partial specific volume, $\bar{\nu}$, (ml/g)[b]	0.80 ± 0.02 (13)	0.77 ± 0.02 (13)
Sedimentation coefficient, $s_{20,w}$ (S)	5.6 ± 0.3 (11)	4.5 ± 0.3 (11)
Stokes radius, a (Å)	67 ± 3 (6)	44 ± 4 (6)
Molecular weight M_r[c]	212000 (191000–233000)[d]	124000 (105000–139000)[d]
Triton X-100 bound, (mg/mg protein)[e]	0.32	0.17
Molecular weight of protein	144000 (130000–158000)	101000 (87000–115000)

[a] The values are calculated from all possible paired combinations of data from H_2O and D_2O gradients, and the results given are the averages ± SE. The number in parenthesis is the sum of experiments in H_2O and D_2O

[b] The values of $\bar{\nu}$ were calculated from H_2O and D_2O gradients by the method of Sadler [24]

[c] Molecular weight was calculated according to the following equation:

$$M_r = \frac{6\pi N \eta_{20,w}}{1 - (\bar{\nu}\rho_{20,w})} a\, s_{20,w}$$

where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20°C, $\rho_{20,w}$ is the density of water at 20°C

[d] Range values were calculated using 1 SE above and below each parameter

[e] Calculated using $\bar{\nu}_{protein} = 0.735$ and $\bar{\nu}_{Triton} = 0.94$ [25]

*GAP-43, a growth cone protein, is not the associating protein based on Western blots of sucrose density gradients of G_o and GAP-43

unlikely to be $\alpha\beta\gamma$ or $\alpha(\beta\gamma)_2$ because the ratio of immunoreactivity of α to β was the same for Triton-soluble G_o, with a larger molecular weight and partially purified smaller G_o.

We next wanted to determine whether the larger form of G_o could be maintained through further steps of purification. One passage over an ion exchange column or repeat sucrose density centrifugation caused dissociation of the complex. The larger Stokes radius was preserved on repeat gel filtration but there was no further purification. These observations show that the complex of G_o and is associated protein is a fragile one which survives few steps of purification.

3.3. *Functional comparison of larger and smaller forms of G_o*

To determine whether there are differences in the function of the larger and smaller forms of G_o we examined two functions unique to G proteins: the pertussis toxin-catalyzed inhibition by GTPγS of ADP-ribosylation [19] and GTPγS-dependent changes in the pattern of cleavage by trypsin [20]. There was no difference between the larger and smaller forms of G_o in the GTPγS dose/response curve for either of these processes. G protein functions such as GTP binding or GTPase activity cannot be compared because of other enzymes in the crude mixture.

4. DISCUSSION

The protein which associates with G_o must be present in brain in an amount at least equal to that of G_o itself. We base this conclusion on the observation that G_o behaves as a single population of molecules with a molecular weight approximately 40 kDa larger than the sum of $\alpha\beta\gamma$. Since G_o makes up about 1% of brain membrane protein [12] the protein that associates with it must also be extremely abundant. Its association with G_o is fragile and the two proteins are separated early in the purification of G_o. Nevertheless, the presumed abundance of the protein makes brain a reasonable source for its eventual purification.

Early studies of the structure of unpurified G_s** also gave values for the molecular weight which were 30–100 kDa larger than the value we can now calculate for $\alpha_s\beta\gamma$ [21–23]. G_s is a quantitatively minor membrane protein so any proteins associated with it might also be rare and consequently hard to identify. Our studies now show that even when the G protein is extremely abundant, its initial molecular weight is larger than that of the pure protein. We predict that other G proteins are also solubilized as specific complexes with another protein.

There is a growing body of evidence that signal transduction systems are more complicated than originally proposed. The results reported here indicate that there is an association between G_o and another brain protein with a total molecular weight of approximately 43 kDa. This protein may, of course, itself be made up of several subunits. The role this protein may be playing in modulating the signal transduction system or localizing it to membrane domains has yet to be elucidated. The present studies provide a basis for future experiments to stabilize the interaction and identify the protein.

**G_s is the stimulatory G protein of adenylyl cyclase and some calcium channels

Acknowledgements: The work described was supported by NIH Grant GM36259 to E.J.N. B.D. was supported by a NRSA Fellowship (GM12578).

REFERENCES

[1] May, D.C., Ross, E.M., Gilman, A.G. and Smigel, M.D. (1985) J. Biol. Chem. 260, 15829–15833.
[2] Strittmatter, S.M., Valenzuela, D., Kennedy, T.E., Neer, E.J. and Fishman, M.C. (1990) Nature 344, 836–841.
[3] Asano, T., Ogasawara, N., Kitajima, S. and Sano, M. (1986) FEBS Lett. 203, 135–138.
[4] Salter, R.S., Krinks, M.H., Klee, C.B. and Neer, E.J. (1981) J. Biol. Chem. 256, 9830–9833.
[5] Schlegel, W., Kempner, E.S. and Rodbell, M. (1979) J. Biol. Chem. 254, 5168–5176.
[6] Dufau, M.L., Horner, K.A., Hayashi, K., Tsuruhara, T., Conn, P.M. and Catt, K.J. (1978) J. Biol. Chem. 253, 3721–3729.
[7] Sahyoun, N.E., LeVine, III, H., David, J., Hebdon, G.M. and Cuatrecasas, P. (1981) Proc. Natl. Acad. Sci. USA 78, 6158–6162.
[8] Cherskey, M.B.C., Zadunaisky, J.A. and Murphy, R.B. (1980) Proc. Natl. Acad. Sci. USA 77, 6401–6405.
[9] Insel, P.A. and Kennedy, M.S. (1978) Nature 273, 471–473.
[10] Rasenick, M.M., Stein, P.J. and Bitensky, M.L.J. (1981) Nature 294, 560–562.
[11] Carlson, K.E., Woolkalis, M.J., Newhous, M.G. and Manning, D.R. (1986) Mol. Pharmacol. 30, 463–468.
[12] Huff, R.M., Axton, J.M. and Neer, E.J. (1985) J. Biol. Chem. 260, 10864–10871.
[13] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) J. Biol. Chem. 259, 14222–14229.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Bailey, J.L. (1967) in: Techniques in Protein Chemistry, p. 340, Elsevier, New York.
[16] Laemmli, U.K. (1980) Nature 227, 680–685.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Neer, E.J., Pulsifer, L. and Wolf, L.G. (1988) J. Biol. Chem. 263, 8996–9000.
[19] Tsai, S., Adamik, R., Kanaho, Y., Hewlett, E.L. and Moss, J. (1984) J. Biol. Chem. 259, 15320–15323.
[20] Winslow, J.W., Van Amsterdam, J.R. and Neer, E.J. (1986) J. Biol. Chem. 261, 7571–7579.
[21] Neer, E.J. and Salter, R.S. (1981) J. Biol. Chem. 256, 12102–12107.
[22] Howlett, A.C. and Gilman, A.G. (1980) J. Biol. Chem. 255, 2861–2866.
[23] Kaslow, H.R., Johnson, G.L., Brothers, V.M. and Bourne, H.R. (1980) J. Biol. Chem. 255, 3736–3741.
[24] Sadler, J.E. (1979) J. Biol. Chem. 254, 4443.
[25] Steele, J.C.H., Tanford, C. and Reynolds, J.A. (1978) Methods Enzymol. 48, 11–23.