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Partial characterization of mitochondrial G proteins in adrenal cells

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Abstract

Four low molecular mass G proteins have been identified in mitochondrial membranes from bovine adrenal cortex. These proteins (referred to as proteins 1 to 4) showed molecular masses of 28, 27, 26 and 24 kDa with isoelectric points (p*I*) of 8.1, 5.6, and 6.3 respectively for proteins 1, 2 and 4. Protein 3 was shown to be heterogeneous, with isoelectric points of 5.0–6.1. Proteins were identified by binding of [α-^{32}P]guanosine triphosphate (GTP) after separation by 12% SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose. Competitive binding by unlabelled competing nucleoside phosphate ligands showed specificity for guanosine triphosphate (GTP) and guanosine diphosphate (GDP) with little binding of guanosine monophosphate and no detectable binding with adenosine nucleoside phosphates. Binding was less than 10% with 100-fold excess GDP and GTP which showed equal intensities of binding. Inhibition of binding by 1000-fold cytidine triphosphate and uridine triphosphate was approx. 10%. Magnesium (Mg^{2+}) stimulated binding of GTP by all four proteins. The effect of Mg^{2+} was essentially the same for proteins 1, 2 and 3, while protein 4 was less sensitive to Mg^{2+} at concentrations $< 10^{-3}$ M. Centrifugation of sonicated mitochondrial membranes through sucrose density gradients showed the presence of all four proteins in contact points. The presence of lower concentrations (expressed per mg protein) of the proteins in inner and outer membranes suggests that either small amounts of these membranes are part of contact points as presently prepared or that the proteins occur in contact points and to a much smaller extent in inner and outer membranes. It is proposed to examine a possible role for these proteins in transport of cholesterol from outer to inner mitochondrial membranes.

Keywords: G protein; Adrenal cortex; Mitochondria; Cholesterol transport; Steroidogenesis

1. Introduction

It is now generally agreed that the rate-limiting step in steroidogenesis involves the transport of cholesterol from depots in the cytoplasm to the inner mitochondrial membrane [1,2]. This transport process is stimulated by adrenocorticotrophic hormone (ACTH) and cyclic adenosine monophosphate (cAMP) [1,2]. It proceeds in at least two steps, namely transport to mitochondria and transport within mitochondria to the inner mitochondrial membrane [1–5]. This latter process requires synthe-

Abbreviations: GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; UTP, uridine triphosphate; CTP, cytidine triphosphate; ACTH, adrenocorticotrophic hormone; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; p*I*, isoelectric point; Mg^{2+}, magnesium

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sis of new protein [4,5]. It remains unclear whether cholesterol transport proceeds from outer to inner membrane, through the intermembrane space, or perhaps through contact points established between inner and outer membrane. It has been proposed that a number of proteins may be involved in the transport of cholesterol between the membranes, including the steroidogenic acute regulatory (StAR) protein [6], the peripheral benzodiazepine receptor (pBR) [7–9] and endozepine (sometimes called diazepam-binding inhibitor, DBI) [3,10–12]. The transport of cholesterol from outer to inner membrane is a slow process [13] compared with the passage of this substrate to the side chain cleavage enzyme in mitoplasts, i.e. mitochondria from which outer membrane has been removed [14]. Intracellular transport frequently proceeds by the movement of vesicles within the cell from a donor to an acceptor compartment in which membrane fusion occurs through hydrolysis of guanosine triphosphate (GTP) and accompanying changes in one or more G proteins [15]. Moreover, it appears that GTP may cause some stimulation of the first enzymatic step in the steroidogenic pathway, namely conversion of cholesterol to pregnenolone [16–18]. Earlier studies from this laboratory revealed the presence of two nucleotide-binding proteins in adrenocortical mitochondria (19 kDa and 45 kDa) [19]. These proteins were shown to bind adenosine as well as guanosine nucleotides. We report here the identification of four proteins that behave as typical small G proteins in contact points between the two mitochondrial membranes and which show specificity of binding to guanosine as opposed to other nucleotides.

2. Materials and methods

2.1. Materials

[α-^{32}P]GTP (specific activity 3000 Ci mmole^{-1}; 10 mCi ml^{-1}) was supplied by ICN; [α-^{35}S]GTP (specific activity 1250 Ci mmole^{-1}; 12.5 mCi ml^{-1}) and [^{32}P]orthophosphoric acid (specific activity 8500–9120 Ci mmole^{-1}; 10 mCi ml^{-1}) were from Dupont NEN (Boston, MA).

2.2. Isolation of mitochondria and submitochondrial fractions

Mitochondria were prepared from bovine adrenal glands and disrupted as described previously [19]. Submitochondrial fractions were separated by a stepwise sucrose gradient in which three layers of sucrose densities (1.192 g l^{-1}, 1.142 g l^{-1}, 1.094 g l^{-1}) were layered on top of each other (8 ml each) and finally 7 ml broken mitochondrial solution layered on top [20]. Gradients were subjected to ultracentrifugation at 4°C for 1 h in a Sorvall superspeed centrifuge (rotor SW27) at 100 000×g. The inner/outer membrane fraction at the 1.142/1.092 g l^{-1} interface was collected. Further separation occurred by layering this solution (5 ml) on a 15–50% linear sucrose gradient, followed by ultracentrifugation at 4°C in a SW27 rotor for 20 h at 100 000×g. The gradient was collected from the bottom in 20×1.4 ml fractions. In some studies, preparation of mitochondrial and submitochondrial fractions occurred in the presence of a cocktail of protease inhibitors (aprotinin (45 μg ml^{-1}), antipain (2.5 μg ml^{-1}), chymostatin (1.25 μg ml^{-1}), leupeptin (5 μg ml^{-1}), pepstatin (5 μg ml^{-1}) and phenylmethylsulphonyl fluoride (PMSF) (100 μg ml^{-1}).

2.3. Protein and enzyme assays

Concentrations of protein were measured by means of the Pierce (Rockford, IL) bicinchoninic acid (BCA) protein assay kit. Cytochrome oxidase (EC 1.9.3.1) activity was measured according to Cooperstein and Lazarow [21]. Activity of hexokinase (EC 2.7.1.1) was measured according to Bucher et al. [22]. Rotenone-insensitive NADH cytochrome *c* reductase (EC 1.6.2.2) was assayed according to Sottacasa et al. [23]. Enzyme activity was calculated and expressed as μmoles activity per minute per mg protein.

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer

Proteins were separated on 12% polyacrylamide gels using the Bio-Rad Protein II Slab Cell and transferred to nitrocellulose (Hoefer Scientific Instru-

ments, San Francisco, CA) with a semidry blotter (W.E.P. Company, Seattle, WA) in the presence of 48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol.

2.5. 2D gel electrophoresis

Pharmacia Immobiline strips (18 cm pH 3-10L) were rehydrated by incubating overnight with 500 μg mitochondrial proteins in rehydration buffer (8 M urea, 2% w/v (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) (CHAPS), 10 mM dithiothreitol (DTT), 2% w/v Resolytes 3-10 (Bio-Rad, Richmond, CA), 0.002% bromophenol blue). 2D gel electrophoresis was carried out using the Pharmacia (Uppsala, Sweden) LKB Multiphor II system in which the first dimension was focused for 100 kVh. Strips were then incubated in reducing solution (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% w/v glycerol, 2% SDS, 2% w/v DTT) for 12 min, followed by 8 min in iodination solution (50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% w/v SDS, 2.5% iodoacetamide). Strips were drained briefly and the second dimension was resolved by SDS-PAGE on linear 12% (or gradient 12–14%) SDS polyacrylamide gels.

2.6. GTP binding assays

Nitrocellulose blots were washed and equilibrated for 10 min in each of two changes of incubating buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.3% Tween 20, 1 mM K_2HPO_4/KH_2PO_4 pH 7.5), followed by 1 h in incubating buffer plus 1 mCi ml^{-1} [α-^{32}P]GTP (0.33 nM). Blots were washed in five changes of incubating buffer (10 min in each), air dried and subjected to autoradiography for 2–16 h at −80°C. The intensity of bands was measured with the Bio-Rad Gel Doc 1000. In some experiments with 2D gels, 2 mCi ml^{-1} [α-^{35}S]GTP was added to the incubation buffer instead of [α-^{32}P]GTP.

2.7. Calculation of free Mg^{2+} concentrations

The concentrations of $MgCl_2$ (Mg_{tot}) required to obtain the desired concentrations of Mg^{2+} under the conditions of EDTA ($EDTA_{tot}$) and GTP (GTP_{tot}) used, were calculated according to the equation:

$$M_{tot} = Mg^{2+}[1 + E_{tot}/(Mg^{2+} + K_{EDTA}) + GTP_{tot}/(Mg^{2+} + K_{GTP})]$$

where K_{EDTA} and K_{GTP} are the equilibrium dissociation constants of the MgEDTA complex (0.4 mM) and MgGTP complex (60 mM) at pH 7.5 [24].

2.8. Tissue culture

Bovine adrenal glands were collected from an abattoir in Baxters 0.9% NaCl at 4°C. Uncut glands were incubated 15 min in DMEM/Ham's F12 medium (Trace Biosciences, Castle Hill, Australia) with 12.5% donor horse serum (Cytosystems, Castle Hill, Australia), 2.5% fetal bovine serum (Trace Biosciences), 100 μg ml^{-1} penicillin (CSL, Parkville, Australia), 2.5 μg ml^{-1} gentamicin (Delta West, Bentley, Australia) and 2.5 μg ml^{-1} amphostat B (Trace Biosciences). The zona fasciculata was excised with a scalpel under aseptic conditions, transferred to medium and chopped into small fragments with scissors. Erythrocytes were removed in several changes of medium. Digestion of tissue was performed by stirring fragments in medium containing 0.1% collagenase type 1A (Sigma, St. Louis, MO), and 0.01% DNase I (Boehringer Mannheim, Germany) at 37°C for 20 min. This solution was decanted off, fresh enzymes dissolved in medium were added and digestion continued for another 20 min. Fragments were then filtered through a 300 mm mesh followed by a 75 mm mesh. Cells were pelleted by centrifugation of filtered solution at $1000 \times g$ for 10 min. Cells were resuspended in medium and viability determined by counting with a haemocytometer in the presence of 0.05% Trypan blue. Cells were plated at a density of 1×10^5 cells ml^{-1}.

3. Results

3.1. Identification of G proteins in mitochondrial membranes from bovine adrenocortical membranes

Mitochondrial membranes were prepared from bovine adrenal cortex as described in Section 2. Mem-

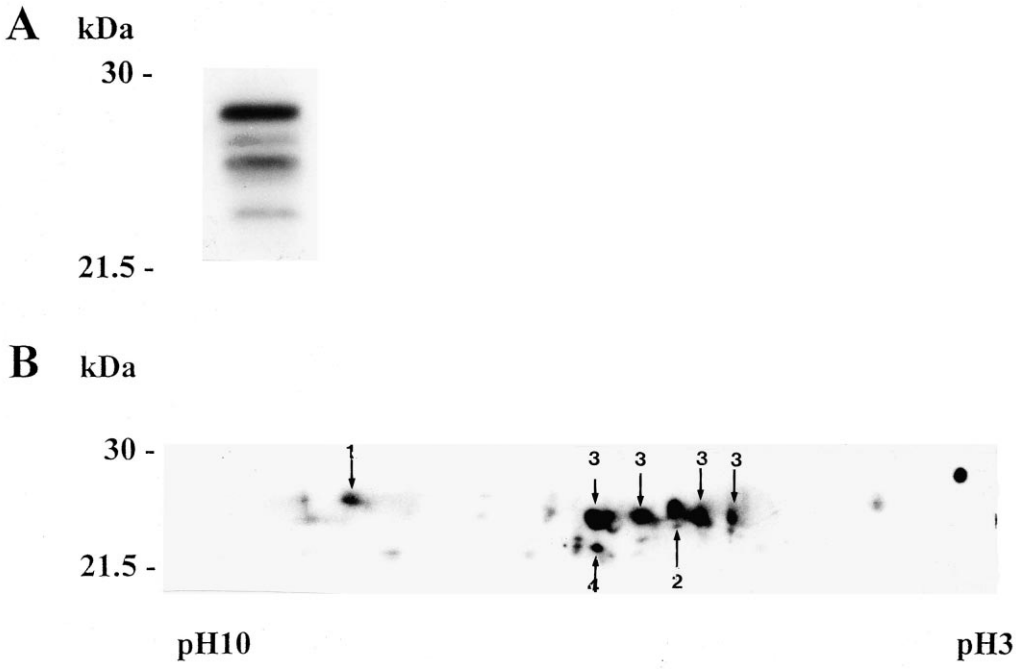

Fig. 1. Identification of proteins (from bovine fasciculata mitochondrial membranes) binding [α-^{32}P]GTP. Mitochondrial membrane proteins were separated by one-dimensional (12%) (A) or two-dimensional (pH 3-10L followed by 12–14% gradient) (B) polyacrylamide electrophoresis, transferred to nitrocellulose and incubated in the presence of [α-^{32}P]GTP. Blots were then subjected to autoradiography.

brane proteins were then separated by one- or two-dimensional gel electrophoresis and transferred to nitrocellulose. Following incubation in buffer containing [α-^{32}P]GTP the membrane was subjected to autoradiography (Fig. 1). The results show that in both one-dimensional (Fig. 1A) and two-dimensional (Fig. 1B) polyacrylamide gels four proteins of molecular masses 28, 27, 26 and 24 kDa were identified (referred to here as proteins 1, 2, 3 and 4, respectively). 2D gel electrophoresis (Fig. 1B) indicated proteins 1–4 showed isoelectric points (p*I*) of 8.1, protein 1; 5.6, protein 2; 5.0, 5.3, 5.8, 6.1, protein 3; and 6.3, protein 4. Protein 3 appeared as a cluster of spots with the same apparent molecular mass but varying in isoelectric points from 5 to 6.1, suggesting either the existence of four distinct proteins with very similar molecular masses, or a protein which exists in different states of glycosylation or phosphorylation (see below).

In some studies mitochondrial membranes were prepared in the presence of a cocktail of protease inhibitors. Under these conditions the same G proteins were isolated as in the absence of the inhibitors (data not shown).

The relative amounts of GTP binding by proteins 1–4 differs from 1D to 2D gel electrophoresis, as shown in Fig. 1A,B. This may be due to effects of the different treatments of mitochondrial membrane samples prior to electrophoresis. For 1D electrophoresis, samples are boiled in SDS sample buffer, whereas for 2D electrophoresis, samples are incubated overnight in rehydration buffer containing urea, CHAPS, DTT and Resolytes.

3.2. *Competitive inhibition of GTP binding to proteins 1–4 by various nucleoside phosphates*

All four proteins were shown to bind GTP and such binding is inhibited by GDP with 50% inhibition at 10-fold concentration (Fig. 2). GTP caused a profound inhibition under these conditions. Negligible inhibition was seen with addition of GMP or adenosine phosphates. Slight inhibition was seen with 1000-fold excesses of either UTP or CTP. It is

noticeable that these patterns of inhibition were largely similar for all four proteins.

3.3. Effect of magnesium ions on GTP binding by proteins 1–4

Mg^{2+} is known to influence the activities of G proteins. Addition of Mg^{2+} increased the binding of GTP by all four G proteins but at significantly different concentrations. Proteins 1, 2 and 3 showed increased activity at 5×10^{-7} M with maximal response at concentrations between 1×10^{-3} and 5×10^{-3} M. By contrast, protein 4 showed relatively small changes in binding of GTP between 5×10^{-7} and 1×10^{-5} M Mg^{2+} with greatest increase in binding observed at concentrations between 1×10^{-5} and 1×10^{-3} M. Twenty millimolar Mg^{2+} inhibited the binding of proteins 2, 3 and 4 to GTP (Fig. 3).

3.4. Intramitochondrial locations of proteins 1–4

The distribution of marker enzymes in adrenocortical mitochondrial membranes after centrifugation through sucrose density gradients (15–50% w/v) is shown in Fig. 4A. The outer membrane marker rotenone-insensitive NADH cytochrome *c* reductase separated readily at the top of the gradient. There was some overlap between the other two fractions further down the gradient. Using hexokinase as a marker for contact points showed that these structures were found on the gradient between inner and outer membrane fractions, consistent with that reported by others [16,25]. We have further identified contact points in adrenocortical mitochondria by electron microscopy [19].

All four proteins were shown to distribute similarly on sucrose density gradients (Fig. 4B). When compared with the distribution of marker enzymes on the same gradients (Fig. 4A), the four proteins were shown to sediment with contact points with some overlap with inner and outer membrane. Contamination with inner and outer membrane is inevitable since each contact point includes some inner and outer membrane in addition to the contact point itself [19,25,26].

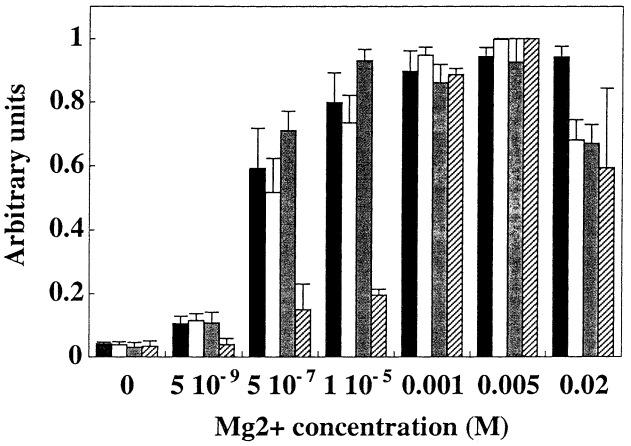

Fig. 3. Effect of magnesium ions on GTP binding. Equal concentrations of mitochondrial membrane proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were incubated for 1 h in the presence of [α-^{32}P]GTP and varying concentrations of free Mg^{2+}. Concentrations of Mg^{2+} (M) were calculated as described in Section 2. Strips were dried, subjected to autoradiography and the resultant bands representing protein 1 (black), protein 2 (white), protein 3 (grey) and protein 4 (striped) quantitated by densitometry using the Bio-Rad GelDoc 1000. (Data represent the mean ± S.E. of three independent studies using three different preparations of mitochondria.)

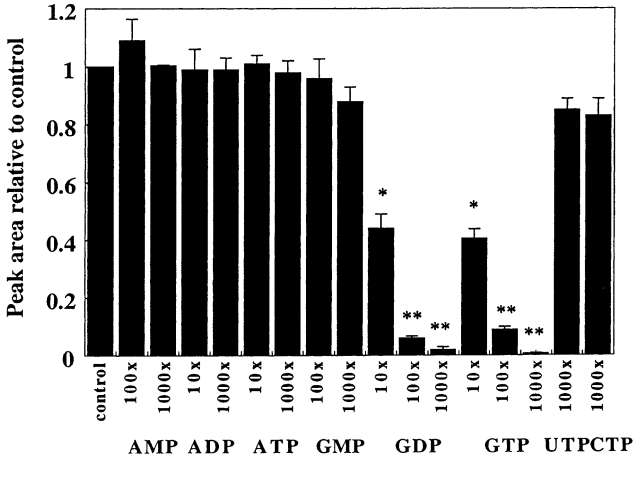

Fig. 2. Specificity of binding of [α-^{32}P]GTP to proteins. Equal concentrations of mitochondrial membrane proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were incubated for 1 h in the presence of [α-^{32}P]GTP and excess unlabelled nucleotides. Strips were then dried, subjected to autoradiography and the resultant bands were quantitated by densitometry using the Bio-Rad GelDoc 1000. Results shown are for protein 1; proteins 2–4 show similar patterns. (Data represent the mean ± S.E. of three independent studies using three different preparations of mitochondria. *Statistically different from control; $P<0.01$, $^{**}P<0.0001$.)

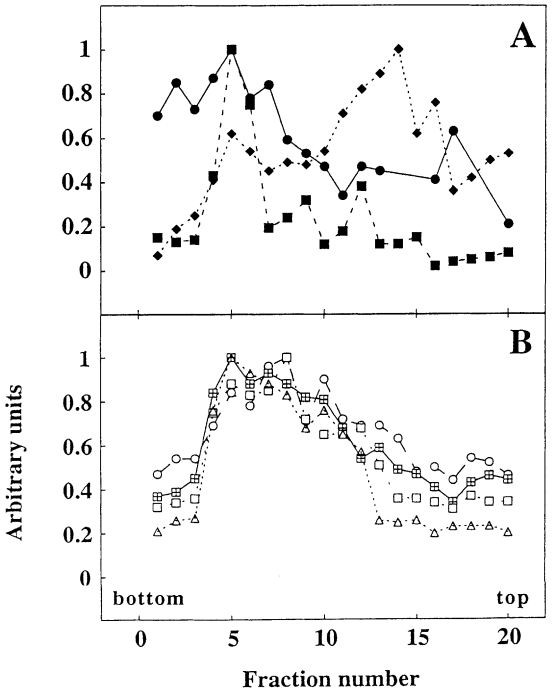

Fig. 4. Localization of G proteins in mitochondrial membranes. Sonicated mitochondrial membranes were separated on a 15–50% sucrose gradient and 20 fractions collected from the bottom of the tube. (A) Fractions were identified as being either inner or outer membrane or contact points by means of assays of marker enzymes. Cytochrome oxidase (●) was used as a marker for inner membrane, hexokinase (■) for contact points, and rotenone-insensitive NADH cytochrome *c* oxidase (◆) for outer membrane. Values were expressed as μmoles of enzyme activity per minute per mg protein, but are shown as relative arbitrary units. (B) Equal concentrations of protein from the same membrane fractions were separated by 12% SDS-PAGE, transferred to nitrocellulose and incubated with [α-^{32}P]GTP as described. Nitrocellulose was subjected to autoradiography and the subsequent bands representing protein 1 (○), protein 2 (plus in box), protein 3 (□), and protein 4 (△) quantitated with the Bio-Rad GelDoc 1000. (Results are representative of three independent experiments using three different preparations of mitochondria.)

3.5. Phosphorylation of adrenal cell proteins

In order to determine whether the four G proteins were phosphorylated as a result of stimulation by ACTH, bovine adrenal fasciculata cells were incubated with [^{32}P]inorganic phosphate for 1 h followed by incubation with or without ACTH for 1 h. Mitochondrial proteins were then separated by two-dimensional polyacrylamide gels and subjected to autoradiography to identify phosphorylated proteins. GTP binding assays were then performed on these blots. None of the phosphorylated proteins colocalized with GTP binding proteins 1–4.

4. Discussion

The adrenocortical mitochondria used in these studies were prepared by a well characterized method which has been shown to yield organelles of high purity [19]. Membranes were prepared from mitochondria by sonication which would be expected to disrupt the organelles. The membrane proteins were separated by one- or two-dimensional gel electrophoresis and G proteins were identified by transfer to nitrocellulose followed by overlay of the nitrocellulose in buffer containing [α-^{32}P]GTP, with and without competing ligands, followed by autoradiography. The addition of excess unlabelled ligands in the form of nucleoside triphosphates demonstrated the specificity of the proteins for the guanosine nucleotides GDP and GTP, thereby demonstrating the identities of proteins 1–4 as G proteins. [α-^{32}P]GTP was used in these studies rather than [γ-^{32}P]GTP in order to avoid contamination by kinase enzymes, hydrolase enzymes and proteins other than G proteins that might bind GTP phosphate groups.

G proteins fall into two main groups, namely heterotrimeric G proteins of which the α subunits bind GTP and so-called small G proteins which are monomeric and fall in the molecular mass range of 20–36 kDa. Gα subunits are of somewhat higher molecular mass. On this basis alone, the present proteins are likely to be small G proteins although this suggestion will clearly require further confirmation.

Earlier reports have demonstrated the existence of GTP-binding proteins in mitochondria [26–28]. Lithgow et al. [26] identified a 52 kDa GTP-binding protein in contact points of mitochondria from rat liver, and a similar protein of almost identical molecular mass in mitochondria of yeast. This larger protein is clearly different from proteins 1–4 described here. Takeda and colleagues [27,28] studied GTP-binding proteins in Ehrlich ascites tumour cells and found only a single G protein in a mitochondrial fraction. The molecular mass of this protein is 33 kDa, larger than any of the proteins found here. Based on molecular masses alone, the GTP-binding proteins iden-

tified here in adrenocortical mitochondria are clearly different from these previously identified proteins. Three additional proteins of lower molecular masses (23 kDa and 26 kDa) were also reported, but it is uncertain with which organelle they are associated [28] and the lack of any further characterization makes it impossible to determine whether they are the same as the proteins we have here identified.

It is worth considering whether the four proteins represent four unrelated proteins or are somehow related. The response to Mg^{2+} is certainly similar in proteins 1–3 but qualitatively different in 4. Competitive binding with unlabelled ligands was almost identical for proteins 1, 2, 3 and only slightly different for protein 4. The distribution of the four proteins in a sucrose density gradient is almost identical across the gradient for all four proteins. One possible explanation of such a situation would be the occurrence of post-translational modification by such processes as proteolysis or phosphorylation. The first of these arguments, however, is not supported by our observation that addition of protease inhibitors did not affect subsequent purification and identification of proteins 1–4. Small G proteins have been shown to be phosphorylated and it has been suggested that such phosphorylation could serve to regulate the activity of the protein through its association with cell membranes [15,29]. However, phosphorylation was not seen in any of the four proteins, again indicating that the similarity in proteins 1–4 was not due simply to this form of post-translational modification. Other possibilities, such as glycosylation, will require further experimentation. However, four separate G proteins would not be unusual as, for example, shown in the recent detection of a similar array of small G proteins with apparent molecular masses ranging from 22 to 31 kDa in rat astrocytes [30] as well as the report of 20 small G proteins in subcellular fractions of rat pancreas, ranging in molecular mass from 20 to 28 kDa and with isoelectric points of 4.8–6.4 [31].

Sonicated mitochondrial membrane fragments in this study were separated based on their relative densities by means of centrifugation on two consecutive sucrose gradients, the first gradient having been shown to remove the possibility of contamination with microsomes [20]. The subsequent distribution of proteins on a linear gradient reveals that within the mitochondria the four G proteins seem most likely to be located in contact points between mitochondrial membranes. However, it is difficult to exclude some of the proteins from the inner and outer membranes occurring in the contact points since as presently prepared these structures inevitably bring tags of inner and outer membrane attached to the contact point itself.

In considering possible functions of these proteins, the role of G proteins in membranes certainly includes intracellular vesicular transport involving membrane fusion. It appears that in liver, the number of contact points between inner and outer mitochondrial membranes increases with increase in metabolic activity [25]. It appears that contact points may be transitory structures created to perform functions associated with high levels of cellular metabolism. It is not unreasonable to consider that cholesterol transport and steroid synthesis may require membrane fusion under the influence of G proteins leading to construction of increased numbers of contact points with subsequent transfer of cholesterol to the inner membrane for side chain cleavage. In support of this hypothesis, earlier work has demonstrated a role for GTP in steroid hormone biosynthesis at the initial step of cholesterol transfer across the mitochondrial double membrane [16–18]. GTP enhances the conversion of extramitochondrial cholesterol to pregnenolone in isolated rat adrenal mitochondria, consistent with the promotion of cholesterol transfer to the inner mitochondrial membrane. Xu et al. [16] also demonstrated the presence of endogenous GTPase activity in isolated rat adrenal mitochondria. Future work is required to confirm the possibility that one or more of the G proteins described in this paper may be responsible for such GTPase activity and may therefore play a role in steroid hormone biosynthesis in adrenocortical cells.

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