Vol. 168, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1990 Pages 99-106

EVIDENCE FOR COMPLEX FORMATION BETWEEN GTP BINDING PROTEIN(Gs) AND MEMBRANE-ASSOCIATED NUCLEOSIDE DIPHOSPHATE KINASE

Narimichi Kimura and Nobuko Shimada

Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, 35-2, Sakaecho, Itabashi-ku, Tokyo 173, Japan

Received February 19, 1990

SUMMARY: When the Gs in rat liver membranes was prelabeled with [^{32}P]NAD and cholera toxin, solubilized with octylglucoside, and then analyzed by sucrose density gradient centrifugation, it was fractionated into two peaks with approximate molecular sizes of 12-13S and 3-4S. Pretreatment without or with GDPβS of the labeled membranes resulted in a larger peak in the high molecular weight region, whereas pretreatment with glucagon plus GTPγS caused almost equal peaks in both regions. The affinity-purified anti-nucleoside diphosphate(NDP) kinase antibodies only precipitated the Gs in high molecular weight region. Under the same condition, small but significant NDP kinase activity was associated with the high molecular weight Gs region although a large portion of the enzyme activity was recovered in fractions where it alone should appear(6.2S). Both Lubrol-PX and digitonin solubilized the Gs in forms insensitive to immunoprecipitation by anti-NDP kinase antibodies although the latter detergent was able to solubilize the Gs in a high molecular weight form, that is, a ternary glucagon-receptor-G protein complex. These results demonstrate that Gs and membrane-associated NDP kinase may exist in part in a complexed form in membranes. Physiological relevance of the complex formation in membrane signal transduction is discussed.

Hormone sensitive adenylate cyclase system consists of a hormone receptor, GTP-binding(G) proteins(Gs, Gi) and an adenylate cyclase catalyst-(1,2). Hormone binding to its receptor induces GDP-GTP exchange reaction, resulting in an activation of G protein and in turn catalyst. Hydrolysis of GTP to GDP on the G protein leads to termination of this activation. Subunit dissociation and reassociation of a trimeric form of the G protein($\alpha\beta\gamma \rightleftharpoons \alpha + \beta\gamma$) proceed along with this activation and inactivation cycle(1).

It has long been known that GDP is as effective as GTP in hormone dependent activation of adenylate cyclase from several tissues even in the absence of an ATP-regenerating system(3-7). It was found that the GDP action

Abbreviations used are: NDP kinase, nucleoside diphosphate kinase; m-, membrane-associated; G protein, GTP-binding protein; octylglucoside, n-octyl-β-D-glucoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-291X/90 $1.50

was manifest only as a result of conversion of the added GDP into GTP by the action of membrane-associated nucleoside diphosphate kinase(mNDP kinase)(8-10). Based on these and other recent studies from this laboratory(11-13), we have proposed that the mNDP kinase may play a role in the hormone-dependent activation of adenylate cyclase by supplying GTP, an essential cofactor, into the G protein(Gs) since this supply seems to occur without complete equilibration with the bulk phase. Some recent works from other laboratories have presented data supporting this view(14,15). The phenomenon is considered to be physiologically relevant because the GTP supply operated by mNDP kinase happens only through receptor activation by hormones(12) and cannot be observed for another GTP binding protein, ARF(ADP ribosylation factor)(13). These observations lead us to speculate that mNDP kinase may somehow interact with the signal transducing machinery.

In a recent study, we have demonstrated that Gs solubilized by certain detergents is precipitable by affinity-purified anti-NDP kinase antibodies-(16), suggesting that the Gs and mNDP kinase may partly form a complex in membranes. The present study was undertaken to further elucidate their possible direct interaction.

MATERIALS AND METHODS

Materials [^{32}P]NAD and [^{32}P]GDP were purchased from New England Nuclear. [^{3}H]GDP and [^{125}I]glucagon were obtained from Amersham. A Yamasa cyclic AMP assay kit was from Yamasa Shoyu Co(Choshi, Japan). GDPβS and GTPγS were from Boehringer Mannheim. Octylglucoside and digitonin were obtained from Wako Pure Chemical Co. Glucagon and Lubrol-PX were from Sigma. Other chemicals were all reagent-grade as described elsewhere(16). Rat liver purified plasma membranes(5) and affinity-purified anti-NDP kinase antibodies(17) were prepared according to the method previously described. The antibodies precipitated neither detergent-solubilized adenylate cyclase activity, Gs nor [^{125}I]glucagon-receptor complex(molecular weight: 59K) preformed by means of a photoreactive crosslinking reagent, N-hydroxysuccinimidyl-4-azidobenzoate.

[^{32}P]ADP ribosylation of Gs in membranes The Gs in membranes was [^{32}P]ADP ribosylated essentially as described previously(16). Briefly, the incubation mixture contained in a total volume of 2ml, 200mM potassium phosphate, pH 7.1, 2μM [^{32}P]NAD(1mCi), 1mM GTP, 1mM ATP, 10mM thymidine, 100μg/ml of preactivated cholera toxin, protease inhibitor mixture(5μM antipain, 5μM leupeptin, 50μg/ml bacitracin), and 2-3 mg of membrane protein. The labeled membranes were washed by repeated centrifugations

Treatment and solubilization of the labeled membranes The labeled membranes were treated without or with 100μM GDPβS alone or 1μM glucagon plus 100μM GTPγS in 100μl of TMEDP(10mM Tris-HCl, pH 7.4, 2mM $MgCl_2$, 1mM EDTA, 1mM DTT and 0.25mM phenylmethylsulfonyl fluoride) for 5min at 30°C. After incubation, the membranes were sediminted by centrifugation and the pellet was resuspended and solubilized with 160μl of 1.5% octylglucoside in TSBP(50mM Tris-HCl, pH 7.4, 0.1M NaCl, 0.2% bovine serum albumin and 0.25mM phenylmethylsulfonyl fluoride) for 30min at 0°C. After the samples were ultracentrifuged at 100,000rpm for 20min in a TL 100.2 rotor, the supernatant was diluted with TSBP to lower the detergent concentration into 0.825%.

Sucrose density gradient centrifugation The solubilized samples with protein markers(~110μl) were applied on a 2ml linear sucrose density gradient(5-20%

sucrose in TMEDP, 0.1M NaCl, 0.825% octylglucoside), overlaid with liquid paraffin to fill the tubes, and centrifuged in an SW 60 Ti rotor at 60,000 rpm for 105min. The samples were fractionated into ~20 tubes and then subjected to various analyses. When digitonin and Lubrol-PX were used, they were included at 1.2 and 1.0% for solubilization and 0.05 and 0.1% for sucrose density gradient centrifugation, respectively. To determine distribution of Gs after centrifugation, aliquots from fractions were subjected to SDS-PAGE, followed by autoradiography. To examine interaction of Gs with mNDP kinase, fractions 7-11(high molecular weight complexed form of Gs) and 14-18(low molecular weight form of Gs; probably free Gs and/or Gsα subunit) were pooled separately, and then treated with affinity-purified anti-NDP kinase antibodies diluted with TBS(50mM Tris-HCl, pH 7.4, 200mM NaCl) containing 3% bovine serum albumin and 0.825% octylglucoside for 2h at 0°C. Immune complexes were adsorbed to Pansorbin(Calbiochem-Behring), washed once with TBS-3% bovine serum albumin-0.825% octylglucoside, detached in SDS sample buffer with boiling, and then subjected to SDS-PAGE.

Enzyme assays Adenylate cyclase activity was determined by measuring cyclic AMP formed by radioimmunoassay after succinylation as described previously (12). NDP kinsse activity was determined by isotopic method(17) using [^{32}P]GDP or [^{3}H]GDP as substrate.

Solubilization and characterization of glucagon receptor Purified membranes (~300μg) were incubated with [^{125}I]glucagon(~2000Ci/mmol, 1μCi) in TMEDP (250μl) containing 0.2% bovine serum albumin for 20min at 30°C, washed by repeated centrifugation, and then solubilized by detergents. The solubilized samples were treated without or with 100μM GDPβS or GTPγS for 5min at 0°C before sucrose density gradient centrifugation. The radioactivity in each fraction was counted.

Miscellaneous Protein concentration was determined as described by Lowry et al.(18) using bovine serum albumin as a standard.

Densitometry was done using Shimazu CS-910 TLC scanner by monitoring absorption at 600nm.

Autoradiography was carried out using Kodak XAR film with intensifying screens.

RESULTS AND DISCUSSION

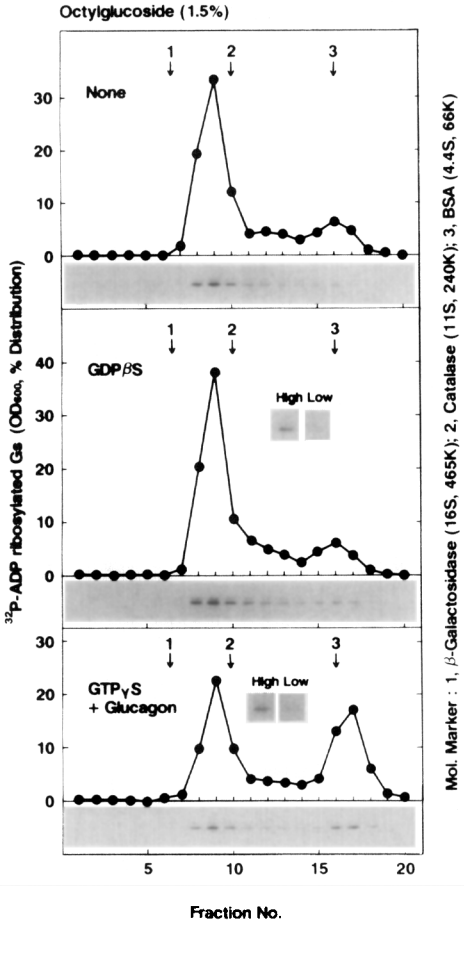
In a previous study(16) we found that octylglucoside was able to solubilize Gs in a form precipitable by anti-NDP kinase antibodies. Whether the immunoprecipitable Gs was actually a complexed form was examined by estimating its molecular size on sucrose density gradient centrifugation(Fig. 1). When prelabeled membranes were incubated without or with 100μM GDPβS, followed by washing and solubilization, most of the solubilized [^{32}P]ADP ribosylated Gs was sedimented at a position having molecular size of 12-13S(high molecular weight complexed form of Gs) and a small portion was recovered from fractions corresponding to free Gs and/or Gsα subunit(3-4S)(low molecular weight form of Gs)(Fig. 1, top and middle panels). On the other hand, when membranes were treated with glucagon(1μM) plus GTPγS(100μM), the solubilized Gs was distributed at these two positions almost equally(Fig. 1, bottom panel), suggesting that the Gs(or Gsα) might be released from the complex through receptor activation.

In order to test the possibility that the high molecular weight Gs complex contains mNDP kinase, fractions containing high and low molecular

weight forms of Gs were separately pooled and then reacted with affinity-purified anti-NDP kinase antibodies. As shown in the inset in Fig. 1(middle and bottom panels), only the Gs with high molecular weight was precipitated by the antibodies. Taking it into account that the purified mNDP kinase has molecular size of 6.2S(17), the present observation suggests that the high molecular weight form of Gs may represent Gs-mNDP kinase complex. Whether the fractions corresponding to high molecular weight form of Gs actually contained NDP kinase activity was further examined under the same condition(Fig. 2, lower panel). A large portion of the NDP kinase activity was recovered in fractions with molecular size of the purified enzyme(6.2S). However, small but significant amount of the activity was detected in fractions corresponding to the high molecular weight form of Gs(12-13S). These results strongly suggest that Gs and mNDP kinase may exist, at least in part, in a complexed form in membranes.

Concentrations of glucagon receptor, Gs and mNDP kinase in liver membranes were reported to be approximately 1-2pmol/mg(19), 0.2-2pmol/mg(20), and 1pmol/mg(17), respectively. Whether these proteins interact one another stoichiometrically remains to be determined. In our experiments, irrespective of whether the solubilized Gs was unfractionated or fractionated on sucrose density gradient centrifugation, only a small portion(~5%) of the Gs preparation used was precipitated by anti-NDP kinase antibodies. This may occur not only due to lability of the Gs-mNDP kinase complex after solubilization but also due to the possible presence of complexes between Gs and other membrane components. The latter possibility is shown in Fig. 2(upper panel) where the distribution of detergent-solubilized adenylate cyclase activity on sucrose density gradient centrifugation was examined. Main peak of the enzyme activity preactivated by glucagon plus GTPγS sedimented at the same position as the high molecular weight form of Gs, suggesting that the Gs with high molecular weight may be comprised of heterogenous complexes formed between Gs and different types of membrane protein components including mNDP kinase and adenylate cyclase.

It is known that glucagon receptor can be solubilized by digitonin in a guanine nucleotide sensitive form(a ternary hormone-receptor-G protein complex)(21). Therefore, we tested the possibility that this complex contained mNDP kinase. As shown in Fig. 3(middle panel), the detergent solubilized the glucagon receptor from membranes into two forms(10S and 6-7S). The larger molecular weight form seems to represent the glucagon-receptor-Gs ternary complex as evidenced by the observation that the glucagon binding activity with larger molecular weight decreased by the addition of GTPγS or GDPβS into the solubilized preparations prior to sucrose density gradient centrifugation. Under the similar conditions, the Gs sedimented at two positions having molecular size of 9-11S and 3-4S(Fig. 3, upper panel). Thus,



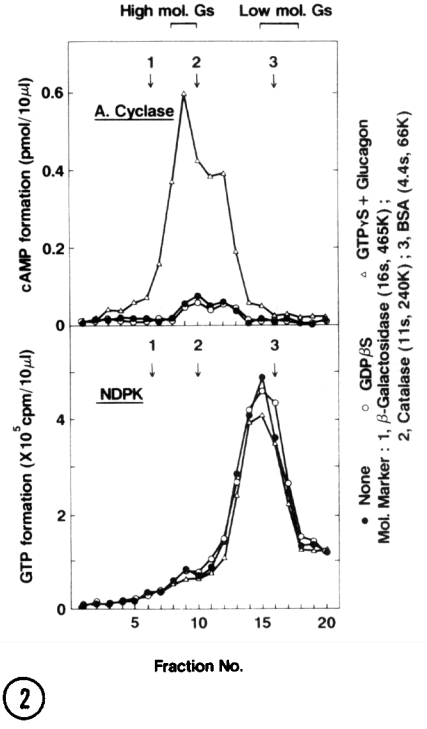


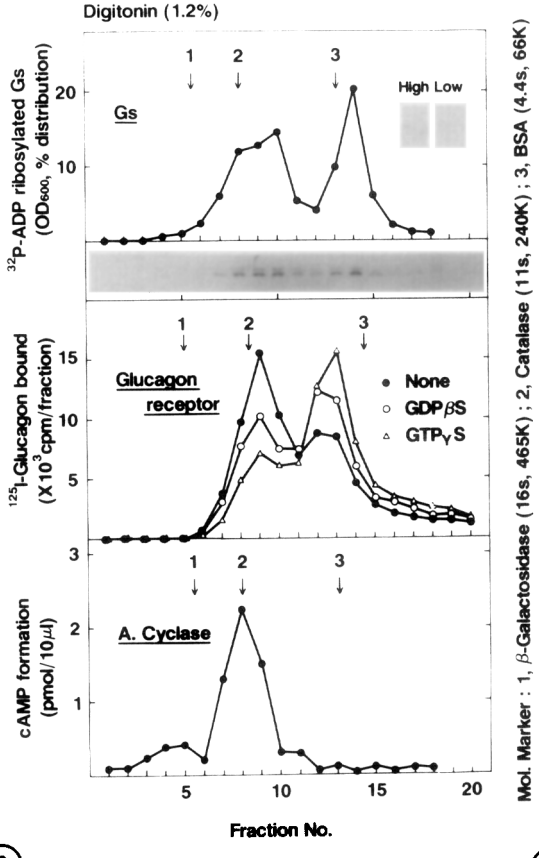
Fig.1 Distribution of [^{32}P]ADP ribosylated Gs solubilized by octylglucoside on sucrose density gradient centrifugation.

The Gs in membranes were [^{32}P]ADP ribosylated, treated with or without glucagon and/or guanine nucleotides, solubilized with 1.5% octylglucoside, and then analyzed by 5-20% sucrose density gradient centrifugation. After centrifugation, tubes were punctured by a needle and 8-drops each was collected from the bottom. Aliquots of each fraction were subjected to SDS-PAGE, followed by autoradiography. Amounts of Gsα were quantitated by scanning these bands at 600nm and is shown in % distribution above autoradiograms. GDPβS, GTPγS and glucagon were included at 100, 100 and 1μM, respectively. Numbers with arrows in the figure show the position of marker proteins shown on the right margin. (Insets) Fractions 7-11(high molecular weight complexed form of Gs) and 14-18(low molecular weight form of Gs) were separately pooled and treated with anti-NDP kinase antibodies for 2h at 0°C. Immune complexes were adsorbed to and eluted from Pansorbin, and analyzed by SDS-PAGE, followed by autoradiography. "High" and "Low" represent high molecular weight complexed form of Gs and low molecular weight form of Gs(and/or Gsα), respectively.

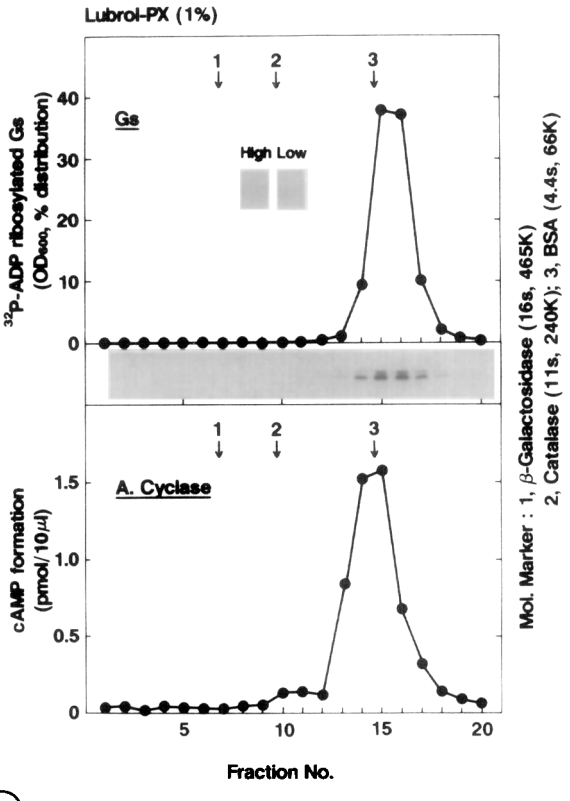
Fig.2 Distribution of adenylate cyclase and mNDP kinase activities solubilized by octylglucoside on sucrose density gradient centrifugation.

Membranes were treated, solubilized and fractionated by the same way as described under legend to Fig. 1. (Upper panel) For adenylate cyclase assay, enzyme activity was determined with no further addition in the assay mixture since the membranes were pretreated with or without glucagon and/or guanine nucleotides. (Lower panel) mNDP kinase activity was measured with 100μM [^{32}P]GDP(0.5μCi) as a substrate.

the Gs with larger molecular size seems to represent one related to the ternary complexes. The two peaks of Gs obtained after sucrose density gradient centrifugation were separately pooled and then treated with anti-NDP kinase antibodies. The result shows that neither the high nor low molecular



(3)



(4)

Fig.3 Distribution of [^{32}P]ADP ribosylated Gs, [^{125}I]glucagon-receptor complex and adenylate cyclase activity solubilized by digitonin on sucrose density gradient centrifugation.

(Top panel) The [^{32}P]ADP ribosylated membranes were treated with 100μM GDPβS for 5min at 30°C and then solubilized with 1.2% digitonin. Inset shows the result of immunoprecipitation of Gs by anti-NDP kinase antibodies. More detailed explanation is given under legend to Fig.1. "High" and "Low" represent complexed and free forms of Gs, respectively. (Middle panel) The solubilized, prelabeled glucagon-receptor complex was treated without or with 100μM GDPβS or GTPγS for 5min at 0°C, and applied on a sucrose density gradient. (Bottom panel) Membranes were treated with 100μM GDPβS for 5min at 30°C and then solubilized. Adenylate cyclase activity in fractions was determined in the presence of 1μM forskolin.

Fig.4 Distribution of [^{32}P]ADP ribosylated Gs and adenylate cyclase activity solubilized by Lubrol-PX on sucrose density gradient centrifugation.

(Upper panel) The [^{32}P]ADP ribosylated membranes were treated with 100μM GDPβS for 5min at 30°C and solubilized with 1% Lubrol-PX. Other explanations are given under legends to Figs. 1 and 3. (Lower panel) Membranes were treated with 100μM GDPβS and then solubilized. Adenylate cyclase activity in fractions was determined in the presence of 1μM forskolin.

weight form of Gs was precipitated, demonstrating that the Gs with larger molecular size seems unlikely to interact with mNDP kinase. On the other hand, Lubrol-PX solubilized the Gs into a single peak with an apparent molecular size of 3-4S(Fig. 4). The radioactivity associated with this peak was not precipitated by the antibodies, ruling out the possibility that the Gs solubilized with Lubrol-PX may interact with mNDP kinase.

The present study demonstrates that Gs and mNDP kinase may exist, at least in part, as a complexed form in membranes. The complex seems to be responsible for effective GTP introduction into the Gs although the physiological significance and mechanism of the complex formation remain to be fully determined. There have been some observations which seem hard to be explained by solely a GDP-GTP exchange reaction generally accepted in recent models. 1) GDP exerts GTP-like effects for adenylate cyclase activation in terms of dose-dependency and magnitude(5,10). 2) GDP provides larger activity ratios(the ratio of adenylate cyclase activities with and without hormone) than GTP and its analog(5). 3) Hormone reduces the inhibitory effect of GDP on the GTP- or its analog-stimulated adenylate cyclase activity(10,12,13). Our proposal including this study on the possible role of mNDP kinase in signal transduction should provide a clue to solve these questions. At present, two possible mechanisms are presumed for the regulation of G protein(Gs) by mNDP kinase. 1) mNDP kinase increases GTP concentration in the vicinity of the Gs, as a result, leading to an increased GDP-GTP exchange reaction on the G protein. 2) mNDP kinase directly phosphorylates bound GDP to GTP on the Gs. These possibilities are currently under investigation.

ACKNOWLEDGMENTS

We are grateful to Dr. Michio Ui(The University of Tokyo) for his encouragement and helpful discussion throughout this work. This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. Gilman, A.G.(1987) Ann. Rev. Biochem. 56, 615-649
2. Ui, M.(1984) Trends Pharmacol. Sci. 5, 277-279
3. Rodbell, M., Birnbaumer, L., Pohl, S.L., and Krans, H.M.J.(1971) J. Biol. Chem. 246, 1877-1882
4. Hanoune, J., Lacombe, M.L., and Pecker, F.(1975) J. Biol. Chem. 250, 4569-4574
5. Kimura, N., and Nagata, N.(1977) J. Biol. Chem. 252, 3829-3835
6. Iyengar, R., and Birnbaumer, L.(1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3189-3193
7. Iyengar, R., Abramowitz, J., Bordelon-Riser, M., Blume, A.J., and Birnbaumer, L.(1980) J. Biol. Chem. 255, 10312-10321

8. Kimura, N., and Nagata, N.(1979) J. Biol. Chem. 254, 3451-3457
9. Kimura, N., and Shimada, N.(1983) J. Biol. Chem. 258, 2278-2283
10. Kimura, N., Shimada, N., and Tsubokura, M.(1985) Biochem. Biophys. Res. Commun. 126, 983-991
11. Kimura, N., and Johnson, G.S.(1983) J. Biol. Chem. 258, 12609-12617
12. Kimura, N., and Shimada, N.(1985) Biochem. Biophys. Res. Commun. 131, 199-206
13. Kimura, N., and Shimada, N.(1986) Biochem. Biophys. Res. Commun. 134, 928-936
14. Ohtsuki, K., Yokoyama, M., and Uesaka, H.(1987) Biochim. Biophys. Acta 929, 231-238
15. Wieland, T., and Jakobs, K.H.(1989) FEBS Lett. 245, 189-193
16. Kimura, N., and Shimada, N.(1988) Biochem. Biophys. Res. Commun. 151, 248-256
17. Kimura, N., and Shimada, N.(1988) J. Biol. Chem. 263, 4647-4653
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J.(1951) J. Biol. Chem. 193, 265-275
19. Rodbell, M., Krans, H.M.J., Pohl, S.L., and Birnbaumer, L.(1971) J. Biol. Chem. 246, 1872-1876
20. Bokoch, G.M., Katada, T., Northup, J.K., Ui, M., and Gilman, A.G.(1984) J. Biol. Chem. 259, 3560-3567
21. Mason, J.C., and Tager, H.S.(1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6835-6839