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MOLECULAR MECHANISMS OF COUPLING IN HORMONE RECEPTOR–ADENYLATE CYCLASE SYSTEMS

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The survey of literature pertaining to this chapter was concluded in March 1980.

I. Introduction

A. ADENYLATE CYCLASE AS A MODEL SYSTEM

Since the original description of the enzyme adenylate cyclase, which catalyzes the conversion of ATP to cyclic AMP (1), a great deal of investigative effort has been directed toward elucidating the mechanisms of its stimulation by hormones. After the initial discovery that epinephrine and glucagon stimulated adenylate cyclase in the liver (2), a wide variety of other hormones and drugs were found to stimulate the enzyme in an extraordinary array of tissues from many species (3). Where the detailed specificity of hormone or drug stimulation could be worked out, as in the case of beta-adrenergic receptors for catecholamines, it was invariably found that the specificity for stimulation of adenylate cyclase was identical to the specificity of stimulation of a particular physiological effect. Findings such as these buttressed the contention that adenylate cyclase was a proximal target of many hormones and drugs and that it functioned as an intermediate in the pathway of physiological hormone or drug response.

Since the enzyme can be studied in cellfree preparations, such as purified plasma membrane fractions, it has provided a simple model system for studying the biochemical basis of hormone and drug action. Moreover, since the enzyme is very intimately related to the hormone receptors and all of the components of the system are lodged in the plasma membrane, such systems also provide useful models for studying transmembrane signaling.

B. HISTORICAL PERSPECTIVE

It is interesting and instructive to review the evolution of concepts concerning the molecular components of hormone-responsive adenylate cyclase systems and their interactions. The earliest conceptions of such systems developed by Sutherland and coworkers (4) featured the presence of one or at most two distinct components (Fig. 1). These were the catalytic unit of the enzyme, which converts ATP to cyclic AMP, and a hormone receptor binding site postulated to exist either as a binding site on the enzyme itself or as a separate molecular entity (4). By the end of the 1960s, this two-component model, consisting of receptor and catalytic moiety, was fairly dominant. Hormones or agonists were felt to induce some conformational or other change in the receptors that was presumably converted to a signal that perturbed the catalytic moiety, thus increasing its enzyme activity.

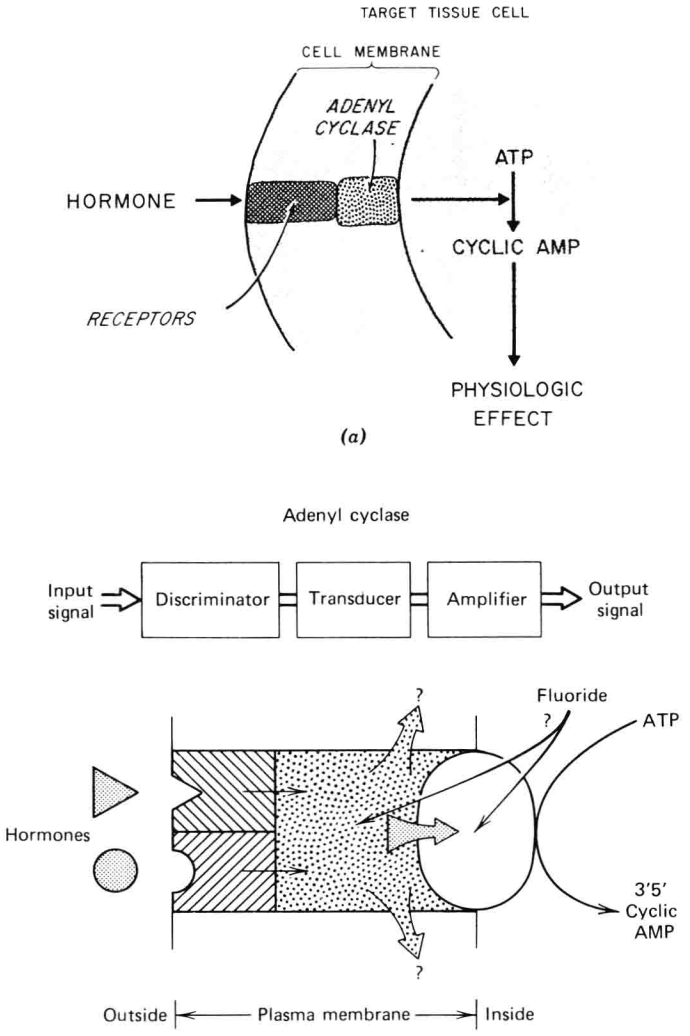

Figure 1. Schematic representation of some early models of hormone stimulation of adenylate cyclase. (*a*) A two-component model including receptor and catalytic moiety. Modified from Reference 3. (*b*) A three-component model including discriminator (receptor), transducer (coupler), and amplifier (catalytic moiety). The possible sites of fluoride stimulation of the enzyme are indicated. Taken from reference 24 as modified from reference 6.

A somewhat different model was initially proposed by Hechter and colleagues in the mid 1960s (5) and subsequently stressed by Rodbell et al. in the early 1970s (6). This model (Fig. 1) featured the existence of some sort of intermediate moiety interposed between receptor and catalytic unit that functions as a transducer or a "coupler" to convey the hormone-induced alteration in the receptor to the catalytic moiety. These earliest concepts of a three-component system consisting of receptor, transducer, and catalytic sites were necessarily vague with respect to the molecular nature of the transducing element. Several early formulations focused on a membrane lipid matrix as perhaps representing the transducing element with receptor-mediated alterations in membrane lipids possibly representing the key transduction event (7).

With the discovery by Rodbell et al. (8), in the early 1970s, of the requirement of hormone-sensitive adenylate cyclase systems for guanine nucleotides, as well as the peculiar effects of guanine nucleotides on hormone receptor binding (9), interest developed in the possible involvement of these nucleotides in the transduction process. As is reviewed below, subsequent studies documented the existence of a specific molecular entity, generally termed "nucleotide regulatory site," that appears to mediate the effects of guanine nucleotides on adenylate cyclase activity and on hormone receptor binding. A number of distinct lines of experimental evidence strongly suggest that this nucleotide regulatory site is, in fact, the transducing or coupling factor hypothesized to exist over the past 10-15 years.

II. Molecular Components of Hormone-Responsive Adenylate Cyclase

The major focus of this chapter is on the molecular mechanisms involved in the coupling of hormone receptors to adenylate cyclase. It is not our purpose here to review in detail what is known about each of the components of the system, namely, receptors, catalytic moiety, and guanine nucleotide regulatory sites. Accordingly, in this section we attempt to *briefly* review information about each of these components. The treatment is not encyclopedic, but rather geared toward laying the foundation for a discussion of what is known about the molecular interactions among the various components. In the material that follows we indicate the evidence for the existence of each of these components as discrete molecular entities and discuss how each can be assayed and studied.

A. RECEPTORS

The concept of specific receptors for hormones and drugs probably dates back at least 80 years and has been popular among endocrinologists and pharmacologists throughout this century. Many investigators never really envisaged such receptors as specific molecular entities, but rather more vaguely as "some pattern of forces" in or on a cell that was in some way complementary to a hormone or drug in structure. As soon as Sutherland discovered the effects of hormones, such as those of epinephrine and glucagon on adenylate cyclase activity, he immediately realized that hormone receptors must be closely associated with, if not be a part of the adenylate cyclase complex (4). However, it was not until about 10 years after this work (around 1970, when direct radioligand binding assays for hormone receptors first became available) that progress in the elucidation of the structure and function of such putative receptors became a reality (10).

Prior to the discovery of adenylate cyclase most studies of polypeptide and catecholamine hormone action relied on the bioassay of a variety of distal physiological effects, such as lipolysis, glycogenolysis, and steroidogenesis. With the discovery of adenylate cyclase a more proximal consequence of hormone action could be studied, namely, the activation of the enzyme. From such studies a variety of useful inferences could be drawn concerning the nature of the presumed receptors that mediated the stimulatory actions of the various hormones. Nonetheless, such inferences remained indirect, since the interaction of the hormone or drug with its physiological receptor was still not being directly measured. However, a great deal of interesting information was obtained from such approaches. For example, it was learned that the catecholamine receptors that lead to the stimulation of adenylate cyclase appeared to have all the properties of typical physiological beta-adrenergic receptors.

Beginning in about 1970 direct binding studies of adenylate cyclase coupled receptors became a reality (10). Such studies involved the binding of radioactively labeled hormones, drugs, and antagonists to membrane-bound sites that could be shown to have all the characteristics expected of the physiological receptors. Such studies have been reviewed in a variety of places and are not extensively covered here (11,12). Suffice it to say that for virtually every known adenylate cyclase coupled hormone and drug receptor appropriate radioligand binding assay techniques have been developed over the past decade. In general, binding has all the properties expected of an interaction with a physiologically relevant receptor. Usually

there is good correlation between the specificity of drugs or hormones interacting with the binding sites and their ability to either stimulate or antagonize stimulation of the enzyme in membrane preparations or whole cells. Such studies have indicated a small number of hormone receptors, in general, 1-2 pmole of receptor per milligram of purified plasma membrane protein from a variety of sources. In the case of the catecholamine beta-adrenergic receptors, which have been, perhaps, the most heavily studied, binding studies have been performed with both agonists and antagonists, and a variety of interesting distinctions between the two have become apparent. These are dealt with in some detail below.

In several cases these receptors have been characterized, solubilized, and partially purified. In no case to date, however, has an adenylate cyclase coupled receptor been purified to homogeneity and definitively characterized.[1] This is a consequence of the fact that the receptors are invariably membrane bound, present in very small concentrations, and often quite labile. In several cases, however, partial purification has been obtained after solubilization. This has been reported for HCG receptors (13), prolactin receptors (14), and beta-adrenergic receptors (15,16), among others. Detergents, such as triton and digitonin have been essential to remove the receptors from the plasma membrane. The technique of affinity chromatography has generally been the key to purification efforts. Since the receptors have not been purified to homogeneity and well characterized, it is not possible to discuss precise molecular weights, subunit composition, and so forth at present. However, in general, the receptors appear to be intrinsic membrane proteins with molecular weights in the range of 50,000-100,000. There is some evidence that they may be lipoproteins, since several phospholipases appear to degrade binding activity in several cases (17). In addition, there is some evidence for their glycoprotein nature, since beta-adrenergic receptors, for example, are bound to lectins such as Con-A (176). To date none of these solubilized receptors has been successfully reincorporated into a natural or artificial lipid membrane in which its physiological function, that is, stimulation of adenylate cyclase, has been restored.

The receptors appear to be exposed at the outer surface of the plasma membrane where they bind drugs and polypeptide hormones. Since, as is discussed below, the enzyme edenylate cyclase, as well as the nucleotide

[1] The hormonal binding subunit of the beta-adrenergic receptor has recently been purified: R. G. L. Shorr, R. J. Lefkowitz, and M. G. Caron, J. Biol. Chem., *256*: 5820 (1981).

regulatory site, appears not to be present at the outer surface of the plasma membrane, the receptor protein probably extends fairly deeply into the plasma membrane, possibly even across its entire width.

Since the beta-adrenergic receptor is the focus of much of this chapter, it is worth briefly summarizing the techniques used for the direct binding study of beta-adrenergic receptors in membrane-bound or soluble and purified form. These have generally involved either antagonist radioligands, namely, (−)[^{3}H] dihydroalprenolol (18) or (±)[^{125}I] iodohydroxybenzylpindolol (19), or an agonist ligand, namely, (±)[^{3}H] hydroxybenzylisoproterenol (20). Solubilization of the receptors has been reported from sources such as frog (15) and turkey (16) erythrocytes, as well as S49 lymphoma cells (21). In the case of the frog and turkey erythrocyte receptors, successful solubilization has been achieved with the plant glycoside digitonin (5,16). After solubilization, the receptors can be studied by binding with (−)[^{3}H] DHA.* Receptors from both of these sources have been purified on affinity columns consisting of alprenolol linked to Sepharose beads. Beta receptors have also been solubilized with Lubrol, from S_{49} lymphoma membranes (21). The lability of the receptors in this system, however, has necessitated that they first be tagged in membranes with the slowly dissociable ligand [^{125}I] HYP prior to solubilization. Attempts to achieve ligand binding to the Lubrol-solubilized receptors have not been successful to this date. In the case of the turkey erythrocyte receptors there appears to be a crucial sulfhydryl group at the ligand binding site that can be inactivated by appropriate group-specific reagents (22). It seems likely that within the next few years complete purification of these adenylate cyclase coupled beta-adrenergic receptors will be achieved.

A final note about the receptors relates to the demonstration that they are, in biochemical terms, quite distinct from the catalytic moiety of adenylate cyclase. This demonstration required the ability to directly assay the hormone receptors by binding techniques. Using such methods it has been demonstrated that after solubilization of membrane fractions the beta-adrenergic receptors can be shown to be distinct from the adenylate cyclase in terms of their elution on gel filtration matrices (23), as well as in terms of their sedimentation behavior on sucrose gradients (21). Evidence such as this establishes, then, that the receptor molecules are distinct

*Abbreviations: [^{3}H] DHA, (−)[^{3}H] dihydroalprenolol; [^{125}I] HYP, (±)[^{125}I] iodohydroxybenzylpindolol; [^{3}H] HBI, (±)[^{3}H] hydroxybenzylisoproterenol; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GTP-γS, guanosine-5′-*o*-(3-thiotriphosphate); Gpp-(CH_2)p, guanyl-5′-yl-methylenediphosphate.

from those molecules that carry out the catalytic function of the enzyme adenylate cyclase.

B. CATALYTIC MOIETY OF ADENYLATE CYCLASE

Hormone-stimulated adenylate cyclase activity has been observed ubiquitously in eukaryotic cells with the possible exception of human erythrocytes and certain mutant cell clones. Because of the pivotal role of cAMP in hormonal regulation of cellular activities adenylate cyclase has been the subject of intense investigation. Although the molecular mechanism of adenylate cyclase activation remains to be defined, it has recently been demonstrated that adenylate cyclase activity requires the concerted functioning of a distinct guanine nucleotide regulatory protein and the catalytic component. Many of the studies reported before 1977, which have been previously reviewed (24,25), focused on the holoenzyme. In this section we briefly summarize this early work on the holoenzyme and then describe the individual catalytic and guanine nucleotide regulatory components.

In general, adenylate cyclase is an intrinsic membrane protein located predominantly, if not exclusively, in the plasma membrane. Evidence for its localization comes from the reported increase in enzyme specific activity in purified plasma membrane preparations (26-28) and the observation that the enzyme copurifies with established plasma membrane marker enzymes such as Na^+, K^+, ATPase, and 5′nucleotidase (29). Although there are reports of adenylate cyclase activity in various cell organelle membrane preparations (30-32) contamination of these preparations by plasma membrane fragments has not been ruled out. The catalytic site of adenylate cyclase is on the inner lamella of the plasma membrane, since it utilizes intracellular ATP as substrate to produce cAMP. Treatment of whole cells or membranes with proteases (17,33) or phospholipase (17,34) uncouples the hormonal response of adenylate cyclase. However, it can be shown that in particulate preparations derived from pretreated cells the enzymatic activity is retained.

The enzyme has been most extensively studied in its particulate state in partially purified plasma membrane preparations. Although these have been derived from diverse cellular sources, many common characteristics have been observed. Adenylate cyclase activity demonstrates a broad pH optimum between 7.0 and 8.5, and although the ionic strength of the assay medium does not appear to be critical, specific ions are required for

activity. The physiologically relevant cation appears to be Mg^{2+} and it must be present in excess of ATP, suggesting that the specific substrate of the enzyme is a Mg-ATP complex. The chelation complex between Mg^{2+} and ATP is important since $ATPH^{3-}$ is a potent competitive inhibitor (35). An additional allosteric role for Mg^{2+} has been postulated since Mg^{2+} can stimulate adenylate cyclase activity at concentrations beyond that necessary to bind the ATP substrate (36,37). Studies employing other metal ions have shown that monovalent cations have no striking effect on the enzyme activity and that Ca^{2+} is usually (37-39), but not always (40,41), inhibitory. Mn^{2+} can substitute for Mg^{2+} at low concentrations (1-5m*M*) and may even increase the v_{max}, possibly through the putative allosteric site. At concentrations above 5 m*M* Mn^{2+} has been found to uncouple the hormonal activation of adenylate cyclase (34,42).

The enzymatic reaction catalyzed by adenylate cyclase converts ATP to cyclic AMP and pyrophosphate according to the scheme:

$$\text{ATP} \xrightleftharpoons[\text{adenylate cyclase}]{} \text{cAMP} + \text{PP}_i$$

Thermodynamic studies indicate that the equilibrium constant is close to unity (43,44)

$$K = \frac{[\text{cAMP}]\ [\text{PP}_i]}{[\text{ATP}]} = 0.065$$

Although the production of ATP from cAMP and pyrophosphate has not been observed (27,45), its formation is favored under standard thermodynamic conditions. Under physiological conditions, however, this reverse enzymatic reaction probably would not occur (44). It is also interesting to note that from thermodynamic considerations the hydrolysis of cAMP to AMP releases about 14.1 kcal/mole of free energy, making cAMP a potential high-energy donor (46).

By using chemical reagents to modify specific amino acid side chains it has been demonstrated that adenylate cyclase activity depends on a highly reactive free sulfhydryl group (47,48). Recent studies have also shown that catalytic activity can be inhibited by phenylglyoxal, which reacts selectively with the guanidino side chain of arginine (49).

The original interest in adenylate cyclase stemmed from the fact that hormone-receptor interactions resulted in an activation of enzymatic activity, but other activators of adenylate cyclase have also been described. Sutherland and coworkers (1,50) showed in the late fifties and early sixties that NaF can stimulate adenylate cyclase activity and that this effect is independent of fluoride inhibition of ATPase. Perkins (24) has pointed out that the NaF stimulation may be an artifact of the particulate enzyme preparation since NaF does not stimulate cAMP production in whole cells even though it probably gets across the plasma membrane. The action of F^- on adenylate cyclase activity has been studied in the hopes of elucidating a general mechanism for activation of this enzyme. The magnitude of F^- stimulation above basal adenylate cyclase activity is variable from system to system. Fluoride activation is dependent on the presence of Mg^{2+} (25), and in some reports an additional requirement for nucleotides has been claimed. The activation of the enzyme by fluoride is apparently irreversible or only very slowly so. The molecular mechanism of adenylate cyclase activation by fluoride has not been precisely defined, but the halide probably interacts with a regulatory subunit of the enzyme (see below).

Rodbell and others (51-53a,53b,53c) have shown that nonhydrolyzable analogs of GTP, such as Gpp(NH)p, Gpp(CH_2)p, and GTP-γS, can also stimulate adenylate cyclase activity and in a quasi-irreversible manner. This and other observations on the regulatory nature of guanine nucleotides on adenylate cyclase systems have provided new insights and new approaches for the investigation of the interactions of the components of the hormone receptor–enzyme complex that are detailed below.

To completely characterize the cyclase enzyme it will be necessary to purify it. To date complete purification has not been accomplished. Purification of adenylate cyclase presents numerous problems, since first the particulate enzyme must be solubilized by detergent out of its normal hydrophobic membrane environment and stabilized for subsequent purification schemes. A number of nonionic detergents have been successfully employed to solubilize adenylate cyclase activity. These include Lubrol PX (21,54-57), Triton X100 (1,54,58), Triton X305 (1,59), and digitonin (23,60). In most cases the enzyme is preactivated in particulate form with fluoride or Gpp(NH)p as the irreversible activator before exposure to the detergent. The subsequently solubilized enzyme maintains much of its activity and has been reported to retain sensitivity to Gpp(NH)p and

fluoride, although to variable degrees (55,56,58). In some cases the solubilized enzyme is unaffected by addition of those activators. A consistent observation has been that after solubilization there is abolition or uncoupling of the hormonal responsiveness of the adenylate cyclase (21,23,53,54,56, 61). There have been a few reports of hormonal activation of solubilized adenylate cyclase (62-64), but these interesting observations have not yet been followed up.

The molecular weight of the prestimulated solubilized adenylate cyclase from many cell types has been estimated by gel filtration (53-58) and sucrose gradient centrifugation (21,54,55,58). These studies indicate that adenylate cyclase binds considerable detergent, consistent with its designation as an integral membrane protein. The molecular weight estimates range between 1.5×10^5 and 7×10^6. Neer (54) reported the resolution of multiple peaks of adenylate cyclase activity solubilized from rat renal medulla and chromatographed on a Sepharose 4B column, suggesting a possible multimeric structure of adenylate cyclase. The wide range of molecular weights reported is probably due to several factors, including the fact that unpurified soluble preparations have been analyzed and the existence of artifacts such as the formation of protein-lipid-detergent complexes. The full characterization of adenylate cyclase awaits definition and purification of the various subunits of the enzyme. At least two distinct components have thus far been identified, a catalytic unit and a nucleotide regulatory component, and application of new chromatographic techniques (65) may facilitate their purification.

C. GUANINE NUCLEOTIDE REGULATORY PROTEIN

Rodbell and coworkers (8,9,66) were the first to recognize the multiple regulatory effects of guanine nucleotides on receptor-coupled adenylate cyclase systems. Through the use of highly purified plasma membrane preparations this group and others have demonstrated that GTP is a physiological regulator of adenylate cyclase activity and that guanine nucleotides are required for hormonal activation of the enzyme (8,67,68). Nonhydrolyzable analogs of GTP, such as Gpp(NH)p and GTP-γS, activate the enzyme independently and in the presence of hormone to a persistently activated state (51-53). This is in contrast to the activation of adenylate cyclase by hormone and GTP, which is readily reversible.

An additional effect of guanine nucleotides is to modulate the binding

affinity of the hormone receptor (9,69,70). Guanine nucleotides reduce the affinity of the receptor specifically for agonist agents without altering the binding affinity for antagonists (69,70).

The description of these multiple regulatory effects of guanine nucleotides on hormone-responsive adenylate cyclase systems initiated an intense investigation into the nature of the component(s) associated with nucleotide binding. The observation that hormonal activation of adenylate cyclase requires guanine nucleotides and that GTP-promoted activation of the enzyme is reversible while the activation by Gpp(NH)p is persistent led to the hypothesis that the hydrolysis of GTP may be associated with adenylate cyclase regulation (53,66,71). Cassel and Selinger (71) were the first to describe a hormone-sensitive GTPase activity in turkey erythrocyte membranes. The GTPase activity could be inhibited by Gpp(NH)p or GTP-γS and the inhibition of GTPase correlated with the ability of these nucleotide analogs to activate the cyclase (72). This suggested that the GTPase was a common site for guanine nucleotide dependent cyclase activation. Cassel and Selinger also reported that cholera toxin, which activates adenylate cyclase irreversibly, also inhibits hormone-specific GTPase activity (73a). Cholera toxin has been an important tool for probing the adenylate cyclase system. Cholera toxin appears to work by catalyzing the covalent transfer of ADP-ribose from its cofactor NAD^+ to a subunit of the adenylate cyclase system (74,75). This covalent modification activates the enzyme to a slowly reversible state dependent on the presence of guanine nucleotides. Enzyme activities measured in the presence of GTP (after cholera toxin treatment) are equivalent to the stimulation observed with Gpp(NH)p or GTP-γS. Cassel and Selinger (73a) proposed that the binding of guanine nucleotide triphosphate activates adenylate cyclase, and subsequently GTPase activity is the shutoff mechanism of the enzyme that returns it to a basal state (Fig. 2). Hormones activate adenylate cyclase by facilitating the binding of guanine nucleotide triphosphates to the regulatory site (76). Adenylate cyclase activity is therefore regulated by the ability of guanine nucleotides to bind to the regulatory component and by the rate of hydrolysis of GTP which deactivates catalytic activity (73a,73b,76,77,87).

Another major focus of investigations of guanine nucleotide regulation of adenylate cyclase has centered on whether the guanine nucleotide binding site resides on an independent component or is an allosteric site of the catalytic unit. Since guanine nucleotides regulate both receptor and

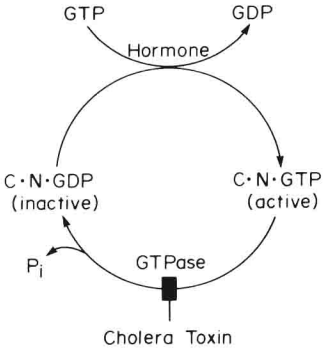

Figure 2. Role of GTPase in terminating activity of adenylate cyclase. C = catalytic moiety of the enzyme, N = nucleotide regulatory protein. Taken from reference 76.

catalytic functions it is necessary to determine how many nucleotide regulatory components exist.

Pfeuffer and Helmreich (53a) suggested that the guanine nucleotide binding site might be located on a separate subunit of the adenylate cyclase complex. In 1977 Pfeuffer (78) reported the development of a GTP photoaffinity probe that labeled several discrete proteins in membranes or in Lubrol-solubilized adenylate cyclase preparations. After partial purification of adenylate cyclase activity on sucrose gradients, he determined that a specifically labeled 42,000 molecular weight protein was associated with the enzyme activity. Pfeuffer applied a soluble adenylate cyclase preparation to a GTP-Sepharose affinity column and measured adenylate cyclase activity in the pass-through fractions. Both Gpp(NH)p- and fluoride-stimulated enzymatic activity were significantly reduced. The affinity column was eluted with guanine nucleotides and the eluate and pass-through combined to reconstitute both the nucleotide and fluoride response. The factors necessary for reconstitution of the cyclase response were shown to be proteins in both fractions. The reconstituted activity appeared to be dependent on the 42,000 molecular weight protein in the Gpp(NH)p eluate of the affinity resin. Subsequently, Cassel and Pfeuffer (79) collaborated to show that the 42,000 molecular weight protein adsorbed on the GTP affinity resin was the site of covalent modification by