The Hepatic Adenylate Cyclase System

II. SUBSTRATE BINDING AND UTILIZATION AND THE EFFECTS OF MAGNESIUM ION AND pH

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SUMMARY

The kinetic characteristics of substrate utilization by hepatic adenylate cyclase were investigated under a variety of incubation conditions, including variations in pH, [substrate], [Mg2+], and in the absence or presence of glucagon. Activities were compared with ATP and 5'-adenylylimidodiphosphate (App(NH)p) as substrates. The \( K_m \) for both substrates was about 50 \( \mu \)M; \( V_{max} \) given with App(NH)p was about 40% lower than obtained with ATP as substrate.

In the presence of a saturating concentration of substrate (1 \( \mu \)M), basal activity was increased 4-fold by increasing [Mg2+] from 5 to 50 \( \mu \)M. The stimulatory effect of Mg2+ was not due to an allosteric action since basal activity was only marginally enhanced (40%) when the substrate concentration was reduced to 10 \( \mu \)M. As suggested by deHaen ((1974) J. Biol. Chem. 249, 2756), it is likely that Mg2+ increases enzyme activity by decreasing the concentration of an inhibitory, unchelated form of substrate that competes with the productive magnesium-substrate complex at the active site.

Activity-pH profiles differed with ATP and App(NH)p as substrates; a shift in pH optimum was observed which correlated with the different pK\(_a\)'s of the terminal phosphate groups of ATP and App(NH)p, and which reflect the concentration of protonated substrate (ATP\(^{2-}\)) present in the incubation medium. Accordingly, protonated substrate is the predominant inhibitory species of unchelated substrate and probably has a considerably higher affinity for the active site than does the magnesium-substrate complex.

Glucagon-stimulated activity was less susceptible to inhibition by protonated substrate than is the basal state as evidenced by lower stimulatory effect when the [Mg2+] was increased from 5 to 20 \( \mu \)M. However, increasing the [Mg2+] from 20 to 50 \( \mu \)M resulted in marked inhibition of glucagon-stimulated activity, particularly in the presence of 10 \( \mu \)M substrate. Conversely, at a fixed [Mg2+], concentrations of substrate at least 20-fold higher than the \( K_m \) were required to achieve maximal hormone-stimulated activity. These findings suggest that the unchelated, fully ionized form of substrate serves as an activating ligand, as has been observed with guanine nucleotides at considerably lower concentrations. Thus, Mg2+ affects adenylate cyclase activity by forming the productive substrate complex and by titrating the inhibitory protonated and activating free forms of substrate. As a result of these effects of unchelated substrate, it proved difficult to evaluate the kinetic parameters involved in substrate binding and utilization and the effects of hormone thereon when substrate was added as the only source of activating ligand. However, linear Michaelis kinetic data were obtained by adding the activating ligand 5'-guanylylimidodiphosphate with glucagon and by making appropriate adjustments of pH and [Mg2+]. \( V_{max} \) was increased 4-fold without changes in \( K_m \) by the actions of 5'-guanylylimidodiphosphate and glucagon.

(\( \alpha,\beta \)Methylene)adenosine 5'-triphosphate and (\( \alpha,\beta \)-methylene)adenosine tetraphosphate, which are not converted to cyclic adenosine 3',5'-monophosphate, competitively inhibited adenylate cyclase with a \( K_i \) of 40 \( \mu \)M and 10 \( \mu \)M, respectively. The closeness of the \( K_i \) for (\( \alpha,\beta \)-methylene)adenosine 5'-triphosphate with the \( K_m \) for ATP suggests that the \( K_m \) approximates \( K_{o} \). If this is the case, a major effect of activating ligands (hormone and guanine nucleotide) is to engender a state of the enzyme that has an increased \( k_{cat} \), the rate constant for product formation by adenylate cyclase; this effect is accompanied by a decrease in affinity for protonated substrate.

In the preceding report (1), it was shown that guanosine 5'-triphosphates bind in their free form to a site, termed nucleotide regulatory site, on the hepatic adenylate cyclase system. Binding of the nucleotides to this site is fundamental to the process of activation of adenylate cyclase; glucagon appears to facilitate this process through its binding and action at the receptor component (1-3).

In this report we consider the interaction of substrate with the catalytic site of the hepatic adenylate cyclase system, the kinetic parameters that may be affected by the activation process, and the problems encountered in evaluating these parameters when guanine nucleotides, essential to the activation process, are not added to the assay medium.

Magnesium ion is required for catalysis of ATP to cyclic
AMP\textsuperscript{4} by adenylate cyclase and it is generally considered that the true substrate of the enzyme is the magnesium-substrate complex (for review see Perkins (4)). If it is assumed that the two ligands (Mg\textsuperscript{2+} and ATP) bind independently to the catalytic site, then to the extent that they compete with the productive magnesium-substrate complex, they will be inhibitory. deHaen (5) tested these assumptions and by a computer-fitting process obtained reasonably close fits with data obtained from the fat cell and ventricular adenylate cyclase systems. He concluded that free ATP (ATP\textsuperscript{4-}) is a competitive inhibitor of the enzyme with binding constants approximately 100-fold higher than the productive magnesium-substrate complex. He concluded also that hormones induce a conformational change in the enzyme which manifests itself in an increase of $V_{max}$ (about 2-fold) and a parallel release of the enzyme from a state of high to low sensitivity toward the inhibitory action of ATP\textsuperscript{4-}. This was considered to be a first approximation since the theoretical curves for the hormone-stimulated states deviated significantly from the experimental points. Interestingly, the computed values for the binding constants of the true substrate were 3- to 5-fold lower for the hormone-stimulated compared to those of the basal state of the enzyme. According to these computations, hormones also engendered a decrease in the affinity of the enzyme for the magnesium-ATP complex.

Two possibilities were not evaluated in deHaen’s model. One possibility is that ATPH\textsuperscript{3-}, referred to here as protonated substrate, is a competitive inhibitor. The concentration of protonated substrate is determined not only by the concentrations of Mg\textsuperscript{2+} and substrate, but also by pH. The other possibility is that free ATP may bind to the nucleotide regulatory site and thus serve as an activator of the enzyme, as seen with guanosine 5’-triphosphates. Since the concentration of free ATP is determined by the concentrations of both ATP and Mg\textsuperscript{2+}, it follows that the activating effects of free ATP will be correspondingly affected by variations in the relative concentrations of substrate and Mg\textsuperscript{2+}.

We have investigated the effects of varying pH and the concentrations of substrate and Mg\textsuperscript{2+} on hepatic adenylate cyclase activity in the absence and presence of glucagon. Activities were compared with ATP and App(NH)\textsubscript{p} as substrates; the latter is not hydrolyzed by nucleotide phosphohydrolases in plasma membranes (6) and has a different $pK_a$ from that of ATP at the terminal phosphate group (7). Advantage was taken of this difference in evaluating the possible role of protonated substrate as inhibitor. In what follows it will be shown that protonated substrate is a potent inhibitor, that basal activity is more susceptible to inhibition by protonated substrate than is hormone-stimulated activity, and that free substrate mimics the activating effects of guanine nucleotides at the nucleotide regulatory site. A complex kinetic pattern develops as a function of pH and the concentrations of substrate and Mg\textsuperscript{2+}. Shown here are the appropriate conditions for obtaining $V_{max}$ and $K_a$ for the basal and hormone-induced states of the enzyme system.

### EXPERIMENTAL PROCEDURES

**Materials**—[a-\textsuperscript{32}P]ATP, [a-\textsuperscript{32}P]App(NH)\textsubscript{p}, App(NH)\textsubscript{p}, and Gpp(NH)\textsubscript{p} were obtained from International Chemical and Nuclear Corp. Cyclic 3’:5’-[\textsuperscript{8-3H}]AMP was from New England Nu-

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1 The abbreviations used are: cyclic AMP, cyclic adenosine 3’:5’-monophosphate; App(NH)\textsubscript{p}, 5’-adenylylimidodiphosphate; Gpp(NH)\textsubscript{p}, 5’-guanylylimidodiphosphate; Ap(Ch)\textsubscript{p}, Ap(Ch)\textsubscript{pp}, and Ap(Ch)\textsubscript{ppp} were from Miles Laboratories. Crystalline porcine glucagon was a gift from Eli Lilly and Co.

**Preparation of Hepatic Plasma Membranes**—Partially purified plasma membranes, prepared from rat liver by a modification of the procedure of Neville (8) as previously described (9), were stored in liquid nitrogen. The adenylate cyclase activities of the plasma membrane preparations varied; therefore, all experiments for direct comparison were performed with the same preparation on the same day. Protein concentration was determined according to Lowry et al. (10) with bovine serum albumin as standard.

**Assay for Adenylate Cyclase Activity**—Assay medium consisted of [a-\textsuperscript{32}P]ATP or [a-\textsuperscript{32}P]App(NH)\textsubscript{p} (30 to 500 cpm/pmol) at concentrations indicated in figures and tables, 5 mM MgCl\textsubscript{2} or as indicated, cyclic AMP (200% of the substrate concentration), 40 mM Tris-HCl, pH 7.6, or as stated, 3 mg/ml of bovine serum albumin, and membranes, in a final volume of 100 μl. Before addition to the incubation medium, hepatic membranes were suspended in Tris-HCl buffer containing 1 mM dithiothreitol and equilibrated for 1 min to the assay temperature (30°C). Where ATP was used as substrate, an ATP-regenerating system consisting of 30 units/ml of creatine phosphate and 50 units/ml of creatine phosphokinase was included. For the pH study, Tris-acetate buffers were used. When indicated, 1 μM glucagon was included in the assay medium.

Cyclic AMP was determined by a recently developed procedure (11). All data were obtained from linear initial rates of cyclic AMP formation determined by following the time course of the reaction. All experiments were repeated at least twice, the values reported are the average of duplicate samples which agreed within 5% in a single experiment.

### Calculations

**pH Dependence of Hepatic Adenylate Cyclase Activity**—Changes in concentrations of hydronium ion can influence several factors which may affect the activity of hepatic adenylate cyclase, including substrate ionization, the catalytic process, environment of the active site, glucagon binding and action in the case of hormone response, and enzyme structure. The fact that the $pK_a$ of the terminal phosphate differs markedly for ATP and App(NH)\textsubscript{p} (7.1 and 7.1, respectively (ii)) presented an opportunity to study the effect of substrate ionization on enzyme activity at a given pH such that all other factors are affected equally.

In solution with moderate excess of Mg\textsuperscript{2+}, substrate (S) is distributed mainly among three species: MgS\textsuperscript{5-}, HS\textsuperscript{4-}, and S\textsuperscript{3-}. Based on the stability constants for MgATP\textsuperscript{2-} and MgApp(NH)\textsubscript{p}\textsuperscript{3-} (7, 12), it was calculated that in the presence of 100 μM substrate and 5 mM Mg\textsuperscript{2+}, the concentration of MgS\textsuperscript{5-} is essentially constant (86 to 99 μM) as is the concentration of S\textsuperscript{3-} (1.0 to 1.16 μM) over a pH range of 6.0 to 9.0. By contrast, the concentration of HS\textsuperscript{4-} changes by about 1000-fold (from 13 μM to 15 nm) over the same pH range (see table I).

At a given pH, the concentration of App(NH)\textsubscript{p}Mg\textsuperscript{2+} is about twice that of ATP\textsuperscript{4-}. Thus, by using either ATP or App(NH)\textsubscript{p} as substrate, it is possible to introduce different concentrations of protonated substrate into the incubation medium under otherwise identical conditions (same enzyme preparation, pH, and concentrations of Mg\textsuperscript{2+} and substrate). This procedure is justified further by the fact that ATP and App(NH)\textsubscript{p} have the same affinity for the active site (see Table II). Accordingly, activities at the active
in the determination of the precise pH optima could account for pK, of ATP (7.1) and App(NH)p (7.7). However, inaccuracies at pH 6.5 and 7.5 with ATP and App(NH)p, respectively. The shift in pH optima is somewhat greater than the difference in optimum for ATP and App(NH)p; 50% of maximum is given at a pH giving 50% that observed at the respective pH is more obvious when activities with the two substrates are compared at a pH giving 50% that observed at the respective pH optimum for ATP and App(NH)p; 50% of maximum is given at pH 6.5 and 7.5 with ATP and App(NH)p, respectively. The shift in pH optima is somewhat greater than the difference in PKa of ATP (7.1) and App(NH)p (7.7). However, inaccuracies in the determination of the precise pH optima could account for the difference.

It will be noted in Fig. 1 that activities given with App(NH)p at its optimal pH were about 50% given with ATP as substrate (see also Table II). If it is assumed that the catalytic process is pH-dependent and that the pH optimum is around pH 7.5, then the shift to a higher pH optimum with App(NH)p due to the higher concentration of protonated substrate could result in lower apparent catalytic efficiency over the entire pH range. Another possible explanation for the different apparent efficiencies in catalysis is that PPi and P(NH)Pi, formed by catalysis of ATP and App(NH)p, respectively, have different binding constants at the catalytic site; a slower rate of dissociation of P(NH)Pi from this site could result in apparent differences in catalytic efficiency. However, this would not explain the differing pH profiles given with the substrates.

The data in Fig. 1 suggest that binding of protonated substrate at the catalytic site is an important controlling factor in the activity of the hepatic adenylate cyclase system. In addition to pH, the concentration of protonated substrate is determined by the relative concentrations of magnesium ion and substrate present in the incubation medium.

Effects of Mg2+—It has been postulated that magnesium ion serves as a regulatory ligand at an allosteric site (10) in addition to its role in forming the productive magnesium-substrate complex. This possibility was tested by increasing the concentration of Mg2+ in the incubation medium and examining the effects of the cation on hepatic adenylate cyclase activity as a function of increasing substrate concentration. It was reasoned that stimulatory effects of Mg2+ at a putative regulatory site should be observed independently of the concentration of substrate. However, as shown in Table I, the stimulatory effects of magnesium ion seen for basal activity were dependent markedly on substrate concentration. Thus, increasing the concentration of Mg2+ 10-fold in the presence of 10 µM App(NH)p as substrate resulted only in a 40% increase in basal activity whereas activities were increased 3- and 4-fold when the concentration of substrate was increased to 0.1 and 1.0 mM, respectively. Therefore, an allosteric action of Mg2+ is unlikely. Since the stimulatory effects of the cation are seen at concentrations far in excess of that required to form the productive Mg2+-substrate complex, it is likely that stimulation of basal activity is due to increased chelation of uncomplexed, inhibitory forms of substrate, as suggested by deHaen for other adenylate cyclase systems (5). As shown above, the inhibitory species is protonated substrate, the concentration of which is reduced by increasing [Mg2+] from 5 to 50 mM.

The effects of [Mg2+] on glucagon-stimulated activity were more complex (Table I). For example, at 5 mM Mg2+, increasing the concentration of substrate from 10 to 1000 µM increased the foldness of hormone-stimulated activity over that of basal activity; foldness was reduced by increasing the concentration of Mg2+ to 50 mM which, furthermore, resulted in inhibition of hormone-stimulated activity with 10 µM substrate. Inhibition
cannot be explained obviously by removal of the inhibitory protonated substrate. Selective inhibition of glucagon-stimulated activity by 50 mM Mg\(^{2+}\) is unlikely since increasing the substrate concentration 100-fold to 1.0 mM, which does not significantly alter the concentration of free Mg\(^{2+}\) at 50 mM, resulted in a 7-fold increase in glucagon-stimulated activity. It is likely, therefore, that glucagon-stimulated activity is dependent on the concentration of free substrate and that the inhibitory effects of Mg\(^{2+}\) are due to titration of free ATP. In this regard, the preceding study (1) showed that the free form of guanine nucleotides is required for binding to the nucleotide regulatory site; activation of the hepatic system by guanine nucleotides is facilitated by glucagon.

From the above findings, the effects of Mg\(^{2+}\) on adenylate cyclase activity can be attributed to chelation of substrate to form the productive Mg\(^{2+}\)-substrate complex, and the titration of the inhibitory protonated substrate and the activating free form of substrate. Titration of the protonated substrate by Mg\(^{2+}\) appears to have a greater effect on basal activity than on the hormone-stimulated state as evidenced by the marked enhancement of basal activity with increasing concentrations of Mg\(^{2+}\) in the presence of 1.0 mM ATP; this is not seen with glucagon (Table I).

**Kinetic Constants with ATP and App(NH)p**—The activating and inhibitory effects of protonated and free forms of substrate obviously may complicate evaluation of the kinetic constants for Mg\(^{2+}\)-substrate complex at the catalytic site. Fig. 2 shows double reciprocal plots of kinetic data obtained when the hepatic enzyme system was incubated with ATP as substrate in medium containing 5 mM Mg\(^{2+}\), ATP-regenerating system, at pH 7.5. The curves obtained were nonlinear, particularly that for basal activity. These curves were obviously too complex for determining \(K_m\) and \(V_{max}\) for the basal and hormone-stimulated states of the enzyme.

Kinetic studies were conducted subsequently with the enzyme incubated in the presence of 50 mM Mg\(^{2+}\) at pH 8.5 (Fig. 3B).

![Fig. 2. Double reciprocal plots for hepatic adenylate cyclase activity with ATP as substrate. The data were plotted from initial velocities (\(v = \) nanomoles of cyclic AMP per min per mg of protein) obtained at various ATP concentrations in the presence of 5 mM Mg\(^{2+}\) at pH 7.5. Activities were measured in the absence (A) and in the presence of glucagon (1 µM) (A).](image)

![Fig. 3. Double reciprocal plots for hepatic adenylate cyclase with App(NH)p as substrate. Assays were carried out with 5 mM Mg\(^{2+}\) (A) or 50 mM Mg\(^{2+}\) (B) at pH 8.5. Velocities (\(v = \) nanomoles of cyclic AMP per min per mg of protein) were measured in the absence (A) and in the presence of glucagon (1 µM) and Gpp(NH)p (0.1 mM): O.](image)
At pH 8.5, the concentration of protonated substrate is 10-fold less than that at pH 7.5 (Fig. 1); the concentrations of free and protonated substrate are decreased 10-fold by increasing the Mg$^{2+}$ from 5 to 50 mM. We used App(NH)$_3$p rather than ATP as substrate in these experiments since the presence of the ATP-regenerating system could be avoided. Creatine phosphate in the regenerating system, although forming weak complexes with Mg$^{2+}$ (14), nevertheless can alter the concentration of Mg$^{2+}$ and, therefore, the concentrations of unchelated species of substrate. Indeed, creatine phosphate inhibits adenylate cyclase activity (15), possibly because of resultant increases in concentration of protonated substrate. Since increasing the [Mg$^{2+}$] also reduces the concentration of free activating nucleotide, evaluation of the kinetic parameters for the activated state of the enzyme was carried out in the presence of saturating concentrations of glucagon (1 nM) and 0.1 mM Gpp(NH)$_3$ as activator, conditions which cause maximal activation of the hepatic adenylate cyclase system (1). Linear double reciprocal plots were obtained for both basal activity and the activated state (Fig. 3B). Fig. 3A shows for comparison the same membrane preparation incubated under identical conditions except that the [Mg$^{2+}$] was reduced to 5 mM as a means of increasing the concentration of protonated and free forms of the substrate. The basal curve was nonlinear which possibly reflects the stimulating effects of free substrate at the nucleotide regulatory site. Addition of both glucagon and Gpp(NH)$_3$ abolished this deviation from linearity. Extrapolation of the linear curves (Fig. 3B) to the abscissa revealed that the $K_m$ for the productive Mg$^{2+}$-substrate complex was the same (50 μM) for both the basal and activated state; $V_{max}$ was increased 4-fold by glucagon and Gpp(NH)$_3$. Thus, the activating ligands engender a state having a lower affinity for protonated substrate, an increase in $V_{max}$ without changing the apparent affinity for the productive form of the substrate.

Effects of Phosphonate Analogues of ATP—Ap(CH$_2$)$_3$pp cannot be converted to cyclic AMP by adenylate cyclase and inhibits competitively interactions of either ATP (A) or App(NH)$_3$ (B) with the enzyme as shown in Fig. 4. The fat cell adenylate cyclase system is also competitively inhibited by Ap(CH$_2$)$_3$pp (16). In the presence of 50 mM Mg$^{2+}$, linear double reciprocal plots were obtained with either ATP or App(NH)$_3$ at their pH optimum (pH 7.5 and 8.5, respectively). The kinetic constants are summarized in Table II for the productive substrates and the inhibitory analogues. As discussed previously, the $V_{max}$ was about 40% lower with App(NH)$_3$ compared with ATP as substrate; both forms of substrate gave essentially the same $K_m$. The methylene analogue of ATP, Ap(CH$_2$)$_3$pp, gave a $K_i$ nearly equivalent to the $K_m$ for the two substrates. Surprisingly, the analogue of adenosine 5'-tetraphosphate, Ap(CH$_3$)$_3$pppp, was the strongest inhibitor; it appears that the active site can accommodate an extra phosphate without reduction in affinity. The inhibition constants were calculated on the assumption that the analogues have the same stability constants with Mg$^{2+}$ as those of the productive substrates (ATP and App(NH)$_3$) and that the $K_i$ reflect the binding of the magnesium complexes rather than the unchelated forms of the nucleotide. Inhibition by the analogues is not likely due to their contribution as protonated sub-

**Figure 4.** Determination of inhibition constants for Ap(CH$_2$)$_3$pp and Ap(CH$_3$)$_3$pppp. Assays were carried out at pH 7.5 with ATP as substrate (A) or at pH 8.5 with App(NH)$_3$ as substrate (B) in the presence of 50 mM MgCl$_2$. Velocities ($v$ = nanomoles of cyclic AMP per min per mg of protein) were measured in the absence ( ) and in the presence of either Ap(CH$_2$)$_3$pp (180 μM) ( ) or Ap(CH$_3$)$_3$pppp (50 μM) ( ).
strate, which the present study shows to be about 50 PM. In an sence of hormones (1,3).

This inhibitory effect of Mg2+ on glucagon-stimulated activity has been reported previously for the hepatic adenylate cyclase system (9). In the presence of 5 mm Mg2+, 2 mm ATP was required for maximal hormone-stimulated activity; this concentration is at least 3 orders of magnitude higher than that required with GTP or Gpp(NH)p (1 PM) which also activate the enzyme in their free form (1). We have suggested (2) that commercial preparations of ATP or Gpp(NH)p may contain as much as 0.1% contaminating guanine nucleotides which would account for the actions of free substrate in the presence of the hormone.

As shown here, the apparent affinity of the active site for Mg2+-substrate complex is about 50 PM in the absence or presence of glucagon; this value is obtained when appropriate incubation conditions (high pH and [Mg2+] are used for assaying glucagon-stimulated activity. With substrate as the only source of activating free nucleotide, and when lower pH and [Mg2+] are used in the assay medium, nonlinear Michaelis-Menten kinetics were observed as substrate concentration was increased; this is due to the relatively high concentration of unchelated substrate introduced into the incubation medium. Since basal and hormone-stimulated activities are preferentially affected by protonated and free substrate, respectively, it was not feasible to determine the kinetic parameters (Km and Vmax) for the two states of the enzyme system. In this regard, it is of interest that deHaen (5) calculated that the binding constants for Mg2+-substrate complex at the adrenocorticotropic hormone-induced and basal states of the fat cell adenylate cyclase system were about 1.0 mm and 0.17 mm, respectively; a similar reduction in substrate affinity was calculated for the epinephrine-stimulated state of the ventricular enzyme system. Our studies would suggest that these calculated values are erroneous and that the apparent lowering of the affinity of substrate for the hormone-induced states actually reflects the high concentrations of activating free substrate required for hormone-stimulated activities in the absence of guanine nucleotides. As reported elsewhere (17), the nucleotide regulatory site is an ubiquitous feature of adenylate cyclase systems in eucaryotic cells irrespective of the hormone-receptor component; this site is fundamental to the process of activation of adenylate cyclase in the presence or absence of hormones (1,3).

In summary, this study shows that activating nucleotides and glucagon engender a presumed conformational change at the active site which manifests itself in a decrease in affinity for protonated substrate and a 4-fold increase in Vmax. Quantitative analysis of the kinetic parameters at the active site and evidence for a three state model are provided in an accompanying report (3). It is perhaps significant with regard to the increase in Vmax that the apparent Km for Mg2+-substrate complex is close to the Km for the nonproductive phosphate analogue, Ap(CH2)pp; this finding suggests that the Km is equivalent to Keq. If this should prove to be the case from direct binding studies, then activation of adenylate cyclase involves an increase in kcat, the rate constant for product formation.

\[ \text{Table II} \]

Kinetic constants for basal adenylate cyclase activity

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Vmax</th>
<th>Km</th>
<th>( k_1 )</th>
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<tbody>
<tr>
<td>ATP</td>
<td>63 ± 16</td>
<td>55 ± 15</td>
<td>200 ± 0</td>
</tr>
<tr>
<td>App(NH)p</td>
<td>40 ± 9</td>
<td>50 ± 10</td>
<td>400 ± 130</td>
</tr>
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</table>

* Picomoles of cyclic AMP formed per mg of protein per min.

Just as Gpp(NH)p has proven to be a useful analogue of GTP for investigating the role of the nucleotide regulatory site in the activation of adenylate cyclase (1,17), App(NH)p has proven to be valuable for investigating the characteristics of the catalytic site. In addition to its resistance to hydrolysis by potent nucleotide phosphorylases in plasma membranes (6), which allows assays to be conducted in the absence of ATP-regenerating systems, App(NH)p, with its different pHK from that of ATP, provided a means of showing that the protonated form of substrate is a potent inhibitor of the hepatic adenylate cyclase. The same conclusion has been drawn from similar studies of the fat cell adenylate cyclase system. Since the concentration of protonated substrate is very low when the \([\text{Mg}^{2+}]\) is in excess of substrate, it was not feasible to determine the kinetic parameters for the two states of the enzyme system. In this regard, it is of interest that deHaen (5) calculated that the binding constants for Mg2+-substrate complex at the adrenocorticotropic hormone-induced and basal states of the fat cell adenylate cyclase system were about 1.0 mm and 0.17 mm, respectively; a similar reduction in substrate affinity was calculated for the epinephrine-stimulated state of the ventricular enzyme system. Our studies would suggest that these calculated values are erroneous and that the apparent lowering of the affinity of substrate for the hormone-induced states actually reflects the high concentrations of activating free substrate required for hormone-stimulated activities in the absence of guanine nucleotides. As reported elsewhere (17), the nucleotide regulatory site is an ubiquitous feature of adenylate cyclase systems in eucaryotic cells irrespective of the hormone-receptor component; this site is fundamental to the process of activation of adenylate cyclase in the presence or absence of hormones (1,3).

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