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Biochemistry

Aluminum: A requirement for activation of the regulatory component of adenylate cyclase by fluoride

(cyclic AMP/beryllium)

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ABSTRACT **Activation of the purified guanine nucleotide-binding regulatory component (G/F) of adenylate cyclase by F^- requires the presence of Mg^{2+} and another factor. This factor, which contaminates commercial preparations of various nucleotides and disposable glass test tubes, has been identified as Al^{3+}. In the presence of 10 mM Mg^{2+} and 5 mM F^-, $AlCl_3$ causes activation of G/F with an apparent activation constant of approximately 1–5 μM. The requirement for Al^{3+} is highly specific; of 28 other metals tested, only Be^{2+} promoted activation of G/F by F^-.**

The discovery of cyclic AMP (1) was rapidly followed by the first description of adenylate cyclase (2). Although F^- was identified as an activator of adenylate cyclase during the early stages of study of the enzyme (2), the mechanism of this unique stimulation of enzymatic activity has remained a mystery for the past 24 years.

The observation that adenylate cyclase was composed of at least two separable components (3, 4) allowed localization of the site of activation by F^-. Reconstitution experiments revealed that F^- acts on the guanine nucleotide-binding regulatory component (G/F) of the enzyme (5). These studies also implicated both Mg^{2+} and a nucleotide as requirements for activation of solubilized G/F by F^-.

G/F has recently been purified (6, 7) and shown to be activated by F^- in the presence of Mg^{2+} and ATP (7). Further study of this activation has revealed that a contaminant of ATP is responsible for the apparent requirement for nucleotide. These experiments were initially hampered by the fact that disposable glass test tubes also contain significant amounts of an activating factor. Contamination of both commercial preparations of ATP and disposable glass test tubes with Al^{3+} explains these observations.

METHODS AND MATERIALS

Membrane and G/F Preparations. The G/F-deficient variant of the S49 lymphoma cell (cyc$^-$) was used in this study. cyc$^-$ S49 cells were harvested (5) and plasma membranes were prepared (8) as described. G/F was purified from rabbit liver by the method of Sternweis *et al.* (7).

Activation and Assay of G/F. The activation of G/F by F^- is accomplished by incubation in a solution containing 50 mM sodium Hepes (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 0.25% Lubrol 12A9, and various effectors, as indicated. All solutions were made with glass-distilled water and stored in plastic containers. Activations were performed in plastic tubes and were normally initiated by the addition of purified G/F (final concentration about 0.4 μg/ml). After the desired time of incubation, 10 μl of the mixture was added to 90 μl of an assay mixture containing cyc$^-$ membranes (0.5–0.7 mg/ml), which serve as a source of the catalytic component of adenylate cyclase, and the following reagents: 50 mM sodium Hepes (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$, 0.5 mM ATP, [α-^{32}P]ATP (≈10^6 cpm), pyruvate kinase at 10 μg/ml, 3 mM phospho*enol*pyruvate, bovine serum albumin at 0.1 mg/ml, and 0.1 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (concentrations are those in the 100-μl assay). Samples were mixed and assayed at 30°C for 25 min. Cyclic AMP production was determined by the method of Salomon *et al.* (9). Reconstitution of G/F with the catalyst in cyc$^-$ membranes occurs within the first minute of this incubation and the rate of production of cyclic AMP is then linear throughout the course of the assay. The presence of nonactivated G/F can be detected by including 10 mM NaF in the assay mixture.

G/F activity is expressed as quantity of cyclic AMP formed·min^{-1}·mg of G/F^{-1}. Rates of activation of G/F define the time-dependent increase in G/F activity and are thus expressed as quantity of cyclic AMP·mg of G/F^{-1}·min^{-2}.

Factor Preparations. Three sources of factor(s) necessary for activation of G/F by F^- were encountered. Tube factor (TF) was prepared by rinsing the interior surface of glass test tubes (Pyrex borosilicate culture tubes, 13 mm × 100 mm; Corning no. 99445) with either glass-distilled water or 100 mM HCl (acid improves the extraction). The rinses were concentrated by lyophilization and then dissolved in glass-distilled water for use. Factor activity could be observed directly in solutions of ATP (nucleotide factor; NF) and in tap water.

Samples of TF and NF were partially purified and prepared for neutron activation analysis by cation exchange chromatography. ATP (18 g) was dissolved in 2 liters of water and applied to a 100-ml column (2 × 35 cm) of AG 50-X4 (Bio-Rad) in the H^+ form. The column was washed with water (100 ml) and was then eluted in succession with the same volume of 1, 2, 3, 4, and 6 M HCl. Fractions of 12 ml were collected, starting with the water wash. Fractions that contained factor activity (peak about 3 M HCl) were collected and concentrated by lyophilization. Solid residue was dissolved in 0.5 ml of H_2O and transferred to 1-ml polyethylene vials for neutron activation analysis. TF was prepared in a similar fashion. About 1,250 test tubes were extracted with 1 liter of 100 mM HCl. The extract was lyophilized, dissolved in H_2O, and applied to the cation exchange resin. TF bound to the resin and was eluted in a peak with about 3 M HCl; this material was concentrated for analysis. A blank preparation was prepared by eluting the column without application of factor. Fractions in the region where factor

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Abbreviations: G/F, the guanine nucleotide-binding regulatory component of adenylate cyclase; cyc$^-$, the G/F-deficient variant of the S49 lymphoma cell; TF and NF, the factor in preparations derived from test tubes and ATP, respectively, that activates G/F in the presence of F^-.

was ordinarily observed were prepared for analysis. The two different preparations of factor (NF and TF) contained similar amounts of both mass of material (2–3 mg) and activity, whereas the blank sample had essentially none.

Other Methods and Materials. Protein was determined by staining with amido black as described by Schaffner and Weissman (10); bovine serum albumin was the standard. [α-^{32}P]ATP was synthesized by the method of Johnson and Walseth (11).

ATP used as the source of material for neutron activation analysis was obtained from Sigma (product no. A 6144, lot no. 1296-7320). Other lots and grades displayed similar factor activity, as did some preparations of other nucleotides. All inorganic metal compounds used were of at least 99.8% purity or higher and were obtained from Aldrich, Alfa Division of Ventron, Baker, or Fisher. Lubrol 12A9 was obtained from Imperial Chemical Industries (Macclesfield, England).

RESULTS

Requirement for a Factor for Activation of G/F by F^-. Original observations with detergent extracts of plasma membranes from S49 cells suggested that activation of G/F by F^- required or was markedly stimulated by nucleotide (5, 7). At this time it was admitted that the nucleotide specificity was unclear (and variable). When these experiments were repeated with purified G/F from rabbit liver, the requirement for nucleotide was sporadic and the specificity was ill-defined. Further study of the activation thus required stabilization of the requirement for nucleotide. This was eventually achieved by observing two restrictions: (*i*) the use of glass-distilled water to make up all reagents used for activation and (*ii*) manipulation of all solutions and activation reactions in plastic tubes or acid-washed (and extensively rinsed) glass tubes. Under these conditions Mg^{2+} and F^- support only minimal activation of G/F (Fig. 1). If ATP was then added to the incubations, a clear, time-dependent activation of G/F was observed. The low activities

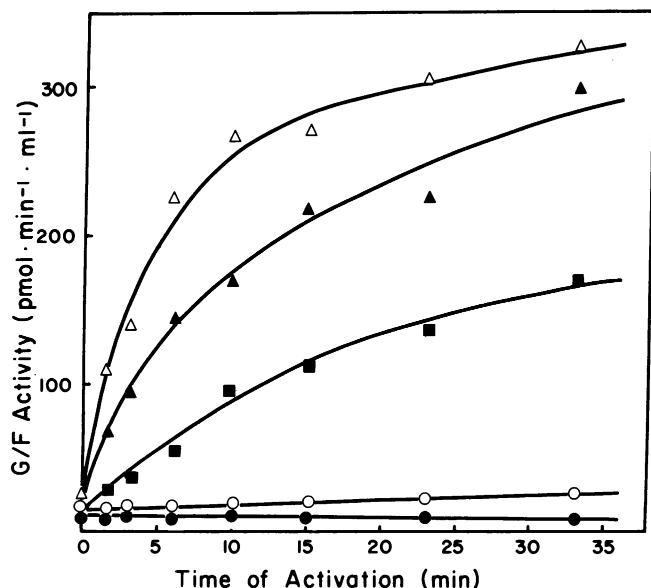

FIG. 1. Requirement for factor(s) for activation of G/F by F^-. Activation of G/F was performed as described. G/F was added at zero time to activation solution (●) or to activation solution that contained 10 mM $MgCl_2$ and 10 mM NaF (○, ■, ▲, △). Indicated reaction mixtures also contained 2 mM ATP (■), a dilute TF preparation, 10% by volume (△), or tap water, 74% by volume (▲). Incubation was at 20°C. The G/F concentration during activation was 0.4 μg/ml. If F^- was included during the assay, activities of G/F were about 150 pmol·min^{-1}·ml^{-1}.

Table 1. Requirements for activation of G/F by F^-

Additions	Initial activation rate, nmol·mg^{-1}·min^{-2}		
	No factor	TF	1 mM ATP
None	<2	<2	<2
10 mM $MgCl_2$	<2	<2	<2
10 mM NaF	<2	<2	<2
10 mM $MgCl_2$ + 10 mM NaF	<2	60	25

G/F was activated as described. $MgCl_2$, NaF, and ATP were added to achieve the indicated concentrations during activation. TF was present during activation as 10% by volume of a dilute TF preparation. Initial rates were determined from the value observed at 3 min of activation.

at zero time indicate that dilution of activating ligands into the assay mixture was sufficient to prevent activation of G/F.

Although a requirement for ATP for activation of G/F was now apparent, other materials could substitute for the nucleotide. Both an extract of glass test tubes or the use of tap water, rather than glass-distilled water, promoted activation of G/F by F^- (Fig. 1).* It thus appeared that an unknown cofactor might be required. (The factors that are used below are referred to simply by their source—i.e., TF from test tubes or NF from nucleotide.) The requirements for activation of G/F by F^- are shown in Table 1. These clearly include three components when activation reactions are conducted as described: Mg^{2+}, F^-, and a third factor. If any of these is missing, activation of G/F is negligible.

Stability of the Factors. Both TF and NF could be concentrated by lyophilization. Unfortunately, the factor activity in tap water was not stable to either boiling or freeze-drying. Because this activity could not be concentrated by these innocuous procedures, we have worked exclusively with TF and NF. Both of these factors were stable to heating at 100°C in 0.5 M HCl, 0.5 M NaOH, or 0.5 M $HClO_4$. Furthermore, if ATP or lyophilized TF was subjected to mild ashing (500°C for 5 hr), factor activity was maintained.

Separation of Factor Activity from ATP. Fig. 2 shows the separation of factor activity from disodium ATP by passage through the cation exchange resin AG 50-X4. While the nucleotide passes through the resin, factor is bound. (The ATP treated in this fashion provides a source of factor-free ATP.) The factor responsible for activation of G/F by F^- can then be eluted with about 3 M HCl. For the purpose of neutron activation analysis, the stepwise elution with HCl also separates Na^+ from the factor. Three comments should be made about this method. (*i*) Application of higher concentrations of ATP (100 mM) does not result in complete removal of factor from the nucleotide. (*ii*) Factor is not removed by the Na^+ form of the resin. (*iii*) The factor is not further removed from ATP when the equivalency of the column is exceeded with Na^+.

TF also binds to AG 50-X4 and is eluted by the same procedure.

Analysis of Factor Preparations. Factor purified by this procedure either from 18 g of ATP or from 1,250 test tubes was subjected to elemental analysis by neutron activation. Elements present above the limits of detection are shown in Table 2. The blank preparation represents material eluted from resin to which no factor had been applied; fractions corresponding to

* We should point out that this experiment was performed with tap water obtained in Charlottesville, VA, and that deionized water derived therefrom was not devoid of activity. Tap water at The University of Texas Health Science Center in Dallas has shown very little activity. No effort has been made to define this variation further.

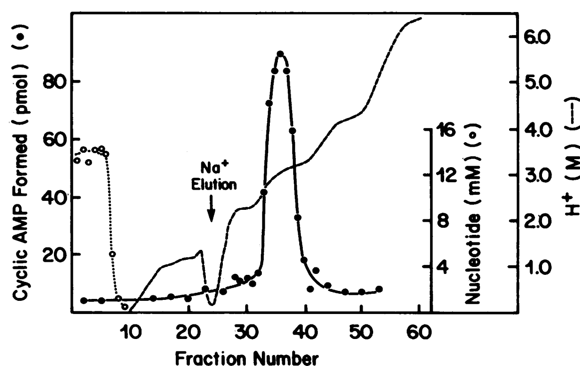

FIG. 2. Separation of factor activity from ATP. ATP (2 Na^+ per mole) was applied to a column of AG 50-X4 and eluted as described. Nucleotide was measured by absorbance at 260 nm; H^+ concentration was measured by titration with NaOH in the presence of bromphenol blue. Factor activity was measured in a portion of each fraction after lyophilization to remove HCl (4 μl of the original fraction was assayed in 100 μl of activation solution containing 10 mM $MgCl_2$ and 10 mM NaF). Activation of G/F was for 3 min at 30°C.

the elution profile of factor activity were analyzed. The major elements in the factor preparations are aluminum and chlorine; Cl^- was expected as the counter ion after elution of the cation exchange column with HCl. Because the two preparations of factor had similar amounts of activity and the blank sample had essentially none, aluminum became the primary candidate for both TF and NF.

Activation of G/F by Al^{3+}. When $AlCl_3$ was added to G/F in the presence of Mg^{2+} and F^-, G/F was activated in the same manner as shown above for crude preparations of factor. The concentration of Al^{3+} required for this activation is shown in Fig. 3. The initial rate of activation and the extent of activation of G/F after 50 min of incubation are shown. Under these conditions, the apparent K_{act} for Al^{3+} is about 5 μM when the initial rate of activation is examined or about 1 μM when the extent of activation is determined. The activation clearly is not a simple process, and these values should be taken only as an estimate of the potency of Al^{3+} in this reaction.

Table 2. Neutron activation analysis of purified factor preparations

Elements	Detection, μg Blank preparation	TF preparation	NF preparation
Al	ND	410	500
Br	0.28	22	0.26
Cl	ND	1,940	2,160
Cr	ND	1.2	0.55
K	7.6	ND	ND
Mn	ND	ND	0.51
Mo	0.63	ND	0.7
Na	11	16	42
Sb	ND	ND	0.01
Zn	1.9	ND	ND

Samples were prepared as described. Neutron activation analysis ("multi-element survey analysis") was performed by General Activation Analysis (San Diego, CA). Samples were irradiated for 1 min and 30 min in a TRIGA Mark I nuclear reactor at fluxes of 2.5×10^{12} neutrons per cm^2·sec and 1.8×10^{12} neurons per cm^2·sec, respectively. They were then subjected to γ-ray spectroscopy at various times and the data were subjected to computer analysis for determination of elemental composition. Further details may be obtained from the authors. ND, not detected.

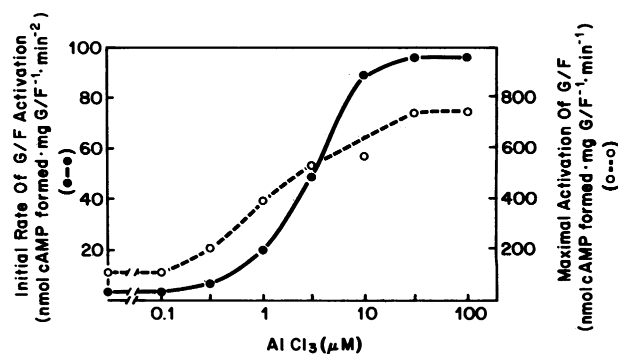

FIG. 3. Activation of G/F by Al^{3+} and F^-. Activation of G/F was performed at 20°C as described. Activation solutions contained 10 mM $MgCl_2$, 5 mM NaF, and the indicated concentrations of $AlCl_3$. Initial rates of activation were determined from early time points during activation (●); maximal activation represents the level of activity observed after 50 min of incubation (○). Slow increases in activity were still seen after 50 min when samples contained low concentrations of $AlCl_3$; maximal levels of activity for these samples are therefore somewhat greater. Initial rates of activation at high concentrations of $AlCl_3$ are based on one or two early time points and may, therefore, be low estimates of the initial rate.

Specificity of the Activation. Several other metallic ions have been examined for their ability to substitute for Al^{3+} in the activation reaction described. These include $BeCl_2$, $CaCl_2$, $ScCl_3$, $CrCl_3$, $MnCl_2$, $FeSO_4$, $FeCl_3$, $Co(NO_3)_2$, $CuSO_4$, $GaCl_3$, YCl_3, $RuCl_3$, $In(NO_3)_3$, $SnCl_2$, $BaCl_2$, $LaCl_3$, $PrCl_3$, $NdCl_3$, $SmCl_3$, $EuCl_3$, $GdCl_3$, $TbCl_3$, $HoCl_3$, $LuCl_3$, $HgCl_2$, $Tl(NO_3)_3$, $Pb(OAc)_2$, and $Bi(NO_3)_3$. Each compound was tested at 1 and 100 μM; at the higher concentration, some compounds caused partial inactivation of G/F. Solutions of the metals tested were made within 3 hr of their use; no precautions were taken to prevent formation of undesired liganded forms of the metal or oxidoreduction reactions. Of the compounds listed, activation of G/F was seen only in the presence of $BeCl_2$ (Table 3). Be^{2+} has a potency similar to that of Al^{3+} at low concentrations of the metal, but the maximal rate of activation of G/F that can be achieved at higher concentrations of Be^{2+} is less than that observed with Al^{3+}. The extent of activation by Be^{2+} also appears to be less than that by Al^{3+}, but further experimentation is required to define these parameters.

Table 3 shows that three different aluminum-containing compounds are equally effective as activators of G/F. Examples of negative results obtained with two other metals are also shown.

Table 3. Metal specificity for activation of G/F by F^-

Compound added	Initial activation rate, nmol·mg^{-1}·min^{-2} — Metal during activation, μM: 1	10	100
$AlCl_3$	25	110	220
$Al_2(SO_4)_3$	20	81	200
$AlCl_3$·$6H_2O$	21	95	—
$BeCl_2$	23	73	120
$ScCl_3$	4	—	4
$LaCl_3$	4	—	4

G/F was activated at 20°C as described. $MgCl_2$ and NaF were present during activation at concentrations of 10 mM and 5 mM, respectively. Initial rates were determined from time courses of activation, except at very high rates where only the first time point (2 min) was used to estimate this rate. The rate of activation in the absence of additional metal was 3 nmol·mg^{-1}·min^{-2}.

DISCUSSION

Preparations of ATP have had an infamous history as sources of compounds other than the indicated nucleotide. This is not the first demonstration that commercial preparations of ATP contain significant amounts of Al^{3+}. Womack and Colowick (12) reported that the inhibition of yeast hexokinase by certain lots of ATP was due to contaminating Al^{3+}. They proposed that AlATP was a potent inhibitor of the enzyme. Tornheim *et al.* (13) performed a more extensive examination of several preparations of nucleotides. They report concentrations of Al^{3+} ranging from 0.21 to 3.4 mmol per mol of ATP. Significant amounts of Al^{3+} were also found in ADP and GTP but not in AMP. This is consistent with our observations of factor activity in preparations of GTP and ADP but not in AMP (data not shown). Our earlier report (5), showing variable efficacies for ATP and ADP as stimulators of activation of G/F by F^-, presumably reflected different concentrations of Al^{3+} in the preparations utilized. Al^{3+}-free ATP does not promote activation of G/F (Fig. 2). In the presence of Al^{3+}, there is no requirement for ATP for the activation of G/F by F^- (Fig. 3).

Viola *et al.* (14) used the inhibition of hexokinase as a means to determine a dissociation constant for AlATP of 0.7 μM at neutral pH. The separation of Al^{3+} and ATP observed on AG 50-X4 presumably reflects acidification of the ATP, release of Al^{3+}, and subsequent binding of the free cation. Higher concentrations of ATP may prevent total removal of Al^{3+} because of equilibrium considerations. As reported here, the exchange resin could prove quite useful for the preparation of large amounts of Al^{3+}-free (and potentially other metal-free) ATP.

The specificity of the requirement for Al^{3+} is remarkable. Of a wide range of metals tested, only Be^{2+} is effective. The specificity of the reaction may result from the interesting chemistry of F^- and the two metals; the bonds between the two elements in AlF_3 and BeF_2 have similar degrees of partially covalent character (15).

The requirement for two effectors invites a question of mechanism. Does Al^{3+} act on G/F at a site separate from F^- or is the activation achieved by a complex of aluminum and fluoride? At this point, the answer is unknown. However, it is probable that F^-, at the concentrations required for activation, will complex with Al^{3+}. Data compiled by Goldstein (16) suggest that Al^{3+} in the presence of 5 mM F^- will exist primarily as AlF_4^-; similarly, Be^{2+} exists primarily as BeF_3^-. The suggestion that a metal liganded with multiple atoms of F^- is the activating species is also attractive in view of the highly cooperative nature of the F^- concentration dependence seen with membrane-bound adenylate cyclase or with G/F (5). Examination of this possibility will require further extensive experimentation.

Postulation of an aluminum–fluoride complex from published data on equilibria suffers from lack of consideration of the other compounds present during activation. Two of these that have to be considered are Mg^{2+} and EDTA. Initial experiments indicate that Mg^{2+} plays a crucial role in preventing chelation of Al^{3+} by EDTA. When activation of G/F is examined in the absence of chelator, the requirement for Mg^{2+} is markedly reduced and may not be absolute (data not shown). Further experimentation with metal-free solutions should clarify this point.

F^- has been used for years as a convenient activator of membrane-bound adenylate cyclase. Initial experiments using Al^{3+}-free ATP reveal a significant effect of Al^{3+} on the activation of the membrane-bound enzyme by F^- (data not shown). However, the effect is most evident as a stimulation of the rate of activation and a reduction in the concentration of F^- required. Activation of membrane-bound adenylate cyclase has not yet been shown to be totally dependent on Al^{3+}. Nor have we assessed the level of contamination of membranes by the metal.

The effect of F^- on adenylate cyclase has provided a nonphysiological, albeit useful, means of activating the enzyme. However, the apparent K_{act} of Al^{3+} is well within the range of usual concentrations of the metal in blood and soft tissues (5–10 μM) (17). Although Al^{3+} does not appear to activate G/F in the absence of F^-, the presence of such an activating site on the protein raises the question of what effect Al^{3+} might have on adenylate cyclase *in vivo*. Is there a physiological cofactor that can promote effects of Al^{3+} or is there a physiological equivalent to an aluminum–fluoride complex? Answers to these questions may provide new insights into mechanisms of regulation of adenylate cyclase.

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