A Direct Pyrophosphatase-coupled Assay Provides New Insights into the Activation of the Secreted Adenylate Cyclase from Bordetella pertussis by Calmodulin*

Continuous recording of the activity of recombinant adenylate cyclase (CyaA) of Bordetella pertussis (EC 4.6.1.1) by conductimetric determination of enzyme-coupled pyrophosphate cleavage has enabled us to define a number of novel features of the activation of this enzyme by calmodulin and establish conditions under which valid activation data can be obtained. Activation either in the presence or absence of calcium is characterized by a concentration-dependent lag phase. The rate of formation and breakdown of the activated complex can be determined from an analysis of the lag phase kinetics and is in good agreement with thermodynamic data obtained by measuring the dependence of activation on calmodulin concentration, which show that calcium increases $k_{on}$ by about 30-fold. The rate of breakdown of the activated complex, formed either in the presence or absence of calcium, has been determined by dilution experiments and has been shown to be independent of the presence of calcium. The coupled assay is established as a rapid, convenient and safe method which should be readily applicable to the continuous assays of most other enzymes that catalyze reactions in which valid activation data can be obtained. Activation either in the presence or absence of calcium is characterized by a concentration-dependent lag phase. The rate of formation and breakdown of the activated complex can be determined from an analysis of the lag phase kinetics and is in good agreement with thermodynamic data obtained by measuring the dependence of activation on calmodulin concentration, which show that calcium increases $k_{on}$ by about 30-fold. The rate of breakdown of the activated complex, formed either in the presence or absence of calcium, has been determined by dilution experiments and has been shown to be independent of the presence of calcium. The coupled assay is established as a rapid, convenient and safe method which should be readily applicable to the continuous assays of most other enzymes that catalyze reactions in which inorganic pyrophosphate is liberated.

The secreted adenylate cyclase (CyaA) of Bordetella pertussis is an important virulence factor and has a number of interesting structural and kinetic properties (1, 2). The N-terminal domain, containing the catalytic and calmodulin-binding domains, is joined to a large RTX (repeats in toxin) domain for membrane translocation (3, 4). Immunological evidence indicates that the catalytic domain may be of eukaryotic origin (5), but the calmodulin binding domain is bipartite and does not resemble classical calmodulin target sites very closely (6). CyaA, and the related edema factor from Bacillus anthracis, are among the most active adenylate cyclases yet characterized and show the largest known responses to calmodulin (4, 7, 8). CyaA is therefore ideal for evaluating new strategies for assay of adenylate cyclase and calmodulin and calmodulin antagonists (9). The fact that the catalytic reaction generates pyrophosphate as a second product has attracted little attention, but offers the possibility of assay by coupled detection of pyrophosphate cleavage; this could, in principle, enable continuous assays to be devised for many other important enzymes.

Radiochemical assays for nucleotide cyclases (10, 11) tend to be used as single time point determinations, and due to logistics of isotope use, especially for $^32$P, there is a tendency to run assays in large batches on collected samples. The aim here was to set up a simple rapid continuous recording method based on conductimetric determination of pyrophosphate cleavage (12) to be readily available for quality control and routine monitoring and which might be useful for assaying calmodulin and its inhibitors. However, the first application of the new method revealed time-dependent features of the kinetics for reactions initiated by addition of calmodulin and drew attention to problems of reaction rate determination at low protein concentration, which had not previously been addressed. Development of the assay method allowed the resolution of various questions relating to the effects of low concentrations of activator.

**Materials and Methods**

**Bacterial Strains and Plasmids**

Adenylate cyclase toxin (CyaA) was produced as a recombinant form after overexpression in Escherichia coli. E. coli BL21/DE3 (F' ompT rpsL mcrA) was used as host strain for production of recombinant proteins. The CyaA pro-toxin, which has adenylate cyclase enzymic activity, and the CyaC protein required for post-translational acylation and activation of cytotoxic activity were expressed from separate compatible plasmids, pGW44 and pGW54, respectively, each under the control of the inducible T7 RNA polymerase promoter (13).

**Preparation of Adenylate Cyclase**

Recombinant CyaA proteins were expressed as inclusion bodies in E. coli and extracts of these were prepared in 8 M urea as described previously (13). The enzyme in the unpurified extract (specific activity in the radiochemical assay $\sim 100$ I.U. per mg of protein (13)) was purified by anion exchange and hydrophobic interaction chromatography (14), giving a single major band on SDS-PAGE (molecular mass 200kDa) with a specific activity of 600 I.U. per mg of protein (15), comparable with that of a similar preparation described elsewhere (16). The time-dependent phenomena described in this paper were observed with both purified and unpurified enzyme preparations.

**Conductimetry**

**Apparatus**—This was an eight-cell system (12, 17) modified for control from a PC serial port with automatic balancing and calibration. The sampling period is 1 s per cell, cycling through from one to eight cells. Incomplete assay mixtures (normally 2 ml) reached thermal equilibrium in a period $\sim 2$ min, and reaction was initiated by addition of up to 20 $\mu$g of either enzyme, substrate, or activator. The effects of addition were tested by controls in which the initiating reagent was added to an
incomplete reaction mixture in synchrony with the test addition to permit valid blank subtraction. Data processing included blank subtraction, on-screen line drawing routines to measure curve slopes and total changes, and the export of standard format data files.

Reagents—Inorganic pyrophosphatase from E. coli (Sigma) was dissolved in water at 500 IU per ml (where 1 IU hydrolyzes 1 μmol/min), stored at -20 °C, and thawed and refrozen repeatedly without noticeable loss of activity. Typically, 0.5 IU was used in each assay. Calmodulin was purified from porcine testicular tissue (17), made up as a stock solution of 7.5 mg/ml in water, and stored at -20 °C.

Assay Methods—The assay buffer was 10 mM Bicine/Na3, pH 8.0, with either 1.5 or 2.5 mM MgCl2 degassed on the day of use by heating to >90 °C and exposing to reduced pressure to promote vigorous boiling for >2 s (18). ATP or other potentially unstable reagents were added to the bulk cooled buffer and dispensed in 2-ml aliquots. Where used, CaCl2 or EGTA was included in the assay buffer.

Calibration—Solution conductance has a near-linear relationship to concentration for small concentration changes (~5%) (19). Because pyrophosphatase hydrolysis is effectively irreversible, reactions linked to it become irreversible, and conductance change can then be calibrated in terms of substrate conversion (rather than specific product formation). Changes were determined in arbitrary “local” units for notional conversion of 1 mM substrate. Total conductance changes and tangents to curves are measured by an on-screen line drawing routine (12). Thus for x a substrate concentration of 1 mM giving a total change of y units and an initial slope of y units per minute, the initial rate is: initial rate = (y/x) μmol/ml/min, which could be used as an internal instrument calibration to display reaction rates in international units.

Blank Subtraction—The addition of highly conductive solutions to the conductivity cells causes an initial incremental change in conductance, which stabilizes within 10–20 s, but partially masks the continuous change caused by the enzyme-catalyzed reaction. The effect can be almost completely (~95%) abolished by addition of the same reagent to two cells and using one as a blank with the blank subtraction procedure. Because cell recordings are made at 1-s intervals, it is possible to add the reagent sequentially to all cells during at most two recording cycles so that a single blank cell can be used for seven reaction cells.

Log Phase Kinetics—The half-time for the duration of lag phases in reaction progress curves was determined by estimating the time at which the tangent to the rate curve reached half its final value. The relaxation time (τ) was calculated from the relationship τ = t0/ln(2).

RESULTS

Pyrophosphatase Assays—Conductimetric determination of enzyme-catalyzed hydrolysis of inorganic pyrophosphatase gives a linear measure of reaction progress provided that initial magnesium ion concentration is in significant excess (>100 μM) over pyrophosphate concentration (12, 19). All common biological buffers in the pH range 6–9 are suitable, but anionic univalent compounds (e.g. Bicine, Tricine) are preferred to cationic buffers (e.g. Tris), because they minimize rising baseline conductances due to CO2 absorption (12). Progress curves for the enzymes from E. coli (Fig. 1a) or Saccharomyces cerevisiae (not shown) are characteristic of enzymes with high affinities for the substrate. The conductance changes were a linear measure of substrate consumption (Fig. 1b), and kinetic analysis2 gives a Ks for pyrophosphate of ~10 μM under these conditions. Variations in long term base-line stability, which determine the practical limit of sensitivity, are equivalent to a change of >0.001%/min. Thus responses causing a change >0.02%/min can be determined with 95% confidence, setting a conservative detection limit for this enzyme at a rate of 5 × 10⁻⁴ μmol/ml/min (i.e. 5 × 10⁻⁴ IU/ml).

The Coupled Conductimetric Assay of Adenylate Cyclase—Typical radiolabeled adenylate cyclase assays use low concentration Tris buffers with 2 mM ATP and up to 10 mM MgCl2 carried out at 30 °C (9–11, 20) with 0.1–1 mg/ml bovine serum albumin, ostensibly to stabilize calmodulin (21). For preliminary investigations of the conductimetric method with CyaA, the pyrophosphatase assay was modified by replacing inorganic pyrophosphate with ATP (0.5–1 mM) in the presence of sufficient inorganic pyrophosphatase to hydrolyze >200 nmol of pyrophosphate/min (i.e. >0.2 IU/ml). The reaction equation, (ATP-Mg)2⁺ + Bicine⁻ → cAMP⁻ + (BicineH) + (PPiMg)2⁺ → 2PPi⁻ + Mg2⁺, predicts little change in conductance unless the pyrophosphate product is cleaved to release the magnesium ion from chelation. Progress curves obtained for pyrophosphatase-coupled assays of CyaA confirmed that this was a simple and practical detection method (Fig. 2a).

The specific activity of highly purified enzyme measured by this assay was found to be 520 ± 20 IU/mg, which is very similar to the value reported using radiochemical assays under these assay conditions (14, 16). A dose-response curve for the activity of the partially purified CyaA extract measured at a single pyrophosphatase concentration demonstrated that coupling between the reactions was efficient up to about 80% of the maximum that can be supported by the inorganic pyrophosphatase (Fig. 2b). The observation of strict proportionality between the observed activity and the CyaA added over the range used in the experiments described in this paper proves that the coupling enzyme does not limit the observed activity of CyaA. A second way in which it was verified that the measured rates were not limited by the concentration of coupling enzyme was by addition of inorganic pyrophosphate after the measuring period and observing the maximum conversion rate. This was a standard procedure for all batches of experiments reported in this paper (data not shown).

From these data the specific activity of the partially purified

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2A. J. Lawrence, J. G. Coote, Y. F. Kazi, P. D. Lawrence, J. MacDonald-Fyall, B. M. Orr, R. Parton, M. Riehle, J. Sinclair, J. Young, and N. C. Price, unpublished observations.
sample was determined as 80 IU/mg of protein. Very similar activity levels were found when Tris buffers (10–60 mM) were substituted for Bicine buffers, but with slight deterioration in signal to noise levels. From these results the limiting useful sensitivity of the present assay is about 10⁻³ IU/ml. Earlier workers had shown that CyaA could be activated by calmodulin in the absence of calcium (20–22), but our initial assumption was that this might be an artifact of a three component system. However the new method gave abundant verification of the earlier results, as the activity was inhibited by EGTA to give a stable plateau level, while activated by low concentrations of calcium and inhibited by higher concentrations (20–22) (Fig. 2c). In our hands there was no effect of 1,10-phenanthroline (0.1 mM), which had been reported to increase CyaA activity by >50% (22); however, albumin decreased CyaA activity to an extent depending on its purity. In the current work, Sigma radioimmunoassay grade albumin at 1 mg/ml gave 15% inhibition, decreasing to 5% inhibition in the presence of 1,10-phenanthroline. Some reagents (e.g. Tris, albumin) may possibly introduce inhibitory cations, but these were absent in the reagents used in the present work.

**Stability of CyaA and Calmodulin—**CyaA in 8 M urea and calmodulin in distilled water were found to be stable indefinitely when stored at −20 °C and subjected to freeze/thaw cycles. CyaA has a tendency to form aggregates in the absence of urea and become inactive as a toxin, but the demonstration of a half-life for the catalytic activity >3 min at 60 °C (23) did not indicate unusual thermolability. Non-activated CyaA was found to be relatively unstable upon incubation under assay conditions (Fig. 3a), losing about 50% of its activity after 10 min at 37 °C or 25% at 30 °C. To test the possible stabilization of calmodulin by albumin (1 mg/ml) (21), calmodulin (20 ng/ml) or CyaA (0.5 µg/ml) was incubated in the presence or absence of albumin for 12 min at 37 °C, the complementary protein added and incubated for 2 min, and the reaction started by addition of ATP. In comparison with controls (in which the components had been mixed immediately before assay) calmodulin retained full activity to activate, while CyaA lost 50% of its activity, but only 23% in the presence of 1 mg/ml albumin. Using the same experimental protocol, it could be shown that CyaA was stabilized by calmodulin, but not by calcium or EGTA (Fig. 3b).

**Initial Lag Phenomena—**Previous workers measured CyaA activation using calmodulin concentrations as low as 1 pm (20) or 10 pm (9), preincubating the proteins for 1 min before initiation of reaction with substrate. Initial lag phases, manifested as sigmoidal progress curves, were observed when whole bacterial membrane preparations were used as the enzyme source, suggesting a latency due to compartmentalization (22, 24). In contrast, progress curves obtained by the present method using soluble CyaA showed clear sigmoidal character. This is a common artifact of coupled assays, caused by insufficient coupling enzyme, but here the lag phase was unaffected by increasing the excess of pyrophosphatase by 10-fold (not shown). When CyaA and calmodulin, at concentrations that produced a pronounced initial lag phase for the catalytic reaction initiated by either protein, were preincubated together and the catalytic reaction initiated by addition of ATP, the lag phase was reduced and, for preincubation times >5 min, was effectively abolished (Fig. 4a). Hence the initial lag phase reflects the progress of CyaA activation. In principle, slow activation could be due to a collision-limited interaction rate, in which case the lag phase duration should depend on protein concentrations, or else it could be due to a slow conformation change after initial complex formation and therefore be concentration-independent. Lag phases were effectively abolished at high calmodulin concentration (Fig. 4b), confirming that the activation is con-

![Fig. 2. Coupled conductimetric assay of CyaA.](image)
EGTA, CaCl₂, CyaA, and calmodulin were 200 μM pyrophosphatase at 37 °C initiated by addition of ATP (final concentration 0.5 mM). Incubations were at 30 °C (●) or 37 °C (○). b, conductivity cells contained 2 ml of 10 mM Bicine/Na₂H₁₁₀₀¹ buffer, pH 8.0, with 1.5 mM MgCl₂ and 0.1 IU inorganic pyrophosphatase. After the specified incubation period, calmodulin (final concentration 0.5 ng/ml) was added, and after a further 2-min incubation, reactions were initiated by addition of ATP (final concentration 0.5 mM). Incubations were at 30 °C (●) or 37 °C (○). b, conductivity cells contained 2 ml of 10 mM Bicine/Na₂H₁₁₀₀¹ buffer, pH 8.0, with 1.5 mM MgCl₂ and 0.1 IU inorganic pyrophosphatase at 37 °C. When added the final concentrations of EGTA, CaCl₂, CyaA, and calmodulin were 200 μM, 20 μM, 0.25 μM, and 1 μg/ml, except for ○, where the calmodulin concentration was 17.5 μg/ml. In all cases reactions were initiated by addition of ATP (final concentration 0.5 mM), 2 min after addition of the last component. The order of additions and incubations was as follows: ●, CyaA plus calmodulin (12-min incubation), CaCl₂; ○, CyaA plus calmodulin plus CaCl₂ (12-min incubation); ▼, CyaA (12-min incubation), CaCl₂ plus calmodulin; ■, CyaA plus EGTA (12-min incubation), calmodulin. The maximum rates for CyaA incubated with calmodulin and for a control in which the components had been added immediately before assay were within ±2% of each other.

Concentration-dependent and is therefore collision-limited under these conditions. Numerical data presented below allow these results to be discussed in terms of the predictions of collision theory. Initial experiments showed that albumin increased the results to be discussed in terms of the predictions of collision theory. Initial experiments showed that albumin increased the activity, because the existing literature has many points of ambiguity, especially for reactions carried out at very low (<10 pm) calmodulin concentrations. Some of the data suggest that CyaA may have multiple binding sites for calmodulin, but it now seems probable that the combined effects of initial lag phases and instability of unactivated CyaA could be responsible for artifactual results. It was also of interest to use the high time resolution of the new assay to investigate the activation of CyaA in the absence of free calcium, because the enzyme is known to activate under these conditions with reduced calmodulin affinity, but significantly increased catalytic activity (20, 22). Thus freshly activated CyaA can be inhibited by EGTA at low calmodulin concentrations (7, 20), but one report (22) suggested that susceptibility to inactivation by calcium sequestration was lost on brief (<10 min) incubation of the activated complex. To avoid some of the complexities of a three-component system we chose to work at 20 μM CaCl₂, which is optimal for CyaA activity (although well above the normal physiological range of calcium) or else at 200 μM EGTA, which is well within the range where inhibition was concentration-independent (Fig. 2c).

Endogenous Activation—CyaA stored in concentrated urea solution and diluted directly into the assay medium shows very low initial activity, and in comparison with the fully activated enzyme, initial rates reveal an activation factor >1000. However when the enzyme is incubated in aqueous buffer at neutral
pH, it tends to activate to a very limited extent (of the order of about 5-fold over a period of 10 min). This is most probably due to contamination with trace amounts of a calmodulin analogue. (It should be noted that an endogenous activator protein has been observed in *B. pertussis* (25), but has not so far been reported in *E. coli*. The slow activation is more noticeable in the presence of albumin, where the degree of activation indicates the presence in albumin of a minimum of 1 ng calmodulin (or analogue)/mg of albumin. It is therefore difficult to obtain satisfactory kinetic behavior of CyaA at calmodulin concentrations less than 100 nM, and the advantage of increased stability obtained in the presence of albumin is partially offset by the increased basal activation. The observation that the rate of activation of CyaA on addition of calmodulin depends on the concentration of calmodulin (see below) makes it very unlikely that any process involving the folding of CyaA in the assay buffer contributes significantly to the kinetics of the activation.

**Kinetics of Activation**—The coupled assay procedure affords the possibility of obtaining the progress curve for product formation. The sigmoidal character of the progress curves meant that it was more reliable to measure maximum rather than initial reaction rates; however, it was also instructive to analyze the concentration dependence of the lag phase duration (Fig. 4b). Double-reciprocal plots of the variation of catalytic activity with calmodulin concentration were satisfactorily linear, consistent with action at a single binding site (Fig. 5, a and b), and the value for $K_d$ for calmodulin in the presence of calcium (0.53 ± 0.05 nM) at 30 °C is in close agreement with an earlier report (4) and rises to 30 ± 5 nM in the absence of calcium. With the exception of $K_d$ measured in the absence of calcium (133 nM), the kinetic parameters show very little dependence on temperature in the range 30–37 °C. These results confirm earlier observations that the enzyme retains activity in the absence of calcium, but with a decrease in apparent cal-

![Fig. 5. Calmodulin dependence of CyaA activity and lag phase duration.](http://www.jbc.org/)

(a) with calcium

(b) with EGTA

(c)

(d)
modulin affinity of about 2 orders of magnitude (20, 22). However the reported increase in catalytic activity of about 1.7-fold in the presence of EGTA and 1,10-phenanthroline at 30 °C (20, 22) was not observed in the present work.

Analysis of Lag Phases—The lag phases observed (Fig. 4b) in the activation of CyaA can be analyzed using equations that describe the rate of approach to equilibrium in terms of the rates of forward and backward processes (27). The dependence of the relaxation time (τ) as a function of the concentration of calmodulin is given by 1/τ = koff + kon [calmodulin]. Plots of 1/τ versus [calmodulin] in the presence and absence of calcium were satisfactorily linear (Fig. 5, c and d) from which the values of kon and koff could be determined; in addition the value of KD (= kon/koff) can be determined from the (negative) intercept at 1/τ = 0. In the presence of a saturating calcium concentration the values of the parameters at 30 °C were found to be kon = 7.9 ± 2 × 10⁶ M⁻¹ s⁻¹, koff = 0.002 ± 0.002 s⁻¹ and KD = 0.76 ± 0.2 nM, (cf. KD = 0.53 nM from activity measurements). The values obtained in the absence of calcium at 30 °C were kon = 2.3 ± 0.5 × 10⁶ M⁻¹ s⁻¹, koff = 0.014 ± 0.003 s⁻¹ and KD = 60 ± 15 nM (cf. 0 nM from activity measurements). Due to the increased instability of CyaA at 37 °C, the lag phase characteristics cannot be measured with adequate precision at this temperature.

The values for kon can be compared with the rate constant calculated for collisions between proteins of masses 200 kDa (CyaA) and 17 kDa (calmodulin), which is of the order of 10¹⁰ m⁻¹ s⁻¹ at 30 °C (26). Thus the association between CyaA and calmodulin has a probability factor of the order of 0.1%, which is reduced to about 0.001% in the absence of calcium. It should be noted that the values observed for kon are within the range found for other protein-protein interactions and reflect the low probability of matching complementary surfaces during collisions between protein molecules (26).

Dissociation of the Activated Complex by Dilution—The kinetic data described above predict that conditions exist where the CyaA-calmodulin complex can be formed either in the presence or absence of calcium and then diluted into an assay medium either to dissociate (+ EGTA) or else remain complexed (+ calcium). It should therefore be possible to obtain an independent estimate of the rate of complex dissociation (koff) and to investigate the effect of calcium on this rate. The expectations were either that bound calcium has free access to the medium and would be sequestered very rapidly by EGTA, resulting in no observable difference in behavior or else that calcium would not have access to the medium and would decrease the rate of dissociation by increasing the binding energy for the CyaA-calmodulin complex. The experimental system required that CyaA be diluted from 8 M urea to 20 mM urea for activation at high calmodulin concentration and then further diluted for assay. Under the conditions used, the enzyme was activated 1.2-fold greater in the presence of calcium than in the presence of EGTA, and this factor was used to normalize the experimental data so that a true comparison of the time courses of the Ca²⁺-activated and EGTA-activated assay data could be made.

The dissociation data (Fig. 6) allow values for koff to be determined with reasonable confidence. In the presence or absence of calcium the values are 0.004 ± 0.001 s⁻¹ at 30 °C and 0.011 ± 0.003 s⁻¹ at 37 °C. In view of the assumptions made and the experimental uncertainties involved (including the significant instability of non-activated CyaA especially in the presence of EGTA (Fig. 3)), the agreement between these values of koff at 30 °C and those determined by analysis of the lag phases can be considered satisfactory. The difference in activity in the presence or absence of calcium (Fig. 6b) indicates that the activity of the CyaA-calmodulin-calcium complex is higher than the CyaA-calmodulin complex. The observation of single kinetic processes both in the presence and in the absence of calcium (Fig. 6b) implies that the bound calcium remains part of the CyaA-calmodulin-calcium complex until the proteins dissociate from each other. While this appears to be at variance with the lack of effect of calcium on V₉₀₀₀₀ (Fig. 5, a and b), we believe that the data can be reconciled because in the present case there is no external calcium, which leads to inhibition (Fig. 2c).

The demonstration that calcium remains bound for the lifetime of the protein complex at 37 °C makes it very probable that it also does so at 30 °C, where the complex is more stable. The most probable interpretation of the observed difference in behavior is that while the catalytic activity shows little variation over this temperature range, inhibition by calcium has a high temperature coefficient. To our knowledge, this represents the first investigation of the behavior of the CyaA-calmodulin-calcium complex in the absence of external calcium.

Dissociation of the CyaA-Calmodulin Complex by EGTA—An earlier report (7) of measurement of the rate of inhibition of pyrophosphatase-coupled CyaA from B. pertussis by EGTA performed by the radiochemical sampling assay method had shown that inactivation was slow, but subsequent work by the same group (22) failed to find any inhibition in response to addition of EGTA 10 min after initiation of reaction. However, their enzyme preparation showed a marked stimulation by 1,10-phenanthroline, suggesting that effects on calcium chelation were masked by the action of a powerful inhibitory ion. The simplicity and high time resolution of the present method allows such experiments to be carried out very easily, and the results confirm that inactivation is slow (Fig. 6c). From the half-times of inactivation taken from these curves, it can be calculated that koff = 0.006 ± 0.002 s⁻¹ at 30 °C, which is in reasonable agreement with the values listed above. The value of koff at 37 °C was 0.02 ± 0.01 s⁻¹ at 37 °C, thus confirming the large temperature dependence of the dissociation process. However in experiments where the susceptibility of CyaA to inactivation by EGTA was tested at various times after the addition of calmodulin, the inhibition factor did not change over a 12-min incubation period, in contrast to results reported previously (22).

DISCUSSION

The pyrophosphatase-coupled conductimetric assay for the adenyly cyclase activity of CyaA has been used extensively in our laboratory since its initial development. It offers a large number of practical advantages over the radiochemical methods. Indeed, the only real advantage of the latter is that a lower limit of detection can be achieved, which in practice is rarely required. The obvious benefits of the conductimetric method are in terms of safety, cost, and the ease of data handling, but the greatest advantages are that assays are both simple and rapid and can be available whenever required, for example to monitor the individual steps during a purification procedure. Scientifically, the most useful feature of the methods is the time resolution, which has enabled valuable information to be obtained by analysis of the shapes of reaction progress curves.

A number of features of the coupled assay should be noted. First, the coupling enzyme (pyrophosphatase) of the required purity and activity is commercially available at reasonable cost. Second, the specific activity of purified CyaA determined by the coupled assay is very similar to that obtained using radiochemical assay method under the same assay conditions. Third, we have conclusively established that over the range of concentrations of CyaA assayed the coupling step is not rate-limiting. Finally, under any other assay conditions, e.g. in the
presence of other metals, it is easy to confirm the validity of the coupled assay method.

Conductimetric assays have a multitude of potential uses and provide a method of choice for very many enzymes (12, 17, 19, 28). However one enzyme for which this assay method is outstandingly suitable is inorganic pyrophosphatase, where the extension to coupled detection methods has wide ramifications. Condensation reactions in which pyrophosphate is released fall into two classes: those in which a mononucleotide is added to a substrate molecule (e.g. DNA and RNA polymerases) and those in which a so-called high energy intermediate is generated (e.g. aminoacyl-tRNA ligases and S-adenosylmethione synthetase). In all cases the condensation reactions have low free energy changes and are driven to near-completion by the special conditions operating within the cell. One of these conditions is the biological instability of inorganic pyrophosphate. This molecule is in fact kinetically highly stable, but in the presence of magnesium it is the target for high affinity cytosolic pyrophosphatases. Thus the coupling of pyrophosphate hydrolysis to a suitable detection method provides an assay for the parent enzymes that closely matches the biological function. No problems were encountered in setting up the linked assay for adenylate cyclase, because the kinetic characteristics of inorganic pyrophosphatases make them adaptable

**Fig. 6. Dissociation of the CyaA-calmodulin complex.**

(a) CyaA (10 mg/ml in 8 M urea) was diluted 400-fold into 10 mM Bicine/Na+ buffer, pH 8.0, containing 5 μM calmodulin in the presence of either 20 μM CaCl₂ (▼, □) or 200 μM EGTA (▽, ○) incubated at 25 °C for 10 min and then stored on ice. 20-μl aliquots were then diluted for assay in medium containing 0.5 mM ATP with 1.5 mM MgCl₂ and either in the presence of 20 μM CaCl₂ (▼, ▽) or of 200 μM EGTA (□, ○) at 30 °C. To ensure that the final concentrations of EGTA and CaCl₂ were the same in each case, the carryover concentrations were added to the appropriate assay mixtures. The curves are the averages of duplicate runs. The determinations were repeated using assays at 37 °C, and the results are shown with the time axis shifted by 1000 s for clarity. The half-times of the changes in activity for assays in the presence of EGTA were used to determine the rate constant for the dissociation process. b, semilogarithmic plots of data taken from Fig. 6a (37 °C). c, assays were carried out as above in the presence of 20 μM CaCl₂. After 60 s EGTA was added to give a final concentration of 200 μM and the time course recorded until the gradient became constant (after 1000 s). The data were plotted after subtracting the extrapolated late reaction rate to determine the rate of change of activity from the uninhibited to the inhibited state. ▼, EGTA at 37 °C; □, EGTA at 30 °C; △, control sample for 37 °C (no EGTA).
to a wide variety of conditions; it is therefore likely that this would be true for most other possible pyrophosphatase-linked assays.

The results of this study allow us to present a simple model for the behavior of the CyaA-calmodulin complex. The relatively good agreement between kinetic parameters determined by conventional activity measurements and lag phase analysis and those determined directly from the rates of dissociation indicates that the reaction between CyaA and calmodulin is effectively collision-limited at the protein concentrations used in these (and previous) assays.

We have generated the CyaA-calmodulin complex in the presence or the absence of calcium and have shown that both have similar activity at 30 °C, but the former is considerably more active at 37 °C, and this has enabled us to compare the dissociation properties of the two complexes. Our data show that removal of calcium from the medium, which decreases the collision efficiency for reaction between CyaA and calmodulin by more than 30-fold, has no effect on the dissociation rate. The data indicate that the activity of the complexes is determined by a balance between the intrinsic activity and inhibition by a freely exchangeable ion acting outside the calmodulin binding site. In the absence of any indication of inhibition by 1,10-phenanthroline-sensitive ions, we assume that the inhibitor is calcium itself, because the calcium inhibition curve (Fig. 2c) climbs sharply as it approaches zero concentration, conditions only found in assays of calcium-containing complexes in a calcium-free medium.

In contrast to the unexpected absence of any effect of calcium on the dissociation of the CyaA-calmodulin complex, calcium increases the rate of complex formation >30-fold, which is almost adequate to account for the effect on the apparent binding affinity. In view of the large changes in conformation and properties induced by the binding of calcium to calmodulin, this would appear to be a comparatively small effect on the rate of a collision-limited process. The observations can be rationalized by postulating that the primary interaction of calmodulin with CyaA is made by a part of calmodulin that is not greatly affected by calcium binding and that subsequent exploratory conformational changes take place on a time scale that is very much shorter than the time resolution of these experiments. The affinity of CyaA for calmodulin, even in the absence of free calcium, is such that at concentrations in the order of 10 μM found in eukaryotic cell cytosol (9) activation would be rapid and complete.

It should be noted that CyaA has many potential analytical uses, which could be exploited by the coupled assay method; these include the determination of calmodulin or calmodulin inhibitors and screening procedures for mutants containing cyA translational fusions. Although the latter approach has been developed to identify genes encoding surface-exposed and secreted proteins in Bordetella bronchiseptica (29), the authors comment that its usefulness is limited by the difficulties posed by the conventional radiochemical assay. Our assay method should be of value in this regard. The catalytic and regulatory properties of CyaA reside in the N-terminal domain (4), while those that contribute to its limited solubility (and hence instability) in aqueous solutions almost certainly reside in the C-terminal domain. Thus, it is probable that the practical utility and mechanistic studies of the system could be greatly increased by use of a truncated N-terminal (and hence presumably water-soluble) protein. This represents a goal for future research. It should be noted that in the case of the edema factor from Bacillus anthracis, the adenylate cyclase activity resides in the C-terminal portion of the exotoxin. Recent x-ray crystallographic studies of the edema factor in the absence and presence of CaM have given valuable insights into the mechanism of the activation process (8).

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A Direct Pyrophosphatase-coupled Assay Provides New Insights into the Activation of the Secreted Adenylate Cyclase from *Bordetella pertussis* by Calmodulin

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