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HEAT-STABLE, CALCIUM-INHIBITED CYCLIC NUCLEOTIDE PHOSPHODIESTERASE FROM *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa*, Ascomycetes, cyclic nucleotide phosphodiesterase, Ca^{2+} inhibition; heat stability, multiple molecular forms

Abstract—A heat-stable, Ca^{2+}-inhibited cyclic nucleotide phosphodiesterase (cPDE) (EC 3.1.4.17) from *Neurospora crassa* was isolated and could be separated into three aggregation forms (*ca* M_r values of 83 000, 151 000 and 330 000–400 000) by gel-filtration at 25° in the presence of EGTA, which suggests that the enzyme exists in monomeric, dimeric and tetrameric forms. Aggregation was temperature dependent; under similar conditions at 6°, a single form of *ca* M_r 400 000 was present. The enzyme was not activated by calmodulin but was inhibited by micromolar concentrations of Ca^{2+} and could be activated by addition of excess EGTA. Both cyclic AMP and cyclic GMP were substrates for the enzyme, and kinetic studies indicated that both bound at the same catalytic site. The enzyme had complex kinetics with two K_m values. The affinity of the high K_m component for both substrates was the same, whereas the low K_m component had a higher affinity for cyclic GMP than for cyclic AMP. Ca^{2+} was a competitive inhibitor of the high K_m component, whereas inhibition of the low K_m component was of the mixed type, Ca^{2+} caused an increase in both K_m values. The specific activity of the enzyme when cyclic AMP was used as a substrate was always higher than that with cyclic GMP, under all experimental conditions studied. A comparison of the heat-stable cPDE and cPDE activity in unheated extracts was also carried out using gel-filtration and ion-exchange chromatography. A working hypothesis is presented to account for the results.

INTRODUCTION

Cyclic nucleotides have important roles in intracellular and intercellular communication. Both cyclic AMP [1] and cyclic GMP [2, 3] occur in fungi, together with adenylate cyclase, cyclic nucleotide phosphodiesterase (cPDE) and cyclic AMP-dependent protein kinase [1]. Cyclic AMP has been implicated in a number of functional roles in fungi [1], including the control of morphogenesis, development and the cell cycle [4–7].

A number of fungal cPDEs have been reported [1, 8, 9]. In *Neurospora crassa* there are three intracellular and extracellular orthophosphate repressible cPDEs that have low (4.0) pH optima [10], a Mg^{2+}-stimulated cPDE present in crude mycelial homogenates [10], that may be the same as the particulate, EDTA-inhibited enzyme reported earlier [11], and a heat-activated cPDE, also present in crude mycelial homogenates [10]. Two forms of soluble cPDE from *Neurospora* have been resolved by chromatography on DEAE-cellulose [12] and one of these peaks of cPDE activity is activated by calcium and calmodulin [13]. A calmodulin stimulated cPDE has also been partially purified from a soluble extract of *Neurospora* by affinity chromatography [14]. Calmodulin is a small heat-stable protein [15–20], and one of its functions is the mediation of the interaction between Ca^{2+} and the enzymes required for the biosynthesis and degradation of cyclic nucleotides. For example, Ca^{2+} plus calmodulin have been shown to activate both adenylate/guanylate cyclase and cPDEs from a number of sources [21, 22]. However, a number of different types of cPDE have been isolated from various tissues, and not all of these are regulated by Ca^{2+} and calmodulin [23–27]. In contrast to the results described in refs [13, 14], we have reported the presence in *Neurospora* of a cPDE which has the unusual properties of being heat-stable and inhibited by micromolar concentrations of Ca^{2+} [28]. Furthermore, although calmodulin is present in *Neurospora* [29] there is no evidence that the inhibition by Ca^{2+} is mediated by calmodulin [28].

In the present paper, a kinetic study of the inhibition of the heat-stable *Neurospora* cPDE by Ca^{2+} is reported, as well as a comparison of the heat-stable cPDE with cPDE activity in unheated *Neurospora* extracts.

RESULTS AND DISCUSSION

Previously we reported that a *Neurospora* cPDE is heat-stable and inhibited by Ca^{2+} [28]. In the present study, we have investigated the mechanism of Ca^{2+} inhibition of the heat-stable cPDE using a kinetic approach and have also characterized some of its properties.

Abbreviations, cPDE, cyclic nucleotide phosphodiesterase; PMSF, phenylmethylsulphonyl fluoride; Vo, void volume, Ve, elution volume.

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We used the heated 113000 *g* supernatant to investigate the kinetic mechanism of Ca^{2+} inhibition of cPDE, because the manipulations required for further purification resulted in a decline in the ability of Ca^{2+} to inhibit the enzyme. Figure 1 shows Lineweaver–Burk plots of cPDE using cyclic AMP as substrate in the presence of Ca^{2+} or Ca^{2+} plus an 8-fold molar excess of EGTA In Fig 1a, the effect of substrate concentrations above 0 2 μM is shown, Fig 1b is an expansion of the area close to the intersection of the axes of Fig. 1a and shows the effect of substrate concentrations above 200 μM

There is a discontinuity in the Lineweaver–Burk plot at a cyclic AMP concentration of about 1 μM in the presence of Ca^{2+}, and at about 0 25 μM in the presence of Ca^{2+} plus an 8-fold molar excess of EGTA We obtained similar results with cyclic GMP as substrate The calculated K_m and V_{max} values for two substrate concentration ranges are summarized in Table 1 The enzyme had two apparent K_m values and therefore two kinetic components. Such Lineweaver–Burk plots have been interpreted as being due to negative cooperativity [30], or to the presence of multiple forms of cPDE with different K_m values [31, 32].

In Fig 1a, the two plots (dashed lines) intercept the $[S]^{-1}$ axis at different points Thus, the low K_m value was raised by Ca^{2+} and was calculated to be 2 2 μM (Table1) On the other hand, the V_{max} value, as determined by the intercept of the V^{-1} axis, was lowered by Ca^{2+} When high substrate concentrations were used (Fig 1b), Ca^{2+} raised the high K_m value, but did not significantly change the V_{max} Similar results were obtained for both kinetic components when cyclic GMP was used as a substrate (Table 1). However, the specific activity of the enzyme with cyclic AMP as substrate was always greater than that for cyclic GMP under all conditions studied

There are conflicting reports regarding the nature of the activation of cPDE by calmodulin and Ca^{2+}, i.e. whether the K_m, or V_{max} (or both) of the enzyme is affected [22] In the present paper, we have shown that Ca^{2+} inhibition of *Neurospora* cPDE can be due to an affect on either the K_m or the K_m and V_{max} depending on the substrate concentration used

Figure 2 shows the results of determinations in which cyclic AMP conversion was measured with cyclic GMP present in the incubations and vice versa In both cases,

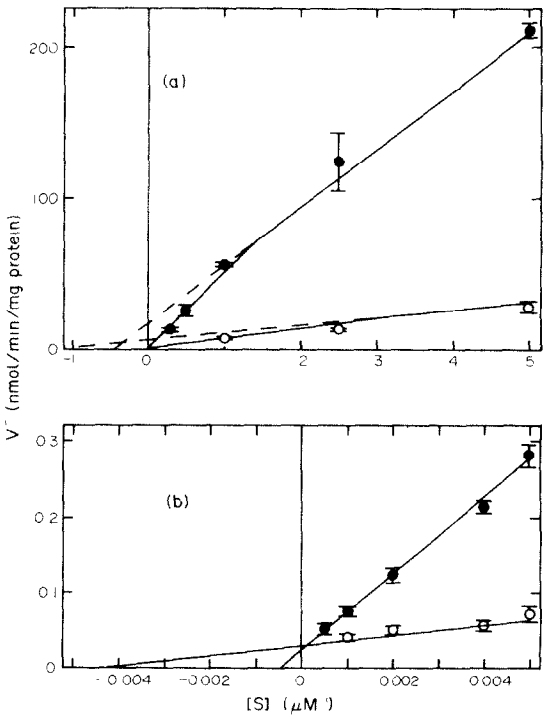

Fig 1 Lineweaver–Burk plots of cPDE activity using cyclic AMP as a substrate showing two different ranges of substrate concentration (a) 0 2 4 0 μm cyclic AMP, (b) 200 μm-2 mM cyclic AMP Assays were done using heat-stable cPDE (113000 *g* supernatant, heated 80 , 5 min), and the substrate concentration in the assays was varied as previously described [14] (● — ●) concentration in the assays of Ca^{2+}, 25 μM, (○ - ○) concentration in the assays of Ca^{2+}, 25 μM and EGTA, 200 μM Lines were fitted to experimental points by linear regression analysis (using points at more substrate concentrations than shown in the figure) Standard deviation is indicated by the error bars

Ca^{2+} was also present Both cyclic AMP and cyclic GMP competitively inhibited the hydrolysis of the other, i.e each raised the K_m for the other, but did not significantly affect the V_{max} The inset in Fig 2 shows the

Table 1 Heat-stable cyclic nucleotide phosphodiesterase from *Neurospora* summary of kinetic determinations

Substrate	Additions	Kinetic component	K_m (μM)	V_{max} (nmol/min/mg protein)
cyclic AMP	Ca^{2+}	High affinity	2 2	0 06
	EGTA	High affinity	0 75	0 15
	Ca^{2+}	Low affinity	2151	43
	EGTA	Low affinity	224	34
cyclic GMP	Ca^{2+}	High affinity	1 2	0 017
	EGTA	High affinity	0 16	0 024
	Ca^{2+}	Low affinity	2144	21
	EGTA	Low affinity	257	23

*The Ca^{2+} concentration in those determinations labelled 'Ca^{2+}' was 25 μM, whereas in those determinations labelled 'EGTA', the Ca^{2+} concentration was 25 μM and the EGTA concentration was 200 μM

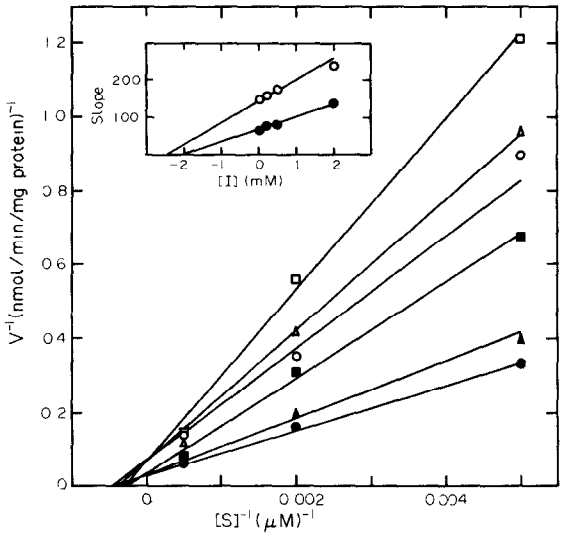

Fig 2 Lineweaver–Burk plots showing cyclic AMP inhibition of cyclic GMP hydrolysis and cyclic GMP inhibition of cyclic AMP hydrolysis Assays were done using heat-stable cPDE (113 000 *g* supernatant, heated 80°, 5 min) The substrate concentrations of cyclic AMP (closed symbols) and the substrate concentrations of cyclic GMP (open symbols) were varied. [8-^{3}H] cyclic AMP was added to the incubations in the former case and [8-^{3}H] cyclic GMP in the latter The concentration of cyclic GMP added as 'inhibitor' was zero (●——●), 500 μM (▲——▲), or 2000 μM (■——■) The concentration of cyclic AMP added as 'inhibitor' was zero (○——○) 500 μm (△——△) or 2000 μM (□——□) In all incubations, the concentration of Ca^2 was 25 μM In the inset, open circles indicate that cyclic AMP was used as an inhibitor in the assays with cyclic GMP as substrate, and closed circles the reverse

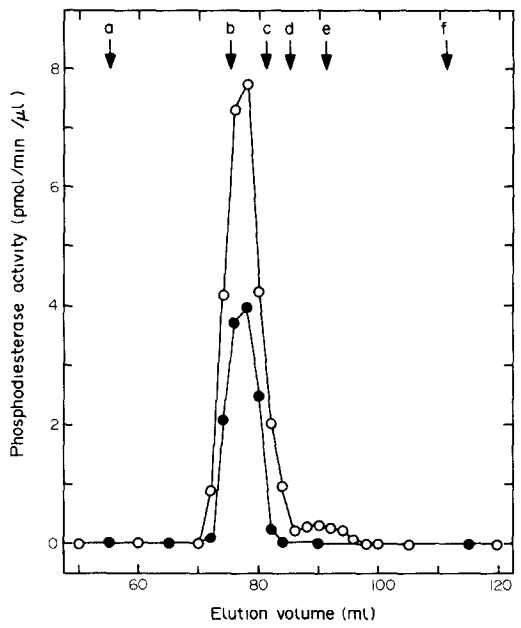

Fig 3 Elution profile of heat-stable cPDE activity from a Bio-Gel A-15 m column Heat-stable cPDE (113 000 *g* supernatant, heated 80°, 5 min) was concentrated to a protein concentration of 10 mg/ml by ultrafiltration One ml of concentrated extract was chromatographed on a Bio-Gel A-1 5 m column at 6° with 0 5 mM EGTA in the elution buffer Assays were done as described [28] with cyclic AMP as substrate (200 μM) (○——○) EGTA concentration 350 μM, (●——●) EGTA concentration 350 μM and Ca^{2+} concentration 1000 μM. The elution positions of the M_r standards are indicated by the arrows (a) blue dextran, (b) ferritin, (c) phycoerythrin, (d) catalase, (e) aldolase, (f) carbonic anhydrase

same plus additional results replotted (slope of Lineweaver–Burk plot versus 'inhibitor' concentration) to give the inhibitor constants (K_i) of cyclic AMP and cyclic GMP. Both had similar K_i values, 2000 μM for cyclic AMP, and 2400 μM for cyclic GMP. These values correspond approximately to their K_m values (high K_m component) in the presence of Ca^{2+} (see Table 1). Therefore, cyclic AMP and cyclic GMP probably competed for the same catalytic site on the enzyme

Properties of the heat-stable cPDE

The results of gel-filtration, at 6° in the presence of EGTA, of a heated cPDE extract (113 000 *g* supernatant, heated 80°, 5 min) are presented in Fig. 3. There was a major peak of cPDE activity which corresponded to a M_r value of *ca* 400 000, and this activity was inhibited by the addition to the assays of a molar excess of Ca^{2+} (relative to EGTA). When a heated cPDE extract was chromatographed on a DEAE-Sephadex column in the presence of Ca^{2+}, a major peak of activity, which can catalyse the hydrolysis of either cyclic AMP or cyclic GMP, eluted at about 0 075 M NaCl (Fig 4). A minor peak of activity was also observed which varied in size from preparation to preparation, but was never larger than the one shown in Fig. 4 The activity in both peaks was stimulated when assayed in the presence of a molar excess of EGTA (relative to Ca^{2+}), but activity in the main peak was not stimulated in the presence of Ca^{2+} plus either bovine calmodulin (0.25 units per assay) or a crude *Neurospora* calmodulin preparation (5 μl per assay, [28]) (results not shown)

When the major peak of activity from the DEAE-Sephadex column was concentrated and subjected to gel-filtration (6°, 0.5 mM EGTA in the eluting buffer), a single peak corresponding to a M_r value of *ca* 400 000 ± 34 000 ($n = 3$) again resulted [Fig 5(i)] However, when the same procedure was carried out at 25°, there were three distinct peaks with M_r values of *ca* 330 000 ± 37 000 ($n = 2$), 151 000 ± 0 ($n = 2$) and 83 000 ± 0 ($n = 2$) [Fig 5 (ii)] which indicated that the aggregation state of the enzyme was temperature dependent. The sharpness of the peaks suggested that the cPDE either dissociated in the sample before application, or very rapidly afterwards, and the M_r values suggested that the enzyme could exist in monomeric, dimeric and tetrameric forms

Gel filtration was also carried out at 6° or 25° with Ca^{2+}, but no EGTA in the eluting buffer [Fig. 5(iii), (iv)]. cPDE activity was spread across a wide range of molecular weights, and at 6° (Fig 5(iii)] cPDE was still eluted at a volume of 120 ml which corresponded to an apparent M_r of 23 000 At 25° [Fig 5(iv)], elution of cPDE activity was retarded to an even greater extent

When all the fractions containing cPDE activity from gel-filtration at 6° with Ca^{2+} in the buffers [Fig. 5(iii)]

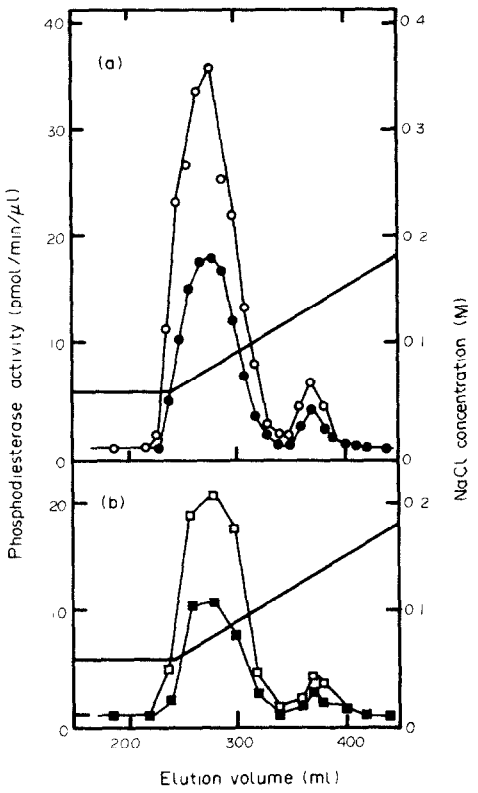

Fig 4 Elution profile of heat-stable cPDE activity from a DEAE-Sephadex A-50 column Heat-stable cPDE (113000 *g* supernatant, heated 80°, 5 min) was chromatographed on a DEAE-Sephadex A-50 column at 6° Assays were carried out as previously described [28] with cyclic AMP as substrate (200 μM) in part a, and cyclic GMP as substrate (200 μM) in part b (●-- --● and ■——■) concentration in the assays of Ca^{2+} (25 μM), (○— —○ and (□ — □) concentration in the assays of Ca^{2+} (25 μM) and EGTA (200 μM)

were pooled, concentrated and rechromatographed by gel-filtration at 6° with EGTA in the buffers, a single peak with a M_r value of *ca* 400000 again resulted (not shown). This result implied that the various forms of heated cPDE activities seen on gel-filtration were interconvertible Heat affects the interconversion of multiple forms of cPDE from human platelets [33], and it has been shown recently [34] that temperature can affect the allosteric and catalytic properties of the cyclic GMP stimulated cPDE from calf liver. It is possible that temperature may have similar effects on the heat stable *Neurospora* enzyme, in addition to affecting its aggregation state This remains to be investigated

Comparison of cPDE activity in heated versus unheated extracts

When the unheated 113000 *g* supernatant was chromatographed by gel-filtration at 6° in EGTA (Fig. 6), a similar peak of activity to that shown by the heated extract (Fig 3) was seen, except that the peak had a slightly higher average M_r *ca* 440000) and was a little broader. cPDE activity in both peaks was inhibited when a molar excess of Ca^{2+} was added to the assays

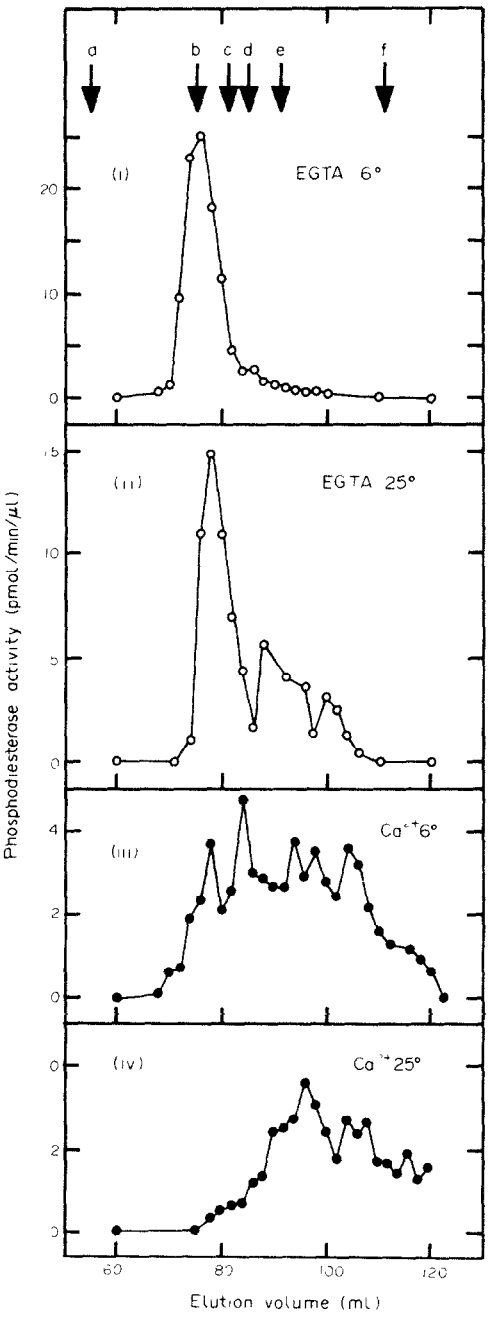

Fig 5 Elution profile of heat-stable cPDE activity from Bio-Gel A-1 5 m columns chromatographed at 6° versus 25° and eluted with either an excess of EGTA or Ca^{2+} in the buffer Heat-stable cPDE (113000 *g* supernatant, heated 80°, 5 min) was chromatographed on DEAE-Sephadex A-50 The main peak of cPDE activity (see Fig 4) was pooled, concentrated to a protein concentration of 1 5 mg ml by ultrafiltration and frozen Aliquots (1 ml) of this fraction were chromatographed on Bio-Gel A-1 5 m columns at either 6° [parts (i) and (iii)] or 25° [parts (ii) and (iv)] and with either 0 5 mM EGTA [parts (i) and (ii)] or 0 1 mM Ca^{2+} [parts (iii) and (iv)] in the elution buffer In parts (i) and (ii), the concentration of EGTA in the assays was 125 μM In parts (iii) and (iv), the concentration of Ca^{2+} in the assays was 25 μM The concentration of substrate (cyclic AMP) was 200 μM in all cases The elution positions of the M_r standards are indicated by the arrows as described in Fig 3

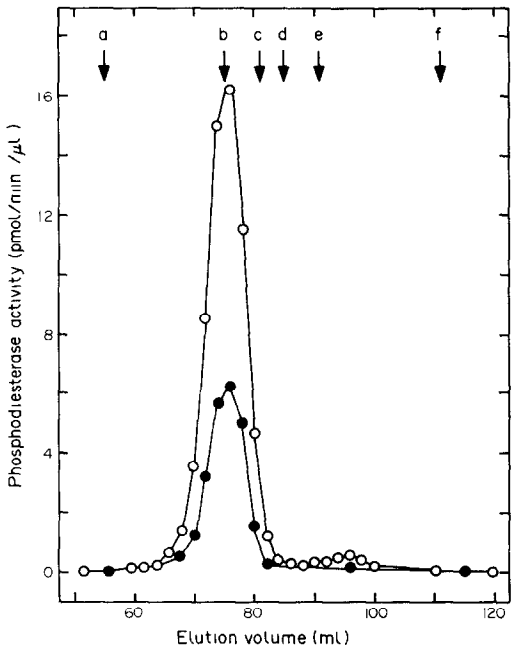

Fig 6 Elution profile of cPDE activity obtained by chromatography of an unheated extract (113 000 *g* supernatant) on a Bio-Gel A-1 5 m column The unheated extract was concentrated to a protein concentration of 75 mg/ml by ultrafiltration and a 1 ml aliquot chromatographed on a Bio-Gel A-1 5 m column at 6°. The elution buffer contained 0 5 mM EGTA Assays were carried out as previously described [28] with cyclic AMP as substrate (200 μM) (○——○) concentration in the assays of EGTA (350 μM), (●——●) concentration in the assays of EGTA (350 μM) and Ca^{2+} (1000 μM). The elution positions of the molecular weight standards are indicated by the arrows as described in Fig 3

When the unheated 113 000 *g* supernatant was chromatographed on ion-exchange and assayed either with cyclic AMP (Fig. 7a) or cyclic GMP (Fig. 7b) as substrates, three major peaks (I, II, III) and several minor peaks of cPDE were eluted This is in contrast to the results obtained using the heated 113 000 *g* supernatant where only one major peak of activity was eluted (Fig. 4) and in a volume close to that of peak I in Fig. 7. The activity of each peak shown in Fig. 7(a, b) was stimulated when assayed in the presence of a molar excess of EGTA. The same was true for the major peak of activity shown in Fig. 4, and in both cases no appreciable stimulation of any of the cPDE peaks of activity occurred when Ca^{2+} plus calmodulin (bovine heart, 0.25 units) were added to the assays (results not shown) When cPDE activity in each of the main peaks shown in Fig. 7 was heated at 80° for 5 min in the presence of Ca^{2+} [28], peaks I and II retained more than 90% of their activity, whereas peak III retained 60% of its activity. When cPDE activity in each of the main peaks was pooled, concentrated and subjected to gel-filtration (6°, EGTA), peaks I and II each gave rise to a single peak with a M_r of 400 000, whereas peak III gave a major peak of *ca* M_r 400 000, plus a leading shoulder with a height of *ca* half of the main peak and with a M_r of *ca* 440 000–500 000 (not shown). These results suggest that the majority of cPDE activity in peaks I and II, and over half the cPDE activity in peak III are constituents of the heat-stable, Ca^{2+}-inhibited cPDE, and that the same enzyme is the major form of cPDE activity in unheated soluble extracts from *Neurospora* mycelia.

Summary and working hypothesis

To account for the properties of the *Neurospora* heat-stable, Ca^{2+}-inhibited cPDE, the following proposal is presented The enzyme can bind Ca^{2+} to negatively charged sites, and as a result inhibition of activity occurs. This effect can be reversed by addition of a molar excess of EGTA which removes the bound Ca^{2+} In ref. [28] we showed that Ca^{2+} conferred additional thermal stability on the heat-stable *Neurospora* cPDE. This may also be a consequence of the binding of Ca^{2+} to the enzyme. We also propose that binding of Ca^{2+} causes the enzyme to become more hydrophobic for the following reasons In the gel-filtration experiments shown in Figs 3 and 5, we included NaCl (0 1 M) in the gel-filtration buffers to reduce non-specific ionic interactions between the gel matrix and proteins [35]. However, some proteins are subject to hydrophobic interaction with the gel matrix on gel-filtration [35, 36]. The progress of such proteins down gel-filtration columns is retarded to a greater extent than their true M_r values would suggest. Consequently, they elute in a volume corresponding to an apparently lower M_r value than their true M_r value. These effects are strengthened by increased temperature and ionic concentration [35] We therefore suggest that the smearing and retardation of cPDE activity seen in Fig. 5(iii and iv), when Ca^{2+} was present in the buffers was due to non-specific hydrophobic interaction between the enzyme and the gel matrix Since these effects were not seen when EGTA was present in the buffers, we propose that Ca^{2+} induced a conformational change in the enzyme, with a resultant increase in its hydrophobic nature.

The proposed Ca^{2+}-binding sites on the heat-stable cPDE could be on catalytic polypeptide subunits of the enzyme or on a tightly bound regulatory polypeptide. This latter possibility raises the question as to whether such a polypeptide could be calmodulin. We cannot unequivocally rule out such a possibility even though we have shown [28] that chlorpromazine, a compound which binds to calmodulin and prevents it from activating cPDE, did not block the Ca^{2+} inhibition of heat-stable *Neurospora* cPDE An analogous example may be rabbit skeletal muscle phosphorylase kinase which contains tightly bound calmodulin [37]. In that case, the calcium dependent activity of the enzyme is unaffected by addition of trifluoperazine.

The unheated enzyme extract contains several forms of Ca^{2+}-inhibited cPDE as indicated by the results obtained by ion-exchange chromatography (Fig. 7). As discussed earlier, these forms (especially peaks I and II) upon heating appear to be converted to the heat-stable Ca^{2+}-inhibited cPDE. This may be due to thermal cleavage of parent PDE species to give an active lower molecular weight species, in an analogous way to the proteolytic cleavage of calmodulin dependent cPDE [38] and the low K_m cyclic AMP PDE from yeast [8] which also result in active cleavage products. More research will be necessary to determine the relationship between each of the Ca^{2+}-inhibited forms of cPDE which have been demonstrated in this investigation. In [28] we speculated about the possible presence of Ca^{2+} inhibited cPDEs in animal tissues similar to the cPDE we had

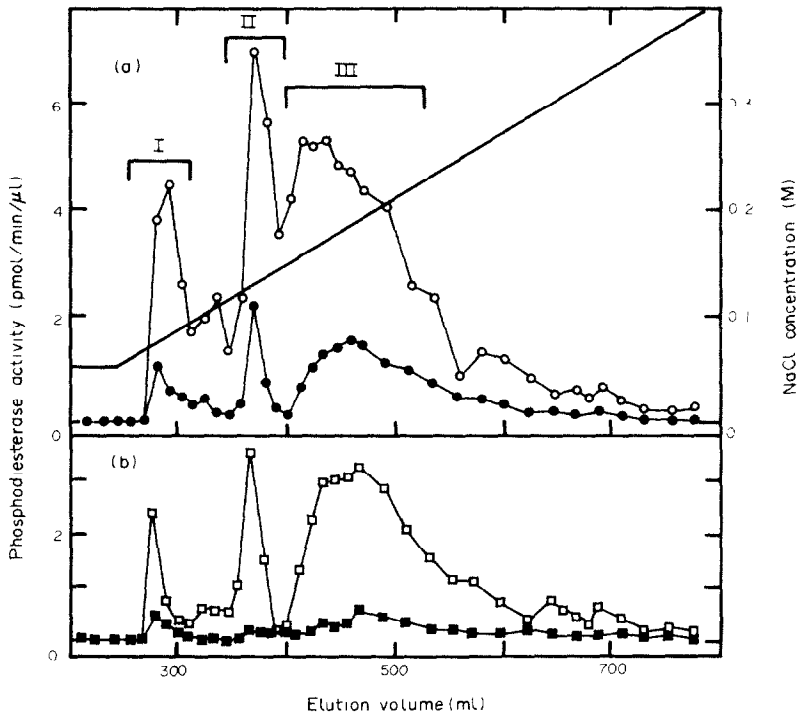

Fig 7 Elution profile of cPDE activity obtained by chromatography of an unheated extract (113 000 *g* supernatant) on a DEAE-Sephadex A-50 column Assays were carried out as previously described [28] with 200 μM cyclic AMP as substrate (part a) and 200 μM cyclic GMP as substrate (part b) (● — -● and ■ - -■) concentration in the assays of Ca^{2+} (25 μM), (○——○ and □ —□) concentration in the assays of Ca^{2+} (25 μM) and EGTA (200 μM).

found in *Neurospora*. Since then we have found a paper [39] which reports Ca^{2+} inhibition (at 250 μM) of several cPDE species from bovine superior cervical ganglia Furthermore, inhibition by Ca^{2+} (20 μM) of a cyclic GMP PDE has also been reported [40] The authors of ref [39] raise the possibility of Ca^{2+}-inhibited cPDEs in animal tissues being overlooked due to the routine addition of calmodulin to all assays in some studies

In our investigation we could not demonstrate any activation of *Neurospora* cPDE by calmodulin plus Ca^{2+} However, such an effect was reported [13, 14] Furthermore, excess EGTA present during the incubation prevented the activation of the enzyme of Ca^{2+} and calmodulin [14] For the *Neurospora* cPDE described in our investigation, excess EGTA stimulated the enzyme activity and Ca^{2+} inhibited. However, the data in ref [14] are not incompatible with the results of the present study because: (i) the calmodulin-stimulated cPDE present in *Neurospora* extracts may be a minor component and, therefore, masked in our study (e g. for the results presented in Fig. 7, endogenous calmodulin could have saturated the calmodulin dependent form, so that when we added calmodulin no more stimulation of that form was seen) and (ii) the authors of ref [14] used a calmodulin-Sepharose affinity column in their purification procedure. Thus they were selecting for an enzyme which binds to calmodulin.

However, our results ([28], and this paper) do disagree in several respects with those in refs [12] and [13] We have shown that unheated *Neurospora* extracts can be resolved on DEAE-sepharose into three major peaks of cPDE activity, each of which is largely heat-stable, inhibited by Ca^{2+}, and not activated by Ca^{2+} plus calmodulin Overall our results lead us to conclude that the heat-stable Ca^{2+}-inhibited cPDE forms the majority of soluble cPDE activity in *Neurospora* mycelia On the other hand, in ref. [13] cPDE was separated into two peaks of activity on DEAE-cellulose, and the enzymes in the main peak of activity was stimulated by Ca^{2+} plus calmodulin, especially after rechromatography on DEAE-cellulose It would be conjecture to attempt to explain the differences between the two studies at present We therefore conclude that the complete isolation and characterisation of the Ca^{2+}-inhibited and calmodulin-stimulated forms of cPDE in *Neurospora* will be necessary to explain the differences between our work and that reported in ref [13] Nevertheless, taken together all the studies so far on cPDEs in *Neurospora* do indicate the complexity of form and control of this enzyme, and will serve as a basis for further research

EXPERIMENTAL

Materials Proteins for calibrating the gel-filtration columns were obtained from Sigma and Nutritional Biochemicals, with the exception of phycoerythrin, which was provided by Dr E Gantt of this Laboratory, [8-^{3}H] cyclic AMP (26 0 Ci/mmol) and [8-^{3}H] cyclic GMP (18 3 Ci/mmol) were from the Amersham Corporation, TLC sheets from E Merck (Darmstadt, F R G), scintillation fluid (Ultrafluor) from National Diagnos-

tics, blue dextran and DEAE-Sephadex A-50 from Pharmacia, Bio-Gel A-1.5 m, 200–400 mesh, was from Bio-Rad Laboratories.

Bovine heart calmodulin and calmodulin deficient bovine cPDE were from Sigma, and a crude preparation of *Neurospora* calmodulin was prepared as in ref [28] One unit of calmodulin activity is defined as that amount which would stimulate 0 016 units of calmodulin deficient bovine heart cPDE to 50% of maximum activity at pH 7 5 and 30° when saturated with calmodulin and 10 μM Ca^{2+}. One unit of bovine heart cPDE was equivalent to the conversion of 1 μmol/min of substrate to product at pH 7 5 and 30°

Preparation of cPDE extracts Wild-type *Neurospora crassa* (strain 74A, Fungal Genetics Stock Center, number 987) was grown, harvested, and 113 000 *g* supernatants prepared as previously described [28] Preparation of heat-stable cPDE from the 113 000 *g* supernatant (heated 80°, 5 min) was also carried out as described in ref [28] *cPDE assays* cPDE assays were carried out as in ref [28] The concentrations of Ca^{2+} and EGTA used in the assays will be indicated For the determination of kinetic values, conditions were selected so that reaction rates were linear with respect to incubation time, and substrate conversion did not exceed 20% of the initial substrate concentration

Ion-exchange chromatography A 2 5 × 28 0 cm bed of DEAE-Sephadex A-50 was equilibrated with buffer containing 100 mM Tris–HCl (pH 7 4), 1 mM PMSF, 2 mM benzamidine, 0 1 mM $CaCl_2$, and 50 mM NaCl (buffer A) The PMSF and benzamidine were included to prevent proteolysis which can readily occur in *Neurospora* extracts if precautions are not taken After loading the sample, the column was washed with buffer A until the absorbance at 280 nm of the column effluent was zero Bound protein was eluted with a linear NaCl gradient. The initial eluent was buffer A, and the final eluent (buffer B) was identical to buffer A except that the NaCl concentration was 400 mM The flow rate was 40 ml/hr, and 10 ml fractions were collected For subsequent gel-filtration chromatography, peaks of activity were pooled and concentrated with an Amicon ultrafiltration apparatus equipped with a type YM 5 membrane.

Gel-filtration chromatography Columns of Bio-Gel A-1 5 m 200–400 mesh (1 25 × 90 cm) were used The elution buffer was 100 mM Tris–HCl (pH 7 4), 1 mM PMSF, 2 mM benzamidine, 100 mM NaCl, and either 0 1 mM $CaCl_2$ or 0 5 mM EGTA as indicated Void volumes were determined using blue dextran, and the following standards were used to calibrate the column ferritin (M_r 440 000), phycoerythrin (M_r 280 000), catalase (M_r 232 000), aldolase (M_r 158 000), carbonic anhydrase (M_r 31 000) Samples were loaded in a volume of 1 ml, the flow rate was 5 ml/hr, and 2 ml fractions were collected Estimates of the M_rs of cPDE were made using a plot of *Ve* over *Vo* of protein standards vs logM_r and when more than one determination was made, the results are expressed ± s.d , where *n* equals the number of determinations

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REFERENCES

1 Pall, M L (1981) *Microbiol Rev.* **45**, 462.
2 Shaw, N M. and Harding, R. W (1987) *Plant Physiol* **83**, 377
3 Rosenberg, G B and Pall, M L. (1979) *J Bacteriol* **137**, 1140
4 Mishra, N. C. (1976) *Naturwissenschaften* **63**, 485
5 Swamy, S , Uno, I and Ishikawa, T (1985) *J Gen Microbiol* **131**, 3211
6 Nurse, P (1985) *Nature* **313**, 631
7 Kunihiro, M , Isao, U. and Ishikawa, T (1985) *Yeast* **1**, 15
8 Suoranta, K and Londesborough, J (1984) *J Biol Chem* **259**, 6964
9 Kerner, N , Moreno, S and Passeron, S (1984) *Biochem J* **219**, 293
10 Hasunuma, K and Shinohara, Y (1986) *Curr Genet.* **10**, 893
11 Scott, W A and Solomon, B (1973) *Biochem. Biophys Res Commun.* **53**, 1024.
12 Tellez-Inon, M T , Glikin, G C. and Torres, H N (1982) *Biochem J* **203**, 611.
13 Tellez-Inon, M T , Ulloa, R. M., Glikin, G C and Torres, H N. (1985) *Biochem J.*, **232**, 425
14 Ortega Perez, R , van Tuinen, D , Marme, D. and Turian, G (1983) *Biochim Biophys Acta* **758**, 84
15 Cheung, W Y (1982) *Fed. Proc* **41**, 2253
16 Klee, C B and Vanaman, T C (1982) in *Advances in Protein Chemistry* Vol 35 (Anfinsen, C B , Edsall, J. T and Richards, F M , eds), pp 213–321 Academic Press, London
17 Brostrom, C O , Brostrom, M A. and Wolff, D J (1982) in *Calcium Regulation by Calcium Antagonists* Vol 201 (ACS Symposium Series) (Rahwan, R G and Witiak, D T , eds), pp 89–103, American Chemical Society, Washington
18 Lin, Y M (1982) *Mol Cell Biochem* **45**, 101
19. Means, A R., Tash, J S and Chafouleas, J G (1982) *Physiol Rev* **62**, 1
20 Klee, C B , Crouch, T H and Richman, P G (1980) *Annu Rev Biochem* **49**, 489
21 Bradham, L S and Cheung, W Y (1980) in *Calcium and Cell Function* Vol. 1 (Cheung, W Y , ed.), pp 109–126, Academic Press, London
22 Lin, Y M & Cheung, W. Y (1980) in *Calcium and Cell Function* Vol 1 (Cheung, W Y , ed.), pp 79–107, Academic Press, London
23 Beavo, J A , Hansen, R. S , Harrison, S. A , Hurwitz, R L , Martins, T J and Mumby, M C (1982) *Mol Cell Endocr* **28**, 387.
24 Vaughan, M., Danello, M A , Manganiello, V. C and Strewler, G. J (1981) *Adv Cyclic Nucleotide Res* **14**, 263
25 Strada, S J and Thompson, W J (1978) *Adv Cyclic Nucleotide Res.* **9**, 265
26 Wells, J N and Hardman, J G (1977) *Adv. Cyclic Nucleotide Res* **8**, 119
27 Appleman, M. M., Ariano, M. A , Takemoto, D J and Whitson, R. H (1982) *Handbook of Experimental Pharmacology* **58**, 261
28 Shaw, N M and Harding, R. W. (1983) *FEBS Letters* **152**, 295
29 Cox, J A , Ferraz, C., Demaille, J G , Ortega Perez, R., van Tuinen, D and Marme, D (1982) *J Biol Chem* **257**, 10694
30 Russell, T. R , Thompson, W J., Schneider, F W and Appleman, M. M. (1972) *Proc Natl Acad Sci U S.A* **69**, 1791
31 Pichard, A. L. and Cheung, W Y (1976) *J. Biol Chem* **251**, 5726
32 Lin, Y. M and Cheung, W Y (1975) *Int J Biochem* **6**, 271.
33 Pichard, A. L , Choury, D. and Kaplan, J. C (1981) *Biochimie* **63**, 603
34 Wada, H , Osborne, J C and Manganiello, V C (1987) *J Biol Chem* **262**, 5139.
35 Scopes, R K (1982) *Protein Purification: Principles and Practice*, pp 141 and 154, Springer, New York.
36. Porath, J (1979) *Biochem Soc Trans* **7**, 1197

37 Shenolikar, S, Cohen, P T W, Cohen, P, Nairn, A C and Perry, S V (1979) *Eur J Biochem* **100**, 329
38 Kincaid, R L, Stith-Coleman, I E and Vaughan, M (1985) *J Biol Chem* **260**, 9009
39 Boudreau, R J and Drummond, G I (1975) *J Cycl Nucl Res* **1**, 219
40 Hidaka, H and Asano, T (1976) *J Biol Chem* **251**, 7508