Cyclic GMP and Cyclic AMP Binding Proteins in *Myxococcus xanthus*

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Three binding proteins, one specific for guanosine 3':5'-monophosphate (cGMP) and two specific for adenosine 3':5'-monophosphate (cAMP) have been partially purified from vegetative cells of *Myxococcus xanthus* M300. The cGMP binding activity was found in the periplasmic shock fluid. Scatchard analysis indicated only a single class of binding sites with high affinity (apparent K_D , 42 nM). The two cAMP binding activities were physically distinct, as indicated by their elution patterns from DEAE-cellulose, K_D values and cellular locations. The cytoplasmic cAMP binding protein, which is probably identical to that previously isolated from developing myxospores of *M. xanthus* had an apparent K_D of 57 nM, whereas the periplasmic cAMP binding protein had an apparent K_D of 1 μ M. During development, the nucleotide binding proteins exhibited changes in activities consistent with their postulated roles during fruiting body development.

INTRODUCTION

A number of reports have suggested an involvement of cyclic nucleotides in morphogenesis in *Myxococcus xanthus* (Campos & Zusman, 1975; Parish *et al.*, 1976; Yajko & Zusman, 1978; Zusman 1978; McCurdy *et al.*, 1978). Consequently, McCurdy *et al.* (1978) proposed a working hypothesis which incorporated a previous suggestion that cAMP acts as a derepressor of development-specific genes (Yajko & Zusman, 1978) and also suggested that cGMP and cGMP phosphodiesterase (cGMP-PD) function in aggregation chemotaxis.

It has since been reported that levels of cAMP, cGMP and their respective PDs exhibit sequential changes during development (Ho & McCurdy, 1980) and that cGMP is indeed a chemoattractant for *M. xanthus* under fruiting conditions (Ho & McCurdy, 1979). Recently, Orlowski (1980) described a cAMP binding protein which he suggested was a control element in the transcription of differentiation-specific operons in *M. xanthus*.

In this report we describe cyclic nucleotide binding activities detected in *M. xanthus* M300 and their behaviour during development.

METHODS

Organism and cultivation. *Myxococcus xanthus* M300 (McCurdy *et al.*, 1978) was used in all experiments. Vegetative cultivation, induction of fruiting body formation and cell harvesting techniques at various stages of development were as described previously (Ho & McCurdy, 1980).

Preparation of periplasmic shock fluid and 'post-shock' pellet crude extract. Periplasmic shock fluid was prepared essentially by the method of Nossal & Heppel (1966), as modified by Burchard (1974). Vegetative cells were collected by centrifugation at 7000 g for 10 min at 4 °C and washed once with T buffer (10 mM-Tris/HCl, pH 7.5). The washed cells were collected by centrifugation and resuspended in 40 vols T buffer. After gentle mixing of the cell suspension for 20 min, an equal volume of 40% (w/v) sucrose in T buffer was added with stirring, and cells were incubated for 10 min with gentle shaking. The bacteria were collected by centrifugation and then shocked with 80 vols cold deionized water containing 0.5 mM-MgCl₂, at 0 °C. The cells were pelleted by centrifugation and the pellet was saved. The shock fluids were routinely concentrated 20-fold by freeze-drying. EDTA was omitted from the shock procedure as it induced lysis in *M. xanthus*. More than 90% of the shocked cells were viable, as determined by plate counts on Sp agar (McCurdy, 1963).

Abbreviation: PD, phosphodiesterase.

The post-shock pellet was suspended in T1 buffer (T buffer containing 5 mM-MgCl₂ and 3 mM-mercaptoethanol) and broken by two 15 s bursts of sonication with a Bronwill Biosonik (Bronwill Scientific, Rochester, NY, USA) set at full power. The cell extract was obtained by centrifugation (7000 g for 10 min, 25 °C). The supernatant fraction was dialysed overnight against 100 vols T1 buffer.

Binding assays. Assays for cyclic nucleotide binding proteins were modifications of the procedure described by Gilman (1970).

The 250 µl reaction mixture used to estimate cGMP binding contained 10 mM-Tris/HCl, pH 7.5; 5 mM-MgCl₂; 3 mM-mercaptoethanol; 3 mM-1-methyl-3-isobutylxanthine (an inhibitor of *M. xanthus* PDs, Ho & McCurdy, 1980); 1 mM-phenylmethanesulphonyl fluoride (a protease inhibitor); 20 nM-tritiated cyclic nucleotide (10000 d.p.m. pmol⁻¹) and 200–800 µg cell protein. The cytoplasmic cAMP binding assay contained the same reaction mixture, except that 200 nM-tritiated cyclic nucleotide (10000 d.p.m. pmol⁻¹) were used.

The reaction mixture was incubated for 10 min at 0 °C and the reaction was terminated by passing the mixture through a membrane filter (Millipore/HAWP, 0.45 µm pore size) and washing immediately with 3 ml ice-cold T1 buffer. The filter was dried under a heat lamp, placed in 10 ml scintillation fluid and counted in a Beckman model 3150P liquid scintillation counter. Controls containing high concentrations of non-radioactive cyclic nucleotides were run in parallel with binding assays. Determinations using boiled proteins were equivalent to controls containing high concentrations of non-radioactive cyclic nucleotides.

Purification procedure. The shock fluid, concentrated by freeze-drying, was passed through a Sephadex G-200 column equilibrated with T buffer. A single broad protein peak was obtained. Fractions containing binding activity were pooled and the pH was adjusted to 4.5 with 7.5% (w/v) acetic acid. The precipitate was separated by centrifugation and the supernatant was loaded into a high flow rate CM-cellulose column (1 ml resin bed volume; 6–8 mg protein to be fractionated; column height:diameter, 3:1) equilibrated with CM buffer (10 mM-sodium acetate, pH 4.5). The column was washed with 3–5 vols 0.3 M-KCl in CM buffer. About 20% of the binding activity was absorbed and subsequently eluted from the column. When repeated, the same recovery was obtained, making it unlikely that there was selective absorption of more than one binding protein. The eluted fractions containing all binding activity were pooled and the pH was adjusted to 7.5 with concentrated aqueous ammonia and then dialysed overnight against T buffer at 4 °C. The dialysate was loaded onto a DEAE-cellulose column (1 ml resin bed volume; 5–7 mg of protein to be fractionated; column height:diameter, 12:1) previously equilibrated with T buffer. The column was washed with 5 bed volumes of cold buffer and activity was eluted with a linear gradient of 0–0.5 M-KCl in T buffer. The flow rate was kept at 8–10 ml h⁻¹ or less. The cAMP binding protein from the post-shock pellet extract eluted between 0.1 and 0.15 M-KCl, whereas the shock fluid cAMP binding protein eluted between 0.17 and 0.23 M-KCl. The cGMP binding protein eluted between 0.15 and 0.17 M-KCl. All active fractions for a given activity were pooled and dialysed overnight against T buffer.

Protein measurements. The method of Lowry was used, with bovine serum albumin as standard.

Biochemicals. The following compounds were obtained from Sigma: cAMP, cGMP, ATP, GTP, AMP, GMP, Tris, 1-methyl-3-isobutylxanthine, 2-mercaptoethanol, phenylmethanesulphonyl fluoride and HEPES.

[8-³H]Adenosine 3':5'-cyclic phosphate and [8-³H]guanosine 3':5'-cyclic phosphate were obtained from Amersham. The DEAE-cellulose, CM-cellulose and Sephadex G-200 were from Whatman.

For liquid scintillation counting, the filmware plastic bags (10 ml) were obtained from Nalge, Rochester, NY, USA. 2,5-Diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP) were purchased from Sigma. Regular grade toluene was obtained from Fisher Chemical Co. The scintillation solution contained 16.5 g PPO, 0.36 g POPOP and 1.01 toluene.

RESULTS

Purification of cAMP and cGMP binding proteins

cAMP and cGMP binding activities present in both the crude shock and post-shock fluids were partially purified as described in Methods; the results are summarized in Table 1. The recovery of periplasmic cAMP binding protein was 26% whereas recovery of periplasmic cGMP binding protein was 7%. The purification of cytoplasmic cAMP binding protein was 5-fold whereas that of the periplasmic protein was 29-fold.

Characterization of cGMP binding protein

Of the total cGMP binding activity, 90% was found in the periplasmic space of *M. xanthus* (Table 2). The binding was complete within 10 s at 4 °C and stable for up to 30 min. No appreciable cGMP hydrolysis was detected under the assay conditions. The cGMP binding activity was proportional to protein added in the range 0–0.3 mg protein per assay and was not inhibited by cAMP, GMP, GTP, AMP, or ATP in competitive binding assays. All the binding

Fig. 1

Fig. 2

Fig. 1. Scatchard plot of [^3H]cGMP binding by shock fluid. The binding assay was performed as described in the text. The 250 μl incubation mixture contained 200 μg protein. B, bound; F, free.

Fig. 2. cGMP binding activity during development of *M. xanthus*. The binding assay was performed as described in Methods. Concentrated periplasmic shock fluids were prepared at the times indicated after inoculation on FM medium (Ho & McCurdy, 1980). The bars show highest and lowest detected values in four or five separate experiments.

Table 1. *Partial purification of binding proteins*

	Vol. (ml)	Total units	Protein (mg ml ⁻¹)	Specific activity [pmol bound (mg protein) ⁻¹]	Purification (fold)	Recovery (%)
Periplasmic cGMP binding protein						
Shock fluid (concentrated)	37	16.00	0.93	0.46	1	100
G-200	20	11.40	0.60	0.95	2.10	71.2
CM-cellulose	10	2.43	0.09	2.70	5.86	15.18
DEAE-cellulose	5	1.13	0.07	3.80	8.26	7.06
Cytoplasmic cAMP binding protein						
Cell-free extract	45	404.1	5.98	1.5	1	100
G-200	25	209.0	3.80	2.2	1.46	51.7
CM-cellulose	10	43.44	1.81	2.4	1.6	10.7
DEAE-cellulose	5	8.4	0.3	5.6	3.73	2.1
Periplasmic cAMP binding protein						
Shock fluid (concentrated)	37	9.6	0.93	0.28	1	100
G-200	20	6.72	0.30	1.12	4.0	70.0
CM-cellulose	10	4.96	0.09	5.52	19.7	51.6
DEAE-cellulose	5	2.49	0.06	8.3	29.6	25.9

Table 2. *Cellular distribution of binding proteins*

	Periplasmic shock fluid	Post-shock pellet	Total
cGMP binding activity (pmol bound)	0.388	0.042	0.43
cAMP binding activity (pmol bound)	1.13	2.03	1.4

activities were corrected for the non-specific binding of labelled cGMP in the presence of a large excess of unlabelled cGMP. Scatchard analysis (Scatchard, 1949) of the binding data for cGMP yielded a straight line, suggesting the presence of a single class of binding sites (Fig. 1). The apparent dissociation constant (K_D) was calculated to be 42 nM.

Fig. 3

Fig. 4

Fig. 3. Scatchard plot of cAMP binding activities. The assay was performed using periplasmic shock fluid (■) and shock pellet cell extract (●) as described in Methods. The amount of cAMP bound (B) was calculated from the amount of radioactivity retained by the filter. The amount of free cAMP (F) was calculated from the total amount of cAMP added to the mixture less the amount bound.

Fig. 4. cAMP binding activities during development of *M. xanthus*. Periplasmic shock fluid (●) and post-shock pellet cell-free extract (■) were obtained from the same batch of cells immediately after harvesting. Binding data represent averages of four separate experiments. The bars indicate the highest and lowest values detected.

During development, *M. xanthus* exhibited two peaks of cGMP binding activity; a minor but reproducible peak was observed at about 18 h and a major peak occurred at around 40 h, the time of maximum aggregation (Fig. 2). Thereafter the binding decreased to initial levels.

Properties of cAMP binding proteins

cAMP binding activity was also detected in periplasmic shock fluid. Binding was proportional to protein concentration in the range 0–0.3 mg protein per assay and complete within 10 s at 4 °C. The binding of labelled cAMP was not inhibited by ATP, cGMP, AMP, GTP or GMP over the concentration range 0–10 μM. Scatchard analysis of the partially purified periplasmic cAMP binding protein indicated the presence of only one class of binding sites with an apparent K_D of 1 μM (Fig. 3).

cAMP binding was also detected in the post-shock pellet. This activity had properties which were virtually identical to the cytoplasmic cAMP binding activity reported by Orłowski (1980), i.e. ATP, AMP, GTP, GMP or cGMP had no significant effects on cAMP binding at concentrations of 50 μM and a Scatchard plot of cAMP binding activity was linear with a K_D of 57 nM (Fig. 3).

During development, cytoplasmic cAMP binding activity exhibited a peak early in development at around 8–18 h and a subsequent decline to relatively constant levels (Fig. 4). The periplasmic cAMP binding activity, however, exhibited a peak around 45–55 h.

DISCUSSION

A cGMP-specific periplasmic binding protein and two cAMP binding proteins distinguishable by their elution patterns on DEAE-cellulose, their K_D values and their cellular locations are present in *M. xanthus*. The cytoplasmic cAMP binding protein has been previously described (Orłowski, 1980).

The properties of these binding proteins are consistent with the premise that they function as receptors which mediate the physiological effects of their ligands. Among these properties are their high specificities and affinities, although the periplasmic cAMP binding protein is marginal in this respect, with a K_D of 1 μM. It is also unlikely that the binding activities are

identifiable with enzymes attacking the ligands. Ligand binding was stable in all cases for at least 30 min with no evidence of hydrolysis and was not affected by the corresponding nucleotide monophosphates. Phosphodiesterases were inhibited in the reaction mixture but in any case exhibited different patterns during development from those of the binding proteins (Ho & McCurdy, 1980). The K_D values of the cAMP binding proteins both differed significantly from the K_D for cAMP-PD (2.4×10^{-7} M, Yajko & Zusman, 1978). It is unlikely therefore that PD interfered with the assay.

It would be consistent with a physiological receptor function if the location and levels of activity of the binding proteins corresponded to the site of action of their ligands as well as changes in their cellular levels. Accordingly, the periplasmic location of the cGMP binding protein would make it accessible to its extracellular and chemotactic ligand (Ho & McCurdy, 1979). Furthermore, as might be expected, it increased in activity concomitantly with the increase in extracellular cGMP concentration which was associated with fruiting body aggregation (Ho & McCurdy, 1980). Similarly, the cytoplasmic cAMP binding protein increased concomitantly with the increased intracellular cAMP levels observed upon fruiting body induction, an observation that supports the premise that it functions with cAMP in the derepression of morphogenesis in a manner analogous to CAP protein in *E. coli* (Zubay *et al.*, 1970).

The periplasmic cAMP binding protein exhibited a peak of activity at the later stages of development which corresponded to a significant increase in the extracellular concentration of cAMP (Ho & McCurdy, 1980). Whether such a correlation is of significance in fruiting body formation is unknown.

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