Rapid, transient methylation of four proteins in aggregative amoebae of *Dictyostelium discoideum* as a response to stimulation with cyclic AMP

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In *Dictyostelium discoideum*, extracellular cAMP induces chemotaxis and cell aggregation. Suspensions of cAMP-sensitive cells are shown to respond to a 10^{-6} M cAMP-pulse with increased methylation of 4 proteins with app. M_r 110000, 46000, 28000 and 16000. The M_r 110000 and 28000 proteins show a triphasic response with maxima 15, 60 and 150–180 s after stimulation. The responses of the M_r 46000 and 16000 proteins are monophasic, maxima being reached 3 and 15 s after stimulation, respectively. Optimal responses of methylation are observed over 10^{-7}–10^{-6} M cAMP. The methylation reaction may be involved in the processing of the chemotactic signal.

Protein carboxymethylation *Chemotaxis* *Cyclic AMP* *Dictyostelium discoideum* *Signal transduction* *Cell aggregation*

1. INTRODUCTION

During the unicellular, amoeboid stage of its life-cycle, the cellular slime mold *Dictyostelium discoideum* lives in the soil and feeds on bacteria. After exhaustion of the food supply, the amoebae pass an interphase, followed by cell aggregation. The cell aggregate becomes a pseudoplasmodium or 'slug', moving over the substrate and finally differentiating into a fruity body, consisting of a stalk with spores embedded in a slime droplet on its top. After dispersal spores germinate, thus forming new amoebae [1].

Cell aggregation is mediated by chemotaxis to cyclic AMP [2], which is detected by cell surface receptors [3–6]. Addition of cAMP to starved cells induces several biochemical responses, such as calcium movements, a transient increase of intracellular cGMP, protein methylation, phospholipid demethylation, accumulation of dephosphorylated myosin heavy chains and signal relay by release of intracellular cAMP (review [7]). Protein methylation is the least well-known of these responses. A protein of M_r 120000 has been shown to respond with increased methylation within 15 s after stimulation with 10^{-6} M cAMP [8,9].

Here, I show that protein methylation in *Dictyostelium* involves at least 4 methyl-accepting compounds. The methylation response of one of these, the M_r 46000 protein, is extremely rapid, reaching peak values within 3 s after stimulation with 10^{-6} M cAMP. Methylation of this protein seems to be one of the very first biochemical events after stimulus administration, preceding both the peak of intracellular cGMP [10–12] and the induction of pseudopod formation [13].

2. MATERIALS AND METHODS

2.1. *Materials*

L-[*methyl*-^{3}H]Methionine (15 Ci/mmol) was obtained from Amersham International (Bucks).

2.2. *Organism*

Dictyostelium discoideum NC-4 (H) was used for all experiments. Cells were grown on a solid

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medium (3.3 g peptone, 3.3 g glucose, 4.5 g KH_2PO_4, 1.5 g $Na_2HPO_4.H_2O$ and 15 g agar/l) and harvested as in [14]. After the cells had been harvested, they were starved by shaking in 10 mM Na,K-phosphate buffer (pH 6.5) (cell density 10^7/ml, duration 5 h at 20°C).

2.3. *Methylation*

Starved cells, preincubated with cycloheximide (250 μg/ml during 1.5 h and prelabeled with L-[*methyl*-^{3}H]methionine (20 μCi/ml) for 0.5 h, were stimulated with different [cAMP] as in [8]. The reaction was stopped after different time intervals by addition of 0.1 vol. 70% $HClO_4$. Perchlorate extracts were centrifuged (12000 rev./min, 5 min, Eppendorf centrifuge) and stored overnight at 4°C. Protein pellets (being equivalent to 2×10^7 cells) were resuspended in 50 μl of a solution containing 0.6 M acetic acid, 5 M urea, 1% 2-mercaptoethanol and 2% *N*-cetylpyridinium-chloride by heating for 5 min at 95°C.

2.4. *Polyacrylamide gel electrophoresis*

Electrophoresis of proteins was performed in 85 × 5 mm rod gels containing 5% acetic acid, 1% *N,N,N',N'*-tetramethylethylenediamine(TEMED), 10% acrylamide, 0.25% *N,N'*-methylene-bisacrylamide, 20% glycerol, 5 M urea and 0.1% ammonium persulfate as in [15]. Gels were manually sliced into 3 mm sections. Sections were incubated overnight in 2 ml Instagel (Packard) and radioactivity was assayed by liquid scintillation counting.

3. RESULTS

Fig. 1 shows the electrophoretic pattern of *Dictyostelium* homogenates after in vivo incubation with 1-[*methyl*-^{3}H]methionine. Both the control and the pattern observed 5 s after stimulation with 10^{-6} M cAMP are presented. At least 4 different peaks show a rapid increase of methylation upon addition of cAMP. Apparent M_r-values of these compounds are 110000, 46000, 28000 and 16000, respectively, the M_r former two peaks being the major components.

Methyl groups incorporated in these methyl-accepting compounds are labile at neutral and alkalie pH. Overnight incubation at 22°C in 0.1 M Tris–HCl (pH 7.5 or 10.5) causes a reduction of radioactivity in the perchloric acid-precipitable

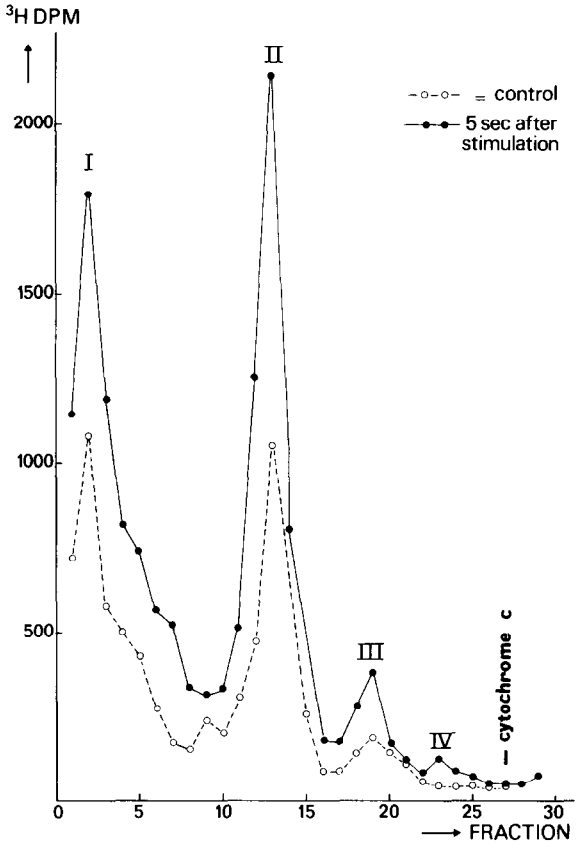

Fig. 1. Electrophoretic pattern of *D. discoideum* homogenates after incubation with 1-[*methyl*-^{3}H]methionine. Both the control pattern and that obtained 5 s after stimulation with 10^{-6} M cAMP are presented. Four components respond with an increased level of methylation: peak I (R_f 0.05 ± 0.02, M_r 110000), peak II (R_f 0.42 ± 0.06, M_r 46000), peak III (R_f 0.64 ± 0.08, M_r 28000) and peak IV (R_f 0.91 ± 0.08, M_r 16000). Proteins were separated in acetic acid/urea-containing polyacrylamide gels as in [15]. R_f-values of proteins were determined with reference to cytochrome *c*.

material to 15% of a control incubated in 6% $HClO_4$ under the same conditions. Radioactivity in peak I (M_r 110000) is reduced to 22%, in peak II (M_r 46000) to 10%, in peak III (M_r 28000) to 11% and in peak IV (M_r 16000) to 15% of the respective control values, suggesting a mechanism of protein carboxymethylation (and not protein or phospholipid *N*-methylation) for all methyl-accepting compounds. The radioactive product of hydrolysis is volatile. Evaporation of the Tris–HCl super-

natant on a hotplace causes a reduction of radioactivity to 12% of a nonevaporated control, suggesting the hydrolytic product to be methanol.
duct to be methanol.

The protein nature of the methyl acceptors has been demonstrated by incubation with the proteolytic enzymes subtilisin and trypsin. Overnight incubation of the perchloric acid-pellet with subtilisin (1 mg enzyme/100 mg cells in 100 mM Tris–HCl (pH 7.5) causes a reduction of radioactivity in peak I to 20% of the level in a control resuspended with buffer only, while similar treatment with trypsin results in a reduction of activity to 24%. Since the proteolytic enzymes cause the fractionation of the M_r 110000 protein in several smaller fragments, the number of peaks in the electrophoretic pattern is greatly increased by proteolysis, making quantification of its effect on the low-M_r methyl acceptors difficult. The protein nature of these compounds is suggested by the observation that proteolysis results in a decrease of peak heights and a shift to greater R_f-values.

The time course of methylation is presented in fig. 2. Methylation of peak I is significantly increased 10 s after stimulation. It reaches a maximum 15 s after administration of 10^{-6} M cAMP and returns to the control level within 30 s. After 1 min, a second maximum is reached, the control level now being regained after 90 s. A third maximum occurs 150 s after stimulus administration. The highest level of methylation is ~1.6-fold above the control value. Finally, methylation returns to the normal state within 210 s and is not increased again. Methylation of peak II rises extremely rapidly, differing significantly from control within 1 second and reaching its maximum 3 s after stimulus administration. The maximum level of methylation is ~3.3-fold above the control value, which is regained after ~90 s. Methylation of peak III shows an almost identical time course as that of peak I, maxima being reached 15, 60 and 150–180 s after stimulation. Just as in peak I, the maximum level of methylation is ~1.65-fold above the control value. Peak IV shows a monophasic response with a maximum 15 s after stimulus administration. The methylation state of this protein returns within 45 s to the control level and the maximum is ~2-fold above the control.

A dose–response curve of methylation for all methyl-accepting compounds is presented in fig. 3.

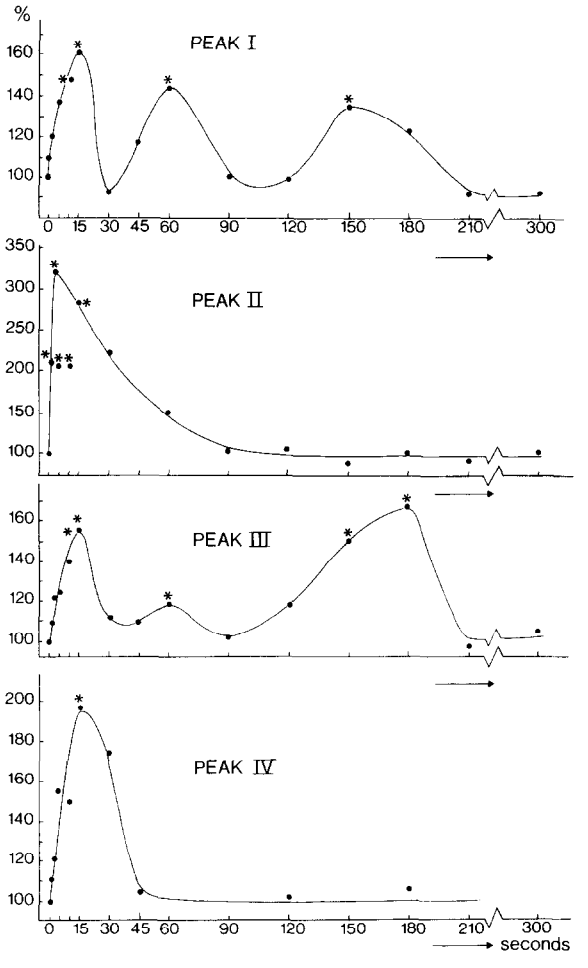

Fig. 2. Time course of protein methylation in *D. discoideum* upon stimulation with 10^{-6} M cAMP. Levels of methylation of the 4 methyl-accepting compounds are presented as percentages of an unstimulated control. Differences between control and experimental points were tested with Wilcoxon's Q-test. Statistically significant differences (at the 5% level) are indicated by asterisks. Each experimental point is a mean of 3–6 independent observations.

The methylation response is shown to be maximal at concentrations between 10^{-7}–10^{-6} M cAMP. cAMP-levels $> 10^{-6}$ M become inhibitory and half-maximal responses are observed at 2×10^{-8}–4×10^{-8} M cAMP.

5′-AMP was unable to elicit any of the responses observed with cAMP.

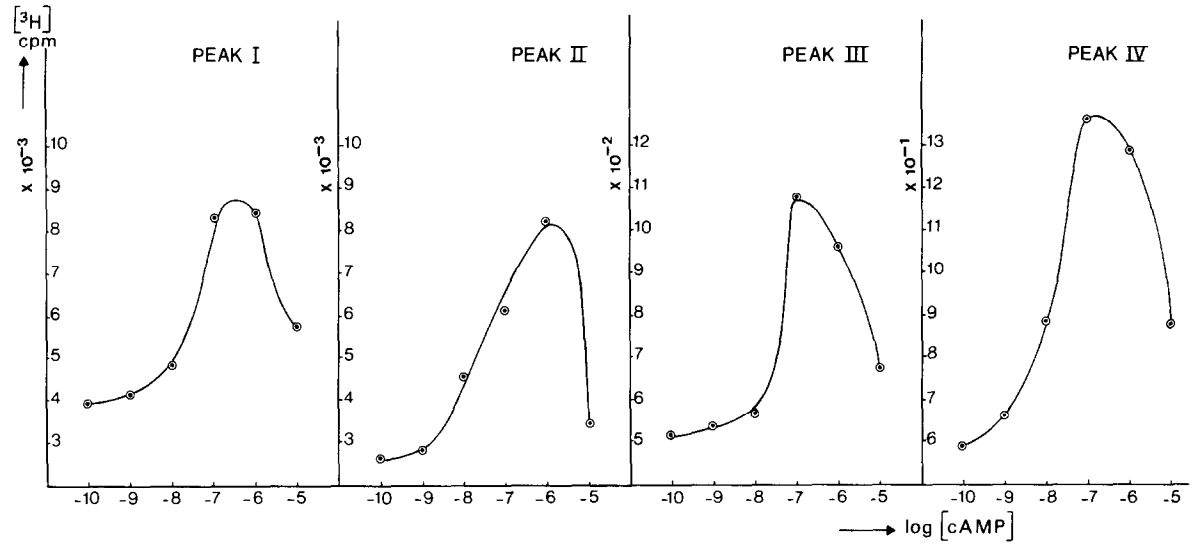

Fig. 3. Dose–response curves of protein methylation in *D. discoideum* stimulated with cAMP. Peak numbers are the same as in fig. 2. Half-maximal stimulation is observed at 4.0×10^{-8} M (peak I), 4.0×10^{-8} M (peak II), 4.5×10^{-8} M (peak III) and 2.0×10^{-8} M cAMP (peak IV). Methylation is optimal at 10^{-7} M cAMP (peaks III, IV), 10^{-7}–10^{-6} M cAMP (peak I) and 10^{-6} M cAMP (peak II), while cAMP $> 10^{-6}$ M becomes inhibitory. Methylation levels were determined 15 s after administration of stimulus.

4. DISCUSSION

Protein methylation during chemotaxis in *Dictyostelium* has been studied in [8,9]. These data both confirm and expand those results. Suspensions of cAMP-sensitive cells of *Dictyostelium discoideum* responded to a cAMP-pulse with increased methylation of a membrane-bound protein of M_r 120000 [8], protein methylation reached a peak 15 s after addition of 10^{-6} M cAMP. Dose–response curves were not presented in [8], but it was stated that no response could be observed at $< 10^{-8}$ M cAMP. The effect of cAMP on the methylation of M_r 120000 protein was re-examined in [9]; the first peak of methylation, occurring 15 s after stimulation with 10^{-7} M cAMP, was followed by a second peak, occurring ~210 s after stimulus administration. In [8,9] methyl-groups incorporated in methyl-accepting protein were very unstable at alkaline pH, especially at high temperature. Proteins were separated in SDS–polyacrylamide gels using an electrode buffer of pH 8.3 in [8,9]. I have examined the suitability of the acetic acid–urea system in [15] for the separation of methyl-accepting proteins in *Dictyostelium discoideum*. Under identical conditions [8], recovery of methyl groups in the M_r 120000 region proved to be 20-fold better in acetic acid–urea gels than in a Tris–glycine buffer system at pH 8.3.

In addition to [8,9], I found here 3 other peaks responding with increased methylation upon stimulation with cAMP. As stated in [16], a true methyl acceptor protein profile from a complex mixture can be obtained only in electrophoretic conditions where the recovery is optimal, since methyl-esters on various proteins have different stability. The M_r 110000 protein observed here is probably identical to the M_r 120000 compound in [8]. Because this protein shows a very low R_f-value in an acetic acid–urea separation system, its M_r could not be determined with great accuracy. Since the M_r 28000 and 110000 compounds showed almost identical time courses of methylation and very similar peak heights, the M_r 28000 protein could be a monomer or disintegration product of the tetrameric M_r 110000 methyl-acceptor. Because the M_r 46000 and 16000 proteins show a unique time course of methylation, they appear to be different compounds with a different accessibility for

protein carboxymethylase.

As observed in [8], protein methylation seems to be stimulated by $\geqslant 10^{-8}$ M cAMP. At $> 10^{-6}$ M, however, methylation is inhibited. cAMP at $> 10^{-6}$ M must be considered unphysiological, since above this level, amoebae show no longer positive chemotaxis to cAMP [17].

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