The EMBO Journal Vol.2 No.4 pp.535 – 542, 1983

Myosin heavy chain kinase inactivated by Ca^{2+}/calmodulin from aggregating cells of *Dictyostelium discoideum*

H.Maruta*, W.Baltes, P.Dieter[1], D.Marmé[1] and G.Gerisch

Max-Planck-Institut für Biochemie, D-8033 Martinsried bei München, and [1]Institut für Biologie III, Universität Freiburg, D-7800 Freiburg/Br., FRG

Communicated by G.Gerisch
Received on 31 December 1982; revised on 9 February 1983

Soluble myosin heavy chain kinases (MHC kinases) were partially purified from growth phase and aggregation-competent cells of *Dictyostelium discoideum*. In the aggregation-competent cells, two MHC kinases were distinguishable. One of these enzymes, called MHC kinase II, was inactivated by Ca^{2+} and calmodulin in a highly temperature-dependent reaction. A MHC kinase found in growth phase cells did not have these regulatory properties. Substrate specificities were analysed for MHC kinase II and for the MHC kinase from growth phase cells. Both enzymes phosphorylated threonine residues of the myosin heavy chains of *D. discoideum* and *Physarum polycephalum*. Phosphopeptide mapping of *D. discoideum* myosin and determination of the stoichiometry of its phosphorylation suggested the presence of two phosphorylation sites per heavy chain. Both sites were contained within a 38-kd chymotryptic fragment. The inactivation of MHC kinase II by Ca^{2+} plus calmodulin suggests this enzyme has a role in the regulation of myosin functions during the chemotactic response of a cell. The phosphorylated myosin had about one third the actin-activated Mg^{2+}-ATPase activity of the non-phosphorylated myosin. Previous findings indicated that stimulation of *D. discoideum* cells with the chemo-attractant cAMP increases the cytoplasmic Ca^{2+} concentration. Under these conditions MHC kinase II might be inhibited and the dephosphorylated, more active form of myosin would accumulate.

Key words: calmodulin/chemotaxis/calcium/myosin phosphorylation/phosphothreonine

Introduction

Phosphorylation regulates the functions of myosins in vertebrate smooth muscle and non-muscle cells. In these cells, myosin light chains are phosphorylated by Ca^{2+}/calmodulin-dependent kinases (Sherry *et al.*, 1978; Hathaway and Adelstein, 1979). Light chain phosphorylation stimulates the actin-activated Mg^{2+}-ATPase activity and the ability of myosin to form bipolar filaments (Adelstein and Conti, 1975; Suzuki *et al.*, 1978; Scholey *et al.*, 1980).

The heavy chains of myosins are phosphorylated in *Acanthamoeba castellanii* (Maruta and Korn, 1977) and *Dictyostelium discoideum* (Rahmsdorf *et al.*, 1978). *Acanthamoeba* contains at least three different myosin isoenzymes. The phosphorylation of myosins IA and IB stimulates the actomyosin Mg^{2+}-ATPase activities (Maruta and Korn, 1977; Maruta *et al.*, 1979), the phosphorylation of myosin II inhibits the Mg^{2+}-ATPase activity (Collins and Korn, 1980). Phosphorylation of *D. discoideum* myosin results in a decrease of the actin-activated Mg^{2+}-ATPase activity and inhibition of the formation of filaments (Kuczmarski and Spudich, 1980).

Myosin heavy chain phosphorylation in *D. discoideum* is of particular interest because it is regulated in response to chemotactic stimulation of the cells by cAMP (Rahmsdorf *et al.*, 1978). Myosin is dephosphorylated in stimulated cells, and myosin heavy chain kinase (MHC kinase) associated with an actomyosin-containing membrane preparation is inhibited through Ca^{2+} plus calmodulin (Malchow *et al.*, 1981). These results are complemented by the finding of an increased Ca^{2+} influx into cAMP-stimulated cells (Wick *et al.*, 1978) and of the presence of calmodulin in *D. discoideum* cells (Bazari and Clarke, 1981), suggesting that the regulation of MHC kinase by Ca^{2+} and calmodulin is involved in the chemotactic response.

Growth phase cells of *D. discoideum* show a very weak chemotactic response to cAMP. Starved cells become highly responsive to cAMP as they acquire the ability to aggregate (Bonner *et al.*, 1969). In these aggregation-competent cells we have identified a soluble Ca^{2+}/calmodulin-inactivated MHC kinase which has not been detected in growth phase cells.

Results

Two MHC kinases partially purified from aggregation-competent cells

Cells were allowed to develop up to the aggregation-competent stage, and MHC kinases were partially purified from the soluble fraction of cell homogenates (see Materials and methods). After removal, by DEAE-cellulose chromatography, of various kinase inhibitors including calmodulin, two MHC kinase activities were separated by fractionated ammonium sulfate precipitation. The material precipitated with 1.5 M ammonium sulfate was called MHC kinase fraction I. After raising the ammonium sulfate concentration to 2.0 M a second precipitate was obtained and designated MHC kinase fraction II. The MHC kinases partially purified from these fractions will be referred to as MHC kinases I and II, respectively. Purification steps included affinity chromatography on Sepharose-bound myosin, calmodulin and histone II-A: at 4°C only MHC kinase I bound to these affinity columns. On Sephadex G-100, MHC kinase II showed an apparent mol. wt. of ~70 000 daltons, while the MHC kinase I activity was eluted in the void volume (Figure 1). Both kinases bound to Blue Sepharose. They phosphorylated myosins from *D. discoideum* and *Physarum polycephalum* specifically on the heavy chains, as shown for MHC kinase II in Figure 2. The preparations proved to be free of detectable MHC phosphatase activity, when tested with ^{32}P-labeled myosin.

Preparation of MHC kinase from growth phase cells

We have not been able to detect substantial MHC kinase activities by applying the purification procedures developed for aggregation-competent cells to the soluble fraction from growth phase cells. Using a modified purification procedure, MHC kinase activity was recovered from growth phase cells in an actomyosin-containing precipitate, as described in

*To whom reprint requests should be sent.

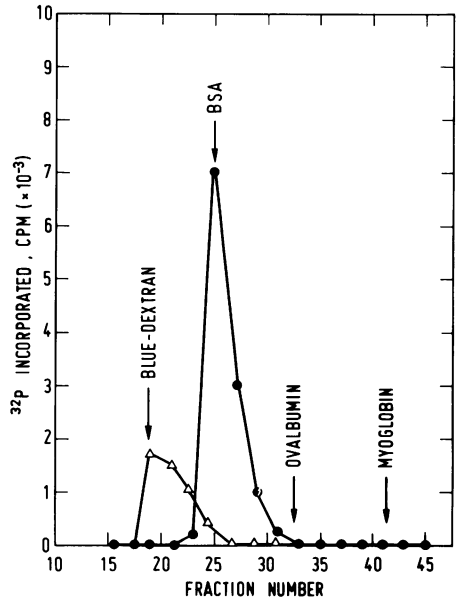

Fig. 1. Gel filtration of MHC kinase I (△) and II (●) of aggregation-competent cells. The partially purified MHC kinases I and II were run separately on Sephadex G-100.

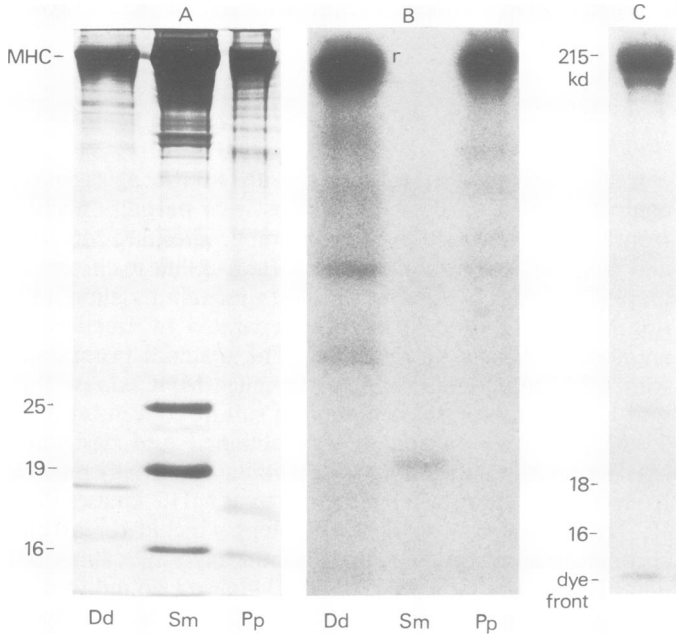

Fig. 2. Phosphorylation of different myosins with MHC kinase II. **A,B,** MHC kinase II partially purified according to procedure 1, as outlined in Materials and methods, up to the Sephadex G-100 step; **C,** MHC kinase II further purified on hydroxylapatite. Myosins phosphorylated with [γ-^{32}P]ATP were run in SDS-polyacrylamide gels for separation of the heavy and light chains. Photographs show polypeptides stained with Coomassie blue **(A)**, or autoradiograms **(B,C)**. Dd, myosin from aggregation-competent cells of *D. discoideum*; Sm, skeletal muscle myosins from rabbit; Pp, myosin from *P. polycephalum*. **A** and **B** show the same gel. The myosin used for **C** was from aggregation-competent cells of *D. discoideum*. In **A** the 16-, 19- and 25-kd light chains of the skeletal muscle myosins are indicated, in **C** positions of the 16- and 18-kd light chains, and of the heavy chains (215 kd), of *D. discoideum* myosins are shown. The figure shows phosphorylation of the heavy chains of *D. discoideum* and *P. polycephalum* myosin, but not phosphorylation of the heavy chains of skeletal muscle myosins. The faint phosphorylation of the 19-kd light chain of skeletal muscle myosins was due to endogenous light chain kinase activity in the myosin preparation purchased from Sigma.

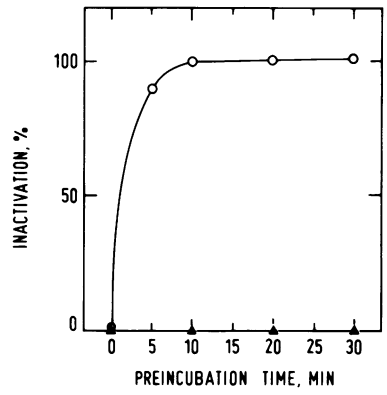

Fig. 3. Effect of temperature on the inactivation of MHC kinase II by Ca^{2+} plus calmodulin. The partially purified kinase was preincubated for the periods of time indicated on the abscissa in buffer D (see Materials and methods) supplemented with 0.2 mM Ca^{2+} and 4 μM calmodulin. ○, preincubation at 35°C; ▲, at 4°C.

Materials and methods. The precipitate was extracted with buffer containing 0.1 M NaCl and the soluble MHC kinase thus obtained was partially purified.

The MHC kinase prepared from growth phase cells bound to calmodulin-Sepharose in the cold, as did MHC kinase I from aggregation-competent cells. On Sephadex G-150, MHC kinase activity from growth phase cells was collected in a major peak corresponding to a protein of ~70 000 daltons. It was only in this respect that the MHC kinase from growth phase cells resembled MHC kinase II.

Inactivation of MHC kinase II by Ca^{2+} plus calmodulin

Inactivation of MHC kinase II was observed when the partially purified enzyme was preincubated with Ca^{2+} and calmodulin before myosin and ATP were added (Figure 3). Inactivation was highly temperature dependent. Ninety per cent inactivation was obtained after 5 min of preincubation at 35°C. At 4°C no inactivation was detected within a period of 30 min. Neither Ca^{2+} nor calmodulin alone inactivated MHC kinase II (Figure 4). Table I shows that neither EGTA nor trifluoperazine (TFP), an inhibitor of calmodulin-mediated processes, immediately reactivated MHC kinase II after preincubation of the enzyme with Ca^{2+} and calmodulin.

MHC kinase I was partially inhibited by calmodulin. This inhibition differed from the inactivation of kinase II in three respects: (1) it did not depend on free Ca^{2+}; (2) it was not blocked by TFP (Table II); and (3) it was faster, i.e., did not require a preincubation period of the order of minutes. Like MHC kinase I, the MHC kinase partially purified from growth phase cells was inhibited by calmodulin in the presence of EGTA, and 0.2 mM Ca^{2+} did not reinforce the inhibition (Figure 5). It is likely that the Ca^{2+}-independent inhibition of these kinases is due to ionic interaction with the negatively charged calmodulin, and the effect might not have a biological significance.

Phosphopeptide maps indicating different MHC kinases have the same substrate specificity

Myosin from either growth phase or aggregation-competent cells was phosphorylated by MHC kinase from growth phase cells or by MHC kinase II. The ^{32}P-labeled myosin heavy chains were then digested by a mixture of trypsin and chymotrypsin, the peptides separated in two dimen-

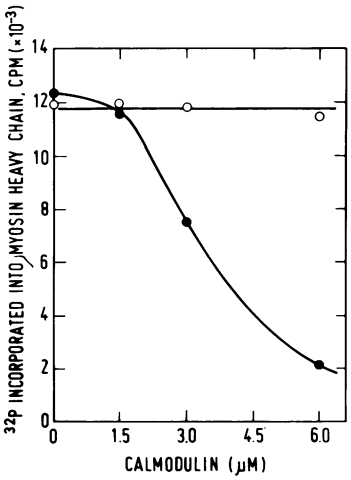

Fig. 4. Requirement of Ca^{2+} and calmodulin for the inactivation of MHC kinase II. The partially purified kinase was preincubated for 5 min at 35°C in buffer D containing calmodulin as indicated on the abscissa, and either 0.2 mM Ca^{2+} (●) or 1 mM EGTA (○).

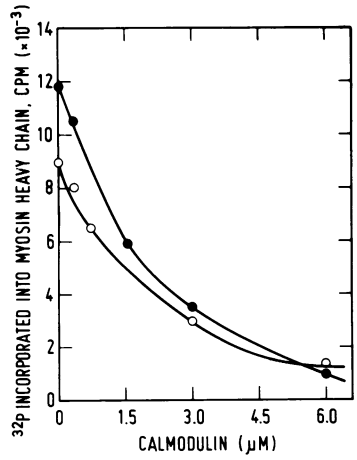

Fig. 5. Inhibition of MHC kinase from growth phase cells by calmodulin in the presence and absence of Ca^{2+}. Activity of the partially purified kinase was assayed by incubation with myosin and [γ-^{32}P]ATP for 15 min at 35°C in the presence of calmodulin as indicated, and either 0.2 mM Ca^{2+} (●) or 1 mM EGTA (○).

Table I. Effect of EGTA and TFP on the Ca^{2+}/calmodulin- dependent inactivation of MHC kinase II

Added before preincubation	Added after preincubation	Percentage of kinase activity
–	–	100[a]
Calmodulin	–	1
Calmodulin and EGTA	–	97
Calmodulin and TFP	–	96
Calmodulin	EGTA	3
Calmodulin	TFP	2

MHC kinase II was preincubated and tested at 35°C in buffer D (see Materials and methods) plus 0.2 mM Ca^{2+}. At the end of a preincubation period of 30 min, 0.2 mM [γ-^{32}P]ATP and 200 μg myosin/ml were added and the reaction mixture incubated for 10 min. Other additions were, as indicated, 4 μM calmodulin from bovine brain, 1 mM EGTA, 0.1 mM TFP. The radioactivity on the myosin heavy chain was determined by scanning the autoradiogram of a 7.5% SDS-polyacrylamide gel.
[a]The 100% value corresponds to the incorporation of ~0.1 mol phosphate/mol of myosin heavy chain during the incubation period.

Table II. Effect of calmodulin on MHC kinase I

Added during the assay	Percentage of kinase activity
EGTA	100
Ca^{2+}	100
Calmodulin + EGTA	40
Calmodulin + Ca^{2+}	36
Calmodulin + Ca^{2+} + TFP	15

1 mM EGTA, 0.2 mM $CaCl_2$, 4 μM calmodulin from bovine brain, and 0.1 mM TFP were added as indicated.

sions by electrophoresis and t.l.c., and the phosphopeptides visualized by autoradiography (Figure 6A,B). All the phosphopeptide maps were superimposable. These results indicate that the myosins prepared from growth phase or aggregation-competent cells did not differ in the region of the phosphorylatable amino acid residues. Furthermore, the identity of phosphopeptide patterns indicates that the amino acid residues phosphorylated by MHC kinase II and those phosphorylated by MHC kinase from growth phase cells were identical or in positions located so closely to each other that neither trypsin nor chymotrypsin could cleave between them.

To investigate further whether the two kinases have similar if not the same substrate specificities, myosin from *P. polycephalum* was phosphorylated (Figure 6C and D). The phosphopeptide maps obtained from the *Physarum* myosin heavy chains were distinct from those of *Dictyostelium*, indicating that the myosins of the two species differed in the phosphorylated portions of their heavy chains.

The phosphopeptide maps of *Physarum* myosin phosphorylated by either MHC kinase from growth phase cells or MHC kinase II were the same. This result shows that, in the case of *Physarum* myosin as well, no cleavage site for trypsin or chymotrypsin was located between the amino acid residues phosphorylated by the two different kinases. Thus, probably, the MHC kinase from growth phase cells has the same substrate specificity as MHC kinase II.

Threonine is the phosphorylated amino acid

^{32}P-labeled myosin heavy chains were acid-hydrolysed and the phosphorylated amino acids identified after two-dimensional separation. Regardless of whether MHC kinase from growth phase cells or MHC kinase II was used, and whether myosin was prepared from growth phase or aggregation-competent cells, the radioactivity was associated only with threonine (Figure 7). Also in myosin heavy chains from *P. polycephalum*, which were phosphorylated by either kinase, the phosphorylated amino acid was identified as threonine. Rabbit skeletal muscle myosin incubated with MHC kinase II was not phosphorylated (Figure 2).

Single or multiple phosphorylation sites?

Peptide mapping has shown that multiple phosphopeptides are derived from the myosin heavy chains. In assessing whether these represent distinct phosphorylation sites, we have subjected the phosphopeptides to more rigorous proteolytic cleavage to see whether a single phosphopeptide might be produced. After two-dimensional separation, the major ^{32}P-labeled peptides obtained with either trypsin or chymotrypsin were scraped off thin layer plates and digested further with the same enzyme as before. Tryptic digestion yielded

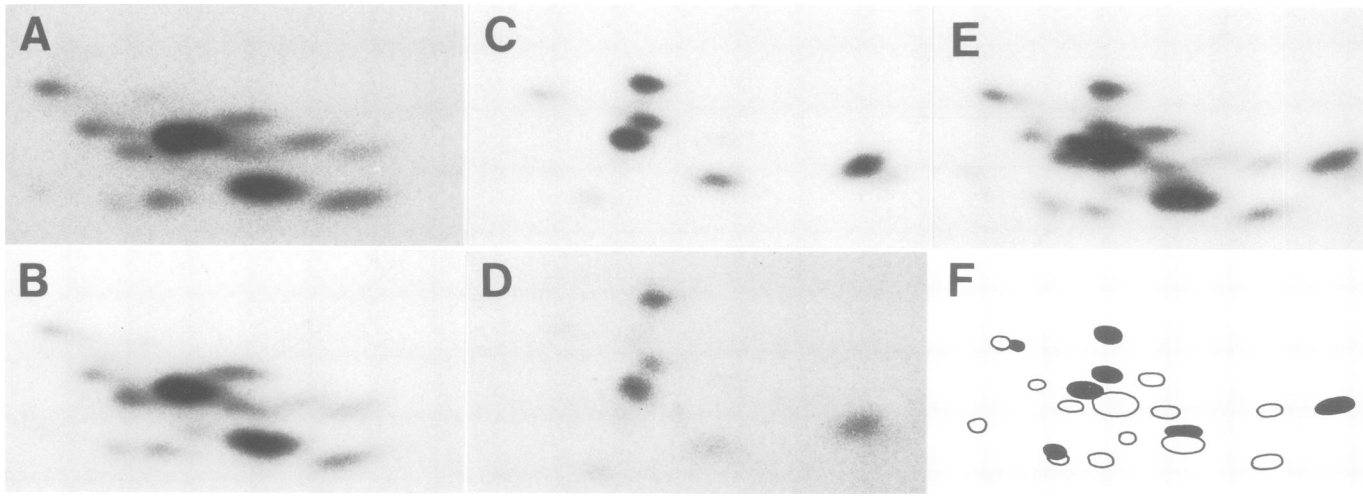

Fig. 6. Phosphopeptide maps obtained after digestion of ^{32}P-labeled myosin heavy chains with trypsin plus chymotrypsin. **A, B,** myosin from aggregation-competent cells of *D. discoideum;* **C, D,** from *P. polycephalum*; **E,** mixture of the two myosins; **F,** diagram indicating the *D. discoideum*-derived phosphopeptides in **E** (open areas), and those from *P. polycephalum* (closed areas). Phosphorylation was performed with MHC kinase from growth phase cells **(A,C,E)** or with MHC kinase II **(B,D)**. Phosphopeptide maps obtained with myosin from growth phase cells of *D. discoideum* were indistinguishable from the ones shown in **A** and **B** (data not shown).

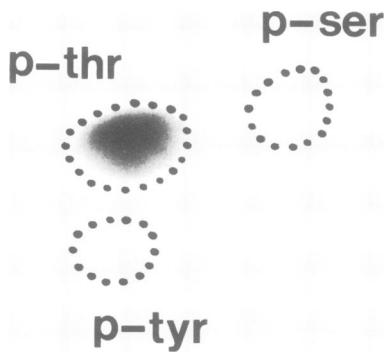

Fig. 7. Identification of phosphothreonine in acid hydrolysates of myosin heavy chains. Myosin of aggregation-competent cells was phosphorylated by MHC kinase II and [γ-^{32}P]ATP. Phosphoamino acids were separated by two-dimensional electrophoresis and the positions of standards indicated by dotted lines. The label seen in the autoradiogram was exclusively associated with phosphothreonine.

three major labeled peptides (nos. 4, 5, 6 in Figure 8) besides a number of minor ones. The major peptides gave rise upon further cleavage to two sets of phosphopeptides; nos. 3, 6, 7, 8, 9, 10 represented one group, nos. 1, 2, 4, 5 the other.

Chymotryptic digestion produced two major phosphopeptides which differed from the tryptic cleavage products (nos. 1 and 5 in Figure 9). But as in the case of trypsin, further chymotryptic digestion converted these products into two distinct sets of phosphopeptides (nos. 1, 2, 3, 4 and 5, 6).

The simplest interpretation of these results is that at least two phosphorylatable threonine residues are located on the myosin heavy chain. We cannot rule out the possibility, however, that the endoproteases used can cleave neighbouring peptide bonds on the myosin heavy chain, giving rise to peptides with different C-terminal or N-terminal ends, that all include a common amino acid sequence. It is also possible that the two phosphopeptides come from different heavy chain isoenzymes.

If there are two different phosphorylatable sites per heavy chain, the question is whether these sites are located close to, or distant from, each other. ^{32}P-labeled heavy chains were, therefore, partially digested with chymotrypsin, the fragments separated by SDS-polyacrylamide gel electrophoresis, extracted from the gel and subjected to phosphopeptide mapping with a mixture of trypsin and chymotrypsin. Two major phosphorylated fragments with apparent mol. wts. of 132 and 38 kd were identified. Both fragments gave the same phosphopeptide pattern as shown in Figure 6A and B for intact heavy chains, indicating that all the phosphorylatable sites are located in the 38-kd fragment which accordingly is derived from the 132-kd fragment.

Stoichiometry of myosin heavy chain phosphorylation by MHC kinase II

Myosin prepared from growth phase cells is phosphorylated to an extent of 0.35 mol of phosphate per mol of heavy chain (Kuczmarski and Spudich, 1980). Myosin prepared from aggregation-competent cells proved to be practically free of phosphate. There were <0.02 mol of phosphate present per mol of heavy chain. We have used this myosin as a substrate in order to determine the stoichiometry of myosin heavy chain phosphorylation by MHC kinase II.

When protein content of the myosin preparation was determined according to Lowry with bovine serum albumin (BSA) as the standard, the maximal amount of phosphate incorporated was 1 mol per mol of heavy chain. When the calculation was based on quantitative amino acid analysis, the ratio was close to 2 mol of phosphate per mol of myosin heavy chain (Figure 10). We assume this latter ratio is more accurate.

Effects of myosin heavy chain phosphorylation on actin-activated Mg^{2+}-ATPase

Dephosphorylated myosin from growth phase cells was reported to have an actin-activated Mg^{2+}-ATPase activity 2- to 3-fold higher than myosin fully phosphorylated by a MHC kinase from growth phase cells (Kuczmarski and Spudich, 1980). Since MHC kinase II phosphorylated the same sites as myosin kinase purified by us from growth phase cells, the effect of heavy chain phosphorylation by the different kinases should be similar. Table III shows that the non-phosphorylated myosin from aggregation-competent cells

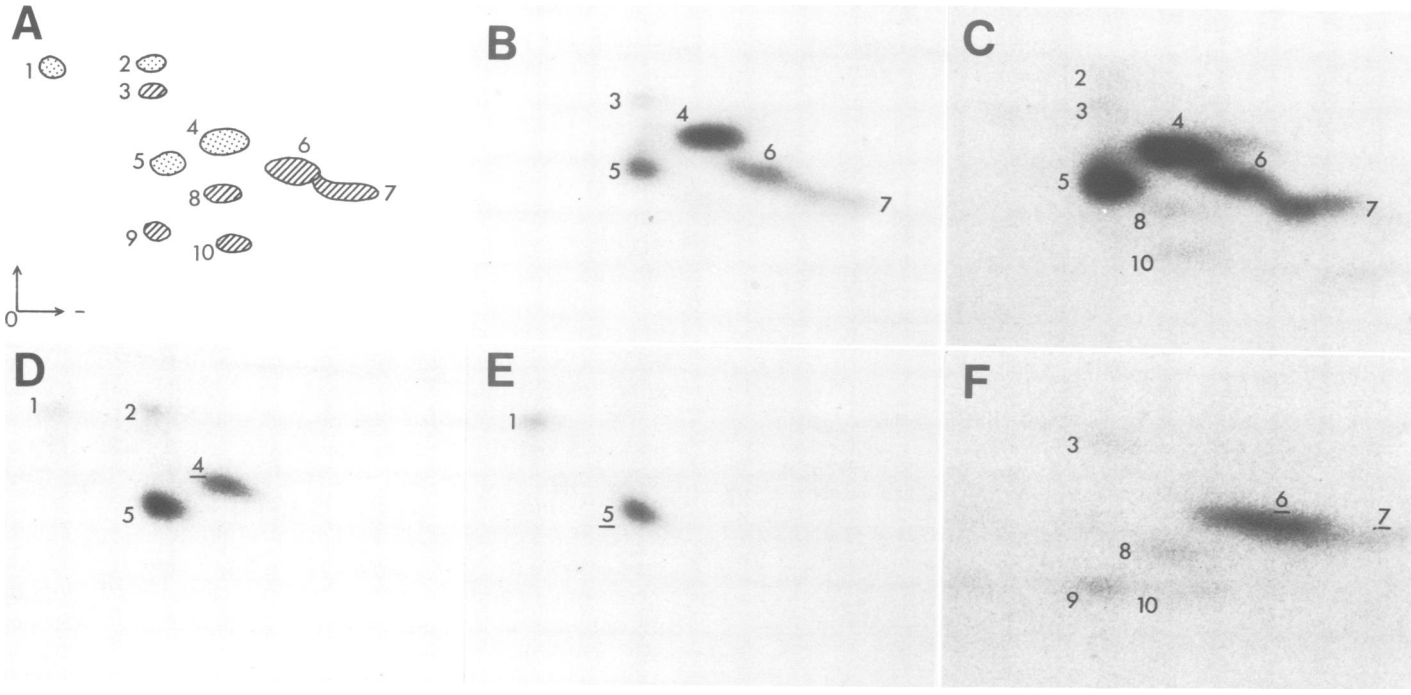

Fig. 8. Autoradiograms of phosphopeptides obtained by tryptic digestion of *D. discoideum* myosin heavy chains. **A**, summary of the two-dimensional phosphopeptide maps. The two separate groups of phosphopeptides obtained are distinguished by dotting and hatching. The origin (○) and direction of electrophoresis is indicated. **B, C**, two-dimensional phosphopeptide maps of two independent preparations. **D,E,F**, products of continued trypsin digestion of the major peptides nos. 4, 5 and 6 plus 7, respectively. The phosphopeptides scraped off the cellulose sheets and subjected to further cleavage are underlined.

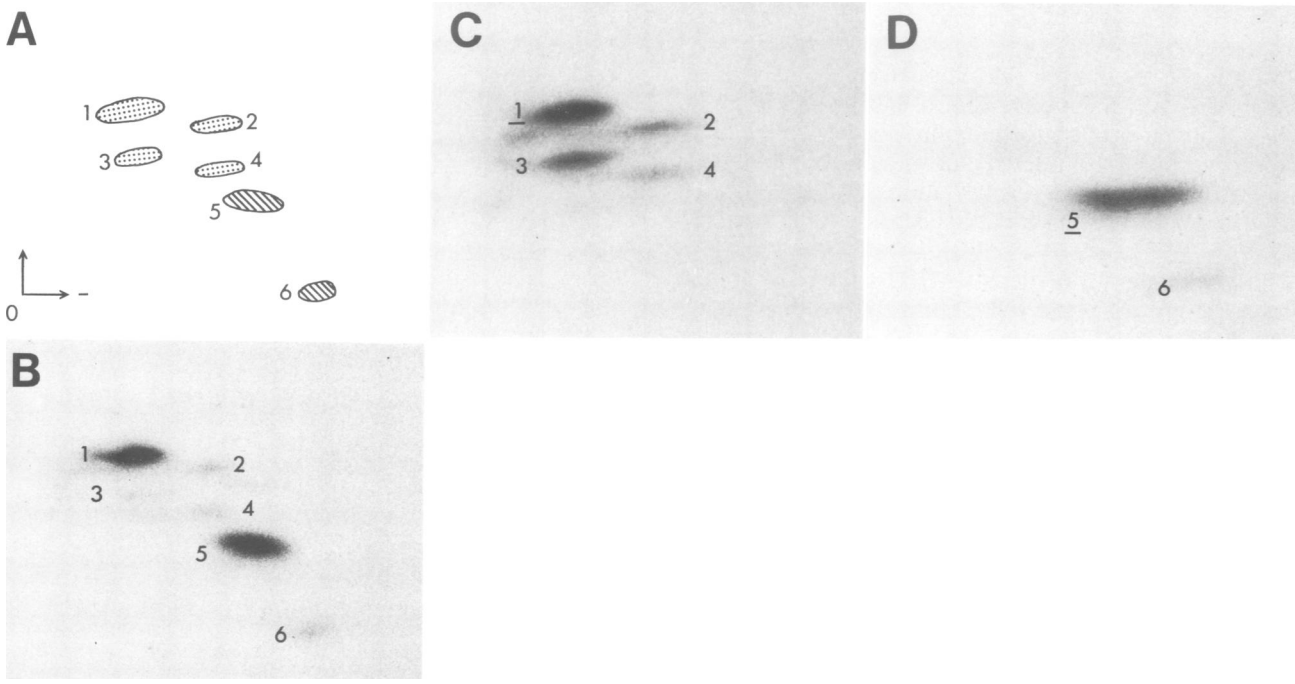

Fig. 9. Autoradiograms of phosphopeptides obtained by chymotryptic digestion of *D. discoideum* myosin heavy chains. **A**, summarizing phosphopeptide maps as in Figure 8. **B**, products of the first digestion. **C,D**, phosphopeptides no. 1 and 5, respectively, as underlined, were subjected to further chymotryptic digestion and the products mapped.

had a 2- to 3-fold higher actin-activated Mg^{2+}-ATPase activity than an aliquot phosphorylated maximally by MHC kinase II.

Discussion

Our studies of myosin phosphorylation in *D. discoideum* indicate the presence of at least two soluble myosin heavy chain kinases, MHC kinase I and II, in aggregation-competent cells. A distinctive feature of MHC kinase II is its inactivation by Ca^{2+} plus calmodulin. The inactivation of this enzyme is strongly temperature dependent. At 4°C the affinity of MHC kinase II to calmodulin-Sepharose was negligi-

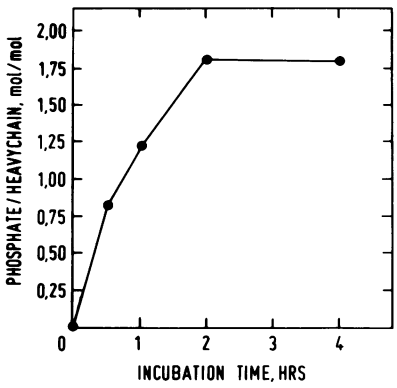

Fig. 10. Stoichiometry of myosin heavy chain phosphorylation. Myosin from aggregation-competent cells of *D. discoideum* was phosphorylated using MHC kinase II. The phosphate incorporated was quantified and the myosin content was estimated as described in Materials and methods.

Table III. Effect of phosphorylation on myosin ATPase activities

Myosin	Ca^{2+}-ATPase	Mg^{2+}-ATPase	
		− Actin	+ Actin
Unphosphorylated[a]	3.4	0.07	0.36
Phosphorylated[b]	3.3	0.07	0.14

[a]Myosin from aggregation-competent cells not treated by MHC kinase II.
[b]Similar myosin fully phosphorylated by incubation with MHC kinase II for 4 h under the same conditions as in Figure 10.
ATPase activities are expressed in μmol ATP hydrolysed/min/mg myosin. The actin used was from rabbit skeletal muscle.

ble. Accordingly, the enzyme was not detectably inactivated at this temperature. The mechanism of inactivation is as yet unknown. Thus it is open to question whether calmodulin reacts directly with MHC kinase II, or with an accompanying protein. Phosphorylation or adenylylation of MHC kinase II are excluded as a basis of inactivation, because ATP is not required.

A kinase with the properties of MHC kinase II has not been found in growth phase cells. From these cells we have obtained another MHC kinase which is probably identical with a MHC kinase partially purified from similar cells by Kuczmarski and Spudich (1980). The presence of MHC kinase II in aggregation-competent cells may be related to the strong chemotactic responsiveness of these cells to cAMP, suggesting a function of MHC kinase II in chemotactic signal processing. It is, however, not established that MHC kinase II is the only enzyme that links the regulation of myosin phosphorylation to Ca^{2+} concentrations. Malchow *et al.* (1981) have found MHC kinase activity in an actomyosin-containing membrane fraction of aggregation-competent cells, which is decreased by ~50% in response to Ca^{2+} plus calmodulin. This membrane-associated MHC kinase activity might be due to a special membrane-associated enzyme, or it might represent MHC kinase II in a membrane-bound state.

Both the MHC kinase from growth phase cells and MHC kinase II specifically phosphorylated threonine residues, and the phosphopeptide maps of myosin heavy chains phosphorylated by either kinase were identical. The phosphorylation of threonine distinguishes the myosin phosphorylation studied in *D. discoideum* from myosin heavy or light chain phosphorylation in other organisms where serine residues are phosphorylated.

It is probable that in *D. discoideum* myosin two threonine residues are phosphorylated both of which reside in a 38-kd fragment obtained by partial chymotryptic digestion of the heavy chains. This fragment reacts with a monoclonal antibody 21-96-3 (K.Pagh, unpublished data) which binds near to the end of the myosin tail (Claviez *et al.*, 1982). These findings establish a similarity between heavy chain phosphorylation in *Dictyostelium* myosin and *Acanthamoeba* myosin II. *Acanthamoeba* myosin II is phosphorylated close to the end of the tail, and peptide mapping revealed three phosphorylated peptides indicating three phosphorylation sites per heavy chain. However, all phosphorylated amino acids in *Acanthamoeba* myosin II are serine residues (Coté *et al.*, 1981; Collins *et al.*, 1982).

The results presented here suggest that MHC kinase II is part of a signal processing pathway involved in the chemotactic response to cAMP. The proposed pathway leads from the activation of cell surface receptors by cAMP to an increase of the cytoplasmic Ca^{2+} concentration and subsequent inactivation of MHC kinase II by Ca^{2+} and calmodulin. Rephosphorylation of myosin which is dephosphorylated by phosphatase is thus suppressed. The higher actin-activated Mg^{2+}-ATPase activity and the filament formation of dephosphorylated myosin (Kuczmarski and Spudich, 1980) should favor contraction of the cell. Contraction, also called 'cringing', has been observed after stimulation of *D. discoideum* cells with cAMP (Futrelle *et al.*, 1980; Gerisch, 1980).

Materials and methods

Cell culture

D. discoideum strain AX2-214 was cultivated in fermenters as described previously (Claviez *et al.*, 1982). Development to aggregation competence was achieved by starving cells in 17 mM Soerensen phosphate buffer pH 6.0 and was enhanced by pulses of 20 nM cAMP applied at intervals of ~6 min for 4–6 h (Gerisch *et al.*, 1975; Darmon *et al.*, 1975).

Buffers

Buffer A: 40 mM Tris-HCl, pH 7.0, 1 mM 2-mercaptoethanol. Buffer B: 20 mM Tris-HCl, pH 7.0, 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.2 M NaCl. Buffer C: 10 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 0.02% NaN_3. Buffer D: 30 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$. Buffer E: 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EGTA, 2 mM $MgCl_2$, 0.02% NaN_3. Buffer F: 40 mM Tris-HCl, pH 7.5, 1 mM DTT, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$. G-Buffer (Gordon *et al.*, 1976). 30 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EGTA, 0.1 mM ATP, 0.5 mM phenylmethyl sulfonylfluoride (PMSF), 0.02% NaN_3. Buffer H: 40 mM Tris-HCl, pH 7.5, 1 mM DDT, 1 mM EGTA, 0.6 M NaCl, 0.5 mM PMSF, 0.02% NaN_3, 30% sucrose. Buffer I: 30 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM Mg^{2+} ATP, 1 M KCl, 0.5 mM PMSF. Buffer K: 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM Mg^{2+}-ATP, 0.6 M NaCl, 0.5 mM PMSF, 0.02% NaN_3. Buffer L: 1 mM NH_4HCO_3, 1 mM 2-mercaptoethanol.

Materials for chromatography

DEAE-cellulose (DE 52) and phosphocellulose (P 11) were purchased from Whatman, DEAE-Sephacel, Blue-Sepharose, DEAE-Sephadex A50 from Pharmacia, hydroxylapatite from BioRad. Calmodulin-Sepharose was prepared according to Dieter and Marmé (1980), and chlorpromazine-Sepharose by coupling 2-chloro-10 (3-aminopropyl) phenothiazine to CNBr-Sepharose 4B (Pharmacia) as described by Jamieson and Vanaman (1979).

The procedure of Khandelwal *et al.* (1976) was used to couple myosin or histone to CNBr-Sepharose which was prepared according to Nevaldine and Kassell (1971). 25 mg of myosin purified from aggregation-competent cells were dissolved in 5 ml of 0.1 M $NaHCO_3$ and 0.5 M NaCl and coupled to 5 g of CNBr-Sepharose. The material was poured into a column and washed with 1 M NaCl in buffer C. 250 mg of histone II-A from calf thymus (Sigma) in 50 ml of 0.1 M $NaHCO_3$ were coupled to 50 g of CNBr-Sepharose and washed as above.

Purification procedures

Purification of myosin. Myosin from growth phase cells was prepared by the method of Clarke and Spudich (1974), modified according to Claviez *et al.*

(1982). Myosin from aggregation-competent cells could not be purified by this procedure, probably because the lack of phosphorylation had changed its solubility. We, therefore, developed the following purification method which is, however, unsuitable for myosin preparation from growth phase cells.

400 ml of densely packed aggregation-competent cells were suspended in 800 ml of G-buffer and homogenized by a Parr bomb. Cell debris were removed by centrifugation for 10 min at 10 000 *g*. The supernatant was centrifuged for 3 h at 100 000 *g* and the sediment, ~100 ml, was resuspended in 270 ml of buffer I. After centrifugation for 3 h at 100 000 *g* the supernatant was collected. Actomyosin was precipitated from the supernatant with 1.5 M ammonium sulfate. The precipitate was dissolved in a mixture of 30 ml of buffer I and 30 ml of 1.2 M KI. After centrifugation for 20 min at 100 000 *g*, the supernatant was fractionated according to Clarke and Spudich (1974) by gel filtration on a BioGel A15m column equilibrated with buffer K. The yield was between 80 and 100 mg of myosin.

Purification of calmodulin. Calmodulin was purified by a modification of the method of Jamieson and Vanaman (1979). 1 kg of bovine brain was extracted with buffer A containing 1 mM EDTA, and the solution applied to 200 g DEAE-Sephacel on a glass funnel. The Sephacel was washed with buffer A containing 1 mM EDTA and 0.1 M NaCl. For elution, the NaCl concentration was raised to 0.5 M. The eluate was applied to a chlorpromazine-Sepharose column in buffer A containing 1 mM Ca^{2+}. After washing, the affinity column was eluted with buffer A containing 0.5 M NaCl and 10 mM EGTA. The eluate was dialysed against buffer L, lyophilized, dissolved in buffer B and fractionated on a DEAE-Sephadex A50 column with a linear 0.2–0.5 M gradient of NaCl in buffer B. Final purification was obtained by gel filtration on Sephadex G-100 in 10 mM NH_4HCO_3.

Partial purification of MHC kinases I and II. (1) Separation of the two kinases: all purification steps were performed at 4°C. 400 ml of densely packed aggregation-competent cells were homogenized in 800 ml G-buffer using a Parr bomb. The homogenate was centrifuged for 10 min at 10 000 *g* and for 3 h at 100 000 *g*. The supernatant was applied to a DEAE-cellulose column (2.6 x 40 cm), equilibrated with buffer C. The flow-through was subjected to differential ammonium sulfate precipitation. First, the concentration was brought to 1.5 M and the precipitate used for partial purification of MHC kinase I. Then, after increase of the ammonium sulfate concentration to 2.0 M, a second precipitate was obtained from which MHC kinase II was further purified.

(2) MHC kinase I: the ammonium sulfate precipitate, dissolved and dialysed against buffer C, was applied to a myosin-Sepharose column (10 ml), and the bound material eluted with 0.5 M NaCl. The eluate was applied to Blue-Sepharose (10 ml) in buffer C, and the bound enzyme eluted with 1 M NaCl in buffer C. The eluate was dialysed against buffer F and applied to a calmodulin-Sepharose column (20 ml). The bound material was eluted with 5 mM EGTA and 0.5 M NaCl in buffer F lacking $CaCl_2$, dialysed against buffer C and applied to histone-Sepharose (20 ml). The bound material was eluted with 1 M NaCl in buffer C, and the eluate fractionated on a Sephadex G-100 column (1.6 x 60 cm) in buffer C containing 0.1 M NaCl. MHC kinase I was recovered in the void volume. The yield was 6 mg protein/liter of packed cells, with a specific activity of 30 pmol phosphate incorporated into myosin/min/mg protein of the partially purified kinase preparation.

(3) MHC kinase II purification procedure 1: the fraction precipitated with 2.0 M ammonium sulfate was dissolved in buffer C and applied to myosin-Sepharose (10 ml). The flow-through was bound to Blue-Sepharose (10 ml), and the bound material eluted with 1 M NaCl in buffer C. The eluate was dialysed against buffer F and applied to calmodulin-Sepharose (20 ml). The flow-through fraction was applied to histone-Sepharose (20 ml) in buffer C, and the flow-through fractionated on a Sephadex G-100 column (1.6 x 60 cm), equilibrated with 0.1 M NaCl in buffer C. MHC kinase II activity was recovered in fractions corresponding to an apparent mol. wt. of 65–70 kd. These fractions were applied to hydroxylapatite (10 ml) in buffer C containing 0.1 M KCl. Most of the kinase activity was eluted by 0.2–0.4 M phosphate in the above buffer. The yield was 0.3 mg protein/liter of packed cells, with a specific activity of 3 nmol phosphate incorporated into myosin/min/mg protein of the partially purified enzyme preparation. MHC kinase II partially purified by the method described above was used for most of the experiments. However, the kinase became unstable after the hydroxylapatite step and lost most of its activity within 1 or 2 days. Therefore, the purification method 2, described below, was developed.

(4) MHC kinase II purification procedure 2: up to ammonium sulfate precipitation, procedure 1 was followed. The precipitate was dissolved in buffer C, dialysed against buffer C containing 15% sucrose and applied to a phosphocellulose column. The kinase was eluted between 150 and 200 mM NaCl, dialysed against buffer C containing 15% sucrose, bound to Blue-Sepharose, eluted with 1 M NaCl in buffer C, and dialysed against buffer C with 15% sucrose. MHC kinase purified by procedure 2 was used in the experiments shown in Figures 4 and 6–9. It resembled the enzyme purified by procedure 1 in Ca^{2+} plus calmodulin-dependent inactivation, in its inability to bind to calmodulin, myosin and histone II-A affinity columns in the cold, and in its apparent mol. wt. on Sephadex G-100.

Partial purification of MHC kinase from growth phase cells. 600 ml of densely packed growth phase cells were homogenized in 1200 ml buffer H using a Parr bomb. The homogenate was centrifuged for 10 min at 10 000 *g*, and for 3 h at 100 000 *g*. To the supernatant, ammonium sulfate was added up to a final concentration of 3 M, the precipitate resuspended in buffer E, and dialysed extensively against the same buffer. The undissolved, actomyosin-containing material was separated by centrifugation for 15 min at 20 000 *g*, extracted with 100 ml of 0.1 M NaCl in buffer E and centrifuged for 3 h at 100 000 *g*. The supernatant was dialysed against buffer E and applied to a Blue-Sepharose column. The bound material was eluted with 1 M NaCl in buffer E, dialysed against buffer C supplemented with 15% sucrose and applied to a DEAE-cellulose column (15 ml) equilibrated with buffer E. The flow-through fraction was applied, after addition of 3 mM $MgCl_2$ and 1.5 mM $CaCl_2$, to calmodulin-Sepharose equilibrated with buffer F. The bound material was eluted with 0.6 M NaCl in buffer F and fractionated on a Sephadex G-150 column (1.6 cm x 63 cm) in buffer E containing 50 mM NaCl. Most of the MHC kinase activity was recovered in fractions corresponding to apparent mol. wts. of ~70 kd. The yield was 2.5 mg protein/liter of packed cells, with a specific activity of 0.22 nmol phosphate incorporated into myosin/min/mg protein of the partially purified enzyme preparation.

Limited chymotryptic digestion

Myosin of aggregation-competent cells was labeled with [γ-^{32}P]ATP using MHC kinase II. For limited digestion (Peltz *et al.*, 1981), 8 mg of the labeled myosin were incubated with 100 μg chymotrypsin (Sigma, type VII, TLCK-treated) for 10 min at 25°C in 2 ml of buffer H. The reaction was terminated by 0.7 mM PMSF. The fragments were separated by SDS-polyacrylamide electrophoresis in 12% gels and stained with Coomassie Blue. The 132-kd and 38-kd bands were cut out and subjected to mapping of phosphopeptides.

Phosphopeptide mapping

The procedure of Gracy (1977) was used for phosphopeptide mapping of either intact ^{32}P-labeled myosin heavy chains, or the 132-kd and 38-kd fragments obtained by limited chymotryptic digestion. Myosin heavy chains were separated by SDS-polyacrylamide electrophoresis using 6% gels, and the Coomassie Blue stained bands cut out from the gels. The polypeptides were extracted and digested at 35°C in 0.5 ml of a solution containing proteolytic enzymes and 1 mM DTT in 0.1 M NH_4HCO_3, pH 8.0 (Huttner and Greengard, 1979). For the experiments shown in Figure 6, 50 μg trypsin plus 50 μg chymotrypsin were used. After 12 h, and again after 24 h, the same amounts of the proteases were added. After a total incubation time of 36 h the material was lyophilized, then redissolved in 20 μl of a solution containing acetic acid (10 vol)/pyridine (1 vol)/water (89 vol), pH 3.5. Aliquots of 5 μl were applied to 20 x 20 cm cellulose thin layer sheets (Polygram Cel 400, Macherey and Nagel) and the phosphopeptides separated in the first dimension by electrophoresis at 400 V for 1.5 h in the solvent. In the second dimension the phosphopeptides were chromatographed in acetic acid (10 vol)/pyridine (33 vol)/butan-1-ol (50 vol)/water (40 vol). The position of the phosphopeptides was visualized by autoradiography.

For the experiments shown in Figures 8 and 9 the procedure was modified. Only trypsin (Sigma, type XI, DPCC-treated) (Figure 8) or chymotrypsin (Sigma, type VII, TLCK-treated) (Figure 9) was added and readded after 12 and 24 h. After two-dimensional separation, the major phosphopeptides were scraped off the cellulose layer, and the whole digestion and separation procedure was repeated.

Identification of phosphorylated amino acids

Myosin was labeled with ^{32}P using either MHC kinase from growth phase cells or MHC kinase II. The heavy chains were separated by SDS-polyacrylamide gel electrophoresis and digested with chymotrypsin as described for phosphopeptide mapping. The digest was lyophilized and hydrolysed with 6 N HCl for 24 h at 120°C under vacuum. The ^{32}P-labeled amino acids were separated by two-dimensional electrophoresis according to Hunter and Sefton (1980). Phosphoserine, phosphothreonine and phosphotyrosine added as references were stained with ninhydrin.

Assays

Protein. Routinely, protein was determined according to Lowry with BSA as the standard. A more precise, absolute quantitation of myosin was required for determining the stoichiometry of phosphate incorporation (Figure 10) and for the assay of specific ATPase activities (Table III). Therefore, all myosin estimates obtained by the Lowry assay were corrected by quantitative amino acid determination. The myosin used was hydrolysed with trifluoroacetic acid/HCl (Tsugita and Scheffler, 1982). Values obtained after hydrolysis of the myosin for either 25 or 50 min were the same. For com-

parison, myosin was hydrolysed under vacuum with 6 N HCl at 110°C (Edman and Henschen, 1975). After 24 h of hydrolysis, 89% of the trifluoroacetic acid/HCl value for the protein content was obtained; and after 48 h, 85% of that value.

ATPase activities. The Ca^{2+}-ATPase and Mg^{2+}-ATPase activities of myosin were determined at 35°C according to Pollard and Korn (1973). The specific activities shown in Table III were based on the quantitation of myosin by amino acid determination after total hydrolysis with trifluoroacetic acid HCl, as described above.

MHC kinase activity. For determining MHC kinase activity, myosin prepared from aggregation-competent *D. discoideum* cells was used as a substrate since, in contrast to myosin from growth phase cells (Kuczmarski and Spudich, 1980), it was almost completely unphosphorylated. The assay mixture contained 20 μg myosin in buffer D and 0.2 mM [γ-^{32}P]ATP (1.0 Ci/mmol) in a total volume of 0.1 ml. Unless otherwise indicated, the assay was carried out at 35°C for 30 min. Under the conditions used, the amount of phosphate incorporated did not exceed 0.1 mol of phosphate per mol of myosin heavy chain.

For quantitation of the phosphate incorporated, the filter paper assay of Pettit *et al.* (1973) was normally used. During purification of the enzymes, when endogenous phosphorylatable proteins were still present, the filter paper assay was not applicable. In these cases the reactions were stopped by boiling in sample buffer containing 1% SDS (Laemmli, 1970), and the myosin heavy chains were separated by SDS-polyacrylamide gel electrophoresis. The radioactivity in the Coomassie Blue-stained heavy chain bands was determined by autoradiography using Kodak NS-2T X-ray film. This procedure was also used for the experiment of Figure 3 performed with the partially purified MHC kinase II. The ^{32}P-incorporation was quantified by scanning of the autoradiograms.

Phosphate content of myosin from aggregation-competent cells. Phosphate was determined in the purified myosin by the sensitive malachite green method of Stull and Buss (1977).

Stoichiometry of phosphate incorporation into myosin. Myosin was phosphorylated with [γ-^{32}P]ATP as described for MHC kinase assays. Incorporation of [^{32}P]phosphate was determined by use of the filter paper assay of Pettit *et al.* (1973). The data shown in Figure 10 were based on quantitation of myosin by amino acid determination after total hydrolysis with trifluoroacetic acid/HCl.

Acknowledgements

We are grateful to Dr.F.Lottspeich for the quantitative amino acid determination and to Dr.W.Huttner for help in phosphopeptide mapping and identification of phosphothreonine. *P. polycephalum* myosin was a gift of Dr.T.Schreckenbach and rabbit skeletal muscle actin of Dr.G.Isenberg. Our work was supported by grants Ge 135-17/18 and SFB 46-Teilprojekt C 4 of the Deutsche Forschungsgemeinschaft and by a grant of the Stiftung Volkswagenwerk to D.Marmé.

References

Adelstein,R.S. and Conti,M.A. (1975) *Nature,* **256**, 597-598.
Bazari,W.L. and Clarke,M. (1981) *J. Biol. Chem.,* **256**, 3598-3603.
Bonner,J.T., Barkley,D.S., Hall,E.M., Konijn,T.M., Mason,J.W., O'Keefe, G.,III and Wolfe,P.B. (1969) *Dev. Biol.,* **20**, 72-87.
Claviez,M., Pagh,K., Maruta,H., Baltes,W., Fisher,P. and Gerisch,G. (1982) *EMBO J.,* **1**, 1017-1022.
Clarke,M. and Spudich,J.A. (1974) *J. Mol. Biol.,* **86**, 209-222.
Collins,J.H. and Korn,E.D. (1980) *J. Biol. Chem.,* **255**, 8011-8014.
Collins,J.H., Cote,G.P. and Korn,E.D. (1982) *J. Biol. Chem.,* **257**, 4529-4534.
Cote,G.P., Collins,J.H. and Korn,E.D. (1981) *J. Biol. Chem.,* **256**, 12811-12816.
Darmon,M., Brachet,P. and Pereira da Silva,L. (1975) *Proc. Natl. Acad. Sci. USA,* **72**, 3163-3166.
Dieter,P. and Marmé,D. (1980) *Cell Calcium,* **1**, 279-286.
Edman,P. and Henschen,A. (1975) in Needleman,S.B. (ed.), *Protein Sequence Determination,* Springer Verlag, NY, p. 232.
Futrelle,R.P., McKee,W.G. and Traut,J. (1980) *J. Cell Biol.,* **87**, CI 415 (Abstract).
Gerisch,G. (1980) *Rheinisch-Westfälische Akad. Wiss.,* Vorträge N 292, Westdeutscher Verlag, pp. 7-38.
Gerisch,G., Fromm,H., Huesgen,A. and Wick,U. (1975) *Nature,* **255**, 547-549.
Gordon,D.J., Eisenberg,E. and Korn,E.D. (1976) *J. Biol. Chem.,* **251**, 4778-4786.
Gracy,R.W. (1977) *Methods Enzymol.,* **47**, 195-204.
Hathaway,D.R. and Adelstein,R.S. (1979) *Proc. Natl. Acad. Sci. USA,* **76**, 1653-1657.
Hunter,T. and Sefton,B.M. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 1311-1315.
Huttner,W.B. and Greengard,P. (1979) *Proc. Natl. Acad. Sci. USA,* **76**, 5402-5406.
Jamieson,G.A.,Jr. and Vanaman,T.C. (1979) *Biochem. Biophys. Res. Commun.,* **90**, 1048-1056.
Khandelwal,R.L., Vandenheede,J.R. and Krebs,E.G. (1976) *J. Biol. Chem.,* **251**, 4850-4858.
Kuczmarski,E.R. and Spudich,J.A. (1980) *Proc. Natl. Acad. USA,* **77**, 7292-7296.
Laemmli,U.K. (1970) *Nature,* **227**, 680-682.
Malchow,D., Boehme,R. and Rahmsdorf,H.J. (1981) *Eur. J. Biochem.,* **117**, 213-218.
Maruta,H., Gadasi,H., Collins,J.H. and Korn,E.D. (1979) *J. Biol. Chem.,* **254**, 3624-3630.
Maruta,H. and Korn,E.D. (1977) *J. Biol. Chem.,* **252**, 6501-6509.
Nevaldine,B. and Kassell,B. (1971) *Biochim. Biophys. Acta,* **250**, 207-209.
Peltz,G., Kuczmarski,E.R. and Spudich,J.A. (1981) *J. Cell Biol.,* **89**, 104-108.
Pettit,F.H., Hamilton,L., Munk,P., Namahira,G., Eley,M.H., Wilms,C.R. and Reed,L.J. (1973) *J. Biol. Chem.,* **248**, 5282-5290.
Pollard,T.D. and Korn,E.D. (1973) *J. Biol. Chem.,* **248**, 4682-4690.
Rahmsdorf,H.J., Malchow,D. and Gerisch,G. (1978) *FEBS Lett.,* **88**, 322-326.
Scholey,J.M., Taylor,K.A. and Kendrick-Jones,J. (1980) *Nature,* **287**, 233-235.
Sherry,J.M.F., Gorecka,A., Askoy,M.D., Dabrowska,R. and Hartstone, D.J. (1978) *Biochemistry (Wash.),* **17**, 4411-4418.
Suzuki,H., Onishi,H., Takahashi,K. and Watanabe,S. (1978) *J. Biochem. (Tokyo),* **84**, 1529-1542.
Stull,J.T. and Buss,J.E. (1977) *J. Biol. Chem.,* **252**, 851-857.
Tsugita,A. and Scheffler,J.J. (1982) *Eur. J. Biochem.,* **124**, 585-588.
Wick,U., Malchow,D. and Gerisch,G. (1978) *Cell Biol. Int. Rep.,* **2**, 71-79.