Demonstration of Two Protein Kinases in Extracts of *Legionella micdadei*

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Protein kinases I (PK I) and II (PK II) were purified 253- and 13.5-fold, respectively, from an extract of sonically disrupted cells of *Legionella micdadei* by ion-exchange chromatography on QAE-Sephadex, by histone affinity chromatography, and by HPLC-gel filtration chromatography. Both enzymes catalysed the phosphorylation of calf thymus histones, with a K_m of 2.7 mg ml⁻¹ for PK I and 2.9 mg ml⁻¹ for PK II. Histone H_{2b} was the best protein kinase substrate for both PK I and PK II. The pH optima were 6.8 and 7.0 for PK I and PK II respectively. The K_m for ATP was 0.29 mM for PK I and 0.33 mM for PK II. PK II activity was stimulated by either cAMP or cGMP, whereas PK I was inhibited by both cyclic nucleotides. The activity of PK I was unaffected by addition of calmodulin, diacylglycerol and mixtures of Ca²⁺ and acidic phospholipids, but these additions increased PK II activity threefold. The activity of PK II was stimulated by spermine and spermidine, but PK I was inhibited by these compounds. PK I and PK II were both strongly inhibited by heparin.

INTRODUCTION

Legionella pneumophila and *Legionella micdadei* cause bacterial pneumonia, but the mechanisms underlying their pathogenicity are largely unknown. The legionellae multiply in host mononuclear phagocytes (Horwitz & Silverstein, 1980; Weinbaum *et al.*, 1984) and are not killed efficiently by polymorphonuclear leucocytes (Horwitz & Silverstein, 1981; Weinbaum *et al.*, 1983). The properties of legionellae which promote their survival in the normally hostile environment within host phagocytic cells are beginning to be explored. In a previous study we found that *L. micdadei* contained an acid phosphatase which inhibited superoxide production by neutrophils following stimulation by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (Saha *et al.*, 1985). Since protein phosphorylation plays an important role in the regulation of enzyme activity in many eukaryotic systems (Greengard, 1978; Rubin & Rosen, 1975), this observation stimulated us to consider the possibility that phosphorylating enzymes in the legionellae might play a role in the pathophysiology caused by this family of organisms. In a preliminary experiment we detected substantial amounts of histone-specific protein kinase activity in extracts of *L. micdadei*.

Protein kinases of various types share the property of catalysing the phosphorylation of specific serine or threonine residues of protein substrates (Knowles, 1980). Evidence demonstrating the presence of multiple species of endogenous phosphorylated bacterial proteins and protein kinases in prokaryotes has been reported for *Salmonella typhimurium* (Wang & Koshland, 1981) and *Escherichia coli* (Enami & Ishihama, 1984; Manai & Cozzzone, 1982). In the case of *E. coli*, it has been demonstrated convincingly (Nimmo *et al.*, 1984; Reeves & Malloy, 1982) that NADP:isocitrate dehydrogenase undergoes phosphorylation and dephosphorylation; interestingly, both reactions are catalysed by a single bifunctional enzyme. In addition,

Abbreviations: PK I, protein kinase I; PK II, protein kinase II.

Kuo & Greengard (1969) partially purified an adenosine 3',5'-cyclic monophosphate (cAMP)-dependent enzyme from *E. coli* that phosphorylated a non-bacterial protein, namely histone. It is unclear whether this enzyme is a protein kinase, or a polyphosphate kinase (Pastan & Adhya, 1976).

Based upon data obtained with another intracellular parasite, *Leishmania donovani* (Das *et al.*, 1986; Gottlieb & Dwyer, 1981; Remaley *et al.*, 1984, 1985), we considered the possibility that protein kinases and phosphatases on the legionella cell surface may play a role in pathogenesis. As a first step in investigating the possible pathophysiological significance of legionella protein kinases, we initiated a study of the enzyme activity in *Legionella micdadei*, an organism which has been the cause of mortality in immunocompromised patients (Pasculle *et al.*, 1980).

METHODS

Materials. [γ - 32 P]ATP (5–10 Ci mmol $^{-1}$; 185–370 GBq mmol $^{-1}$) was purchased from New England Nuclear, QAE-Sephadex from Pharmacia, and a Protein Pak 300 SW column (7.5 $\times$ 600 mm) from Waters Associates. Mixed histones from calf thymus, protamine, casein, phosvitin, calmodulin, cAMP, cGMP, phosphatidylserine, phosphatidylinositol, 1,2-dioleoyl-*racemo*-glycerol (diolein), spermine, spermidine, heparin, phenylmethylsulphonyl fluoride, aprotinin, leupeptin and protein kinase inhibitor from rabbit muscle were obtained from Sigma. Pure histone subtypes were a gift from Dr Sidney Morris, University of Pittsburgh, Pittsburgh, PA, USA. All other chemicals were of reagent grade and purchased from Fisher Scientific Co.

Bacteria. *L. micdadei* strain EK (ATCC 33204), which had been isolated originally in embryonated eggs from lung tissue of a patient with pneumonia (Pasculle *et al.*, 1980), was used in all studies. Pooled yolk material from eggs was grown up on buffered charcoal yeast extract agar (Pasculle *et al.*, 1980), containing 0.1% (w/v) 2-oxoglutarate and 1% (w/v) bovine serum albumin (BCYE agar). Stock cultures, consisting of the growth from several BCYE agar plates suspended in nutrient broth containing 15% (v/v) glycerol, were stored in portions at -70°C . Bacteria for the isolation of protein kinases were obtained by plating an appropriate dilution of the stock culture on BCYE agar plates. These cultures were incubated for 48–72 h at 35°C in ambient air until confluent growth was obtained.

Protein kinase assay. Protein kinase activity was assayed routinely with mixed histones serving as the phosphate acceptor in a 0.1 ml assay composed of 50 mM-Tris/HCl buffer (pH 7.0), 10 mM-MgCl $_2$, 10 mM-NaF, 0.3 mg mixed histones, 1 mM- $[\gamma$ - 32 P]ATP, 50 mM-NaCl and 20 μ l of enzyme solution (0.05–1.0 μ g protein). The reaction was initiated by addition of ATP and incubated at 37°C . After 10 min, 1 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) was added, followed by 20 μ l bovine serum albumin solution (20 mg ml $^{-1}$). The pellet obtained by centrifugation (5000 g, 10 min) was dissolved in 0.2 ml water and precipitated with 10% (w/v) TCA. After two more cycles of TCA precipitation, the precipitate was finally dissolved in 0.1 ml water from which duplicate 40 μ l samples were taken and analysed for radioactivity as described by Corbin & Reimann (1974). The control was prepared with boiled enzyme. One unit of activity was defined as the incorporation of 1 pmol ^{32}P into acid-insoluble product min $^{-1}$. Substrate saturation kinetics were examined with varying concentrations of mixed histones (0.06–0.75 mM); slopes and intercepts were calculated according to the method of Cleland (1979).

Protein determination. Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin as standard.

Purification of protein kinases. All purification procedures were carried out at 4°C unless otherwise indicated. The bacterial cells from 90–100 BCYE agar plates (85 mm diam.) were suspended in 80–100 ml 0.9% NaCl. We obtained 3.4 g (wet weight) of cells from 100 plates. The bacterial suspension was sonicated four times at 150–200 W for 40 s (Braun-Sonic 2000, B. Braun Instruments). The sonication procedure was very efficient; resonation of the resuspended pellet released only 0.2% of protein kinase activity present in the original crude homogenate. Debris from the sonicated cell suspension was removed by centrifugation at 37000 g for 30 min, after which the extract was centrifuged at 100000 g for 60 min.

QAE-Sephadex chromatography. The resulting supernatant fraction was applied to a QAE-Sephadex A-50 column (15 $\times$ 80 mm) that was equilibrated with 10 mM-sodium citrate buffer, pH 6.5, supplemented with 10 mM-2-mercaptoethanol (designated 'standard citrate buffer') and 50 mM each of leupeptin, aprotinin and phenylmethylsulphonyl fluoride, at a flow rate of 0.2 ml min $^{-1}$. Fractions of 2 ml were collected and analysed for protein (absorbance at 280 nm) and protein kinase activity. Approximately 72% of the protein kinase activity appeared in the column breakthrough fractions; this fraction was designated as PK I. The column was then developed with 100 ml of a linear 0–0.5 M-sodium chloride gradient in standard citrate buffer. The conductivity of each fraction was determined using a Radiometer conductivity meter (type CDM 2F). A single peak of protein kinase activity was eluted with approximately 0.25 M-sodium chloride and designated as PK II. The PK I and PK II activity fractions were pooled, and the two pools were dialysed separately against two changes of standard citrate buffer containing 50% (v/v) glycerol.

Histone affinity chromatography. After dialysis, as described above, fractions containing PK I and PK II activity were applied separately to a histone affinity column (15 × 70 mm) prepared by covalently attaching calf thymus histones to Sepharose 4B as described by Wilchek *et al.* (1984). The column buffer was standard citrate buffer. After loading each sample, the column was washed with 50 ml column buffer, followed by 50 ml column buffer containing 0.5 M-sodium chloride to elute protein kinase activity; 2 ml fractions were collected. The histone affinity column can be used at least three times.

HPLC gel filtration chromatography. The pooled fractions from the histone affinity column were dialysed against 10 mM-sodium phosphate buffer, pH 6.5, containing 10 mM-2-mercaptoethanol and 50% (v/v) glycerol and applied to an HPLC gel filtration column (Waters Protein Pak 300 SW, 7.5 × 600 mm). The fractionation range of this column is approximately 40000–150000. The column was fitted to a Waters Associates M-45 solvent delivery system and the eluate was passed through a Waters absorbance detector (model 440) coupled to a Goerz-Metrawatt recorder (model SE 120). The column was equilibrated in 10 mM-sodium phosphate buffer, pH 6.5, containing 10 mM-2-mercaptoethanol and developed at a flow rate of 0.5 ml min⁻¹. Fractions of 1 ml were collected and analysed for protein kinase activity. Fractions constituting the single peak of protein kinase activity were pooled and dialysed against standard citrate buffer containing 50% (v/v) glycerol. The kinase preparations lost less than 10% of their activity when stored for 15 d at 0–4 °C in standard citrate buffer containing 50% (v/v) glycerol.

RESULTS

Purification of protein kinases

The crude sonicate of *L. micdadei* contained a substantial amount of protein kinase activity (Table 1). The resolution and partial purifications of the two *L. micdadei* protein kinases were monitored with mixed histones serving as the exogenous phosphate acceptor in the kinase assay. The 100000 g supernatant fraction of the crude bacterial extract contained more than 85% of the total protein kinase activity. When this material was chromatographed on a QAE-Sephadex column (Fig. 1), about 72% of the protein kinase activity appeared in the breakthrough fractions (PK I) while the remainder (PK II) was eluted by the salt gradient.

The results of the purification schemes for PK I and PK II are summarized in Table 1. Relative to the crude homogenate, PK I was purified 253-fold. However, PK II was purified only 13.5-fold. The specific activities of the final preparations of PK I and PK II were 1.01×10^5 and 1.33×10^3 U (mg protein)⁻¹, respectively, when assayed using mixed histones as the phosphate acceptor. The overall activity yield of these two protein kinases was 38% (Table 1). The purification procedure was performed three times and was very reproducible. The data summarized in Table 1 are representative of these three experiments.

Proof that the ³²P-labelled product generated by PK I was, in fact, ³²P-histone and not polyphosphate (Pastan & Adhya, 1976; Li & Brown, 1973) was obtained by subjecting the incubation medium to SDS-PAGE and chromatography on a Sephadex G-100 column; the radioactive product coelectrophoresed and cochromatographed precisely with histone standard (data not shown).

Substrate specificity

A variety of potential phosphate acceptors were compared in the standard kinase assay (Table 2), namely, mixed histones, protamine, casein and phosvitin. In the case of PK I, only the mixed histones and protamine supported significant activity. Mixed histones were the preferred substrate for PK II. Little incorporation of [γ -³²P]ATP into TCA-precipitable material was obtained when histone was omitted from the kinase assay. The histone subfractions were not equally effective acceptors for PK I or PK II; in each case, histone subtype H_{2b} was the best acceptor and H₁ the poorest.

The K_m values with mixed histones as the substrate for PK I (2.7 mg ml⁻¹) and PK II (2.9 mg ml⁻¹) were indistinguishable. Above 8 mg ml⁻¹ the mixed histones inhibited the activities of both kinases by 30%. The effect of ATP concentration on kinase activity was examined in the range 0.05–3.0 mM-ATP. From the Lineweaver-Burk plots of the data, K_m values of 0.29 and 0.33 mM-ATP were estimated for PK I and PK II, respectively.

Fig. 1. QAE-Sephadex chromatography of *L. micdadei* protein kinase. The protein kinase activity from high-speed supernatant was further purified by chromatography on a 15×80 mm column containing QAE-Sephadex A-50 equilibrated in 0.01 M-citrate buffer, pH 6.5. The column was washed with 5 bed volumes of the same buffer (total volume of breakthrough, 30 ml) followed by elution (flow rate, 0.2 ml min^{-1}) with a 100 ml linear NaCl gradient (0.0–0.5 M-NaCl in standard citrate buffer, pH 6.5) initiated where indicated by the arrow. Fractions (2 ml) were collected and analysed for A_{280} (○), protein kinase activity (●) and conductivity (□). The open bar represents A_{280} , and the hatched bar the protein kinase activity, of the breakthrough fraction. Pools I and II were designated PK I and PK II, respectively. The data are representative of three experiments.

Table 1. Summary of purification of protein kinases I and II from *L. micdadei*

The data are representative of three experiments.

Purification step	$10^{-2} \times$ Total activity (units)*	Total protein (mg)	$10^{-2} \times$ Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
1. Crude homogenate	266	65.0	4.0	1.0	100
2. 100000 g supernatant fraction	231	59.0	3.9	1.0	87
3. QAE-Sephadex					
PK I	166	11.5	14.4	3.6	81†
PK II	52	30	1.7	1.0	
4. Histone affinity					
PK I	146	1.7	85.9	21.4	72†
PK II	47	7.0	6.7	6.8‡	
5. HPLC					
PK I	81	0.08	1012	253	38†
PK II	20	1.5	13.3	13.5‡	

* One unit of kinase activity equals $1 \text{ pmol } ^{32}\text{P}$ incorporated min^{-1} measured using mixed histones as substrate.

† Calculated by adding the activities of the two protein kinases.

‡ Calculated relative to the crude homogenate and assuming that $[52/(52 + 166)] \times 100$ represents the percentage of PK II in the crude homogenate.

Metal ion requirement

The activity of PK II was strongly dependent on the inclusion of NaCl (50 mM), MgCl_2 (10 mM) and NaF (10 mM) in the assay. CaCl_2 (1.0 mM) stimulated PK II activity threefold. Omission of NaCl and NaF reduced PK I activity by 30% and 15%, respectively. Neither exogenous Mg^{2+} nor Ca^{2+} was required for PK I activity. In the absence of Ca^{2+} , both kinases were inhibited about 40% by 1.0 mM- MnCl_2 (data not shown).

Table 2. *Substrate specificity of PK I and PK II*

The protein kinase assay was carried out with the specified amount of substrate in a 0.1 ml assay mixture for 10 min at 37 °C and was terminated by TCA precipitation (see Methods). One unit of kinase activity equals 1 pmol ³²P incorporated min⁻¹. The data were derived from duplicate analysis of a single experiment. The numbers in parenthesis represent one standard error of the mean.

Substrate	Amount of substrate per incubation (μg)	10 ⁻² × Protein kinase activity (units ml ⁻¹)	
		PK I	PK II
None	—	3.2 (0.6)	0.50 (0.25)
Mixed histones	300	33.7 (1.0)	11.5 (1.3)
	100	12.6 (0.8)	5.35 (0.45)
Histone subtype			
H ₁	100	0.21 (0.08)	0.04 (0.02)
H ₃	100	1.84 (0.30)	0.62 (0.25)
H _{2b}	100	4.85 (0.80)	1.66 (0.20)
H _{2a}	100	4.19 (0.75)	1.06 (0.25)
H ₄	100	2.54 (0.50)	1.52 (0.30)
Protamine	300	12.1 (0.75)	1.4 (0.10)
Casein	300	2.4 (0.40)	1.0 (0.25)
Phosvitin	200	1.7 (0.30)	0.90 (0.15)

Table 3. *Effect of various additions on the activities of PK I and PK II*

The phosphorylation reaction was carried out for 10 min at 37 °C as described in Methods, with 20 μl of partially purified protein kinase. The 'control' contained all the components of the standard reaction mixture. One unit of protein kinase activity is equal to 1 pmol ³²P incorporated min⁻¹. The data are representative of two experiments performed in duplicate. The numbers in parenthesis represent one standard error of the mean.

Addition(s) to the assay medium	10 ⁻² × Protein kinase activity (units ml ⁻¹)	
	PK I	PK II
None	26.1 (2.0)	13.6 (1.8)
0.1 mM-cAMP	21.2 (1.3)	16.0 (0.8)
10 mM-cAMP	15.7 (1.0)	26.1 (2.0)
0.1 mM-cGMP	16.0 (0.5)	17.0 (2.5)
10 mM-cGMP	15.5 (1.2)	19.0 (2.1)
3.5 μg calmodulin	20.1 (1.5)	21.5 (2.0)
10 μM-Ca ²⁺ and 3.5 μg calmodulin	29.2 (1.2)	40.2 (2.5)
10 μM-Ca ²⁺ and 10 μg phosphatidylserine	27.5 (2.0)	38.5 (0.8)
10 μM-Ca ²⁺ and 10 μg phosphatidylinositol	29.6 (0.5)	42.3 (2.6)
15 μM-diolein	28.1 (1.0)	39.5 (1.2)
10 μM-Ca ²⁺ , 10 μg phosphatidylinositol and 15 μM-diolein	25.6 (1.5)	51.2 (3.2)

Effects of pH on protein kinase activity

Both protein kinases exhibited maximum activity at pH 6.8–7.0. However, the pH-activity profile of PK I was much sharper than that of PK II (data not shown).

Effects of cyclic nucleotides and other agents on enzyme activity

The activity of PK I was inhibited by 20% and 40% by 0.1 mM- and 10 mM-cAMP, respectively, and 40% by 0.1 or 10 mM-cGMP. The activity of PK II was stimulated by 15% and 48% by 0.1 mM- and 10 mM-cAMP, respectively, and by 25% and 40% by 0.1 and 10 mM-cGMP, respectively (Table 3).

That PK I was not cyclic-nucleotide-dependent was supported by the observation that the protein kinase inhibitor from rabbit muscle (0.5 mg ml⁻¹) had little or no effect on this kinase (data not shown). In contrast, the inclusion of this inhibitor (0.5 mg ml⁻¹) in the assay for PK II reduced kinase activity by more than 50%, both in the presence and absence of cAMP (0.1 mM).

This last observation reinforced the conclusion that PK II was cAMP-dependent. Calmodulin by itself inhibited PK I activity by about 25% and stimulated PK II activity 1.5-fold. However, the Ca^{2+} /calmodulin mixture stimulated PK I activity by only 10%, whereas PK II activity was increased nearly threefold.

PK I activity was unaffected by a mixture of Ca^{2+} (10 μM) and phosphatidylserine (10 μg per assay) and stimulated only 13% by a mixture of Ca^{2+} and phosphatidylinositol (10 μg per assay), while these substances stimulated PK II activity threefold. Diolein (10 μM) stimulated PK I activity by only 7% and the Ca^{2+} /phosphatidylinositol/diolein mixture had no effect. However, both additions stimulated PK II activity three- to fourfold (Table 3).

Since some protein kinases are affected by polyamines (Glover *et al.*, 1983; Tabor & Tabor, 1976) and heparin (Hathaway *et al.*, 1980; Yutani *et al.*, 1982), the effects of these compounds were examined using mixed histones as the phosphate acceptor. PK II activity was stimulated about 1.5-fold by 0.5 mM-spermine or spermidine, and threefold when the concentration of either of these polyamines in the assay medium was 2 mM. In contrast, a 0.5 mM concentration of either of these polyamines inhibited PK I activity about 50%. Both kinases were inhibited by heparin, but their heparin sensitivity differed: 0.4 mg heparin ml^{-1} inhibited PK I activity completely but only inhibited PK II activity by about 50% (data not shown).

DISCUSSION

In this study, two protein kinase activities, PK I and PK II, were resolved and partially purified from an extract of *Legionella micdadei*. As pointed out by Li & Brown (1973), bacterial protein kinases that appear to phosphorylate non-bacterial proteins must be differentiated from polyphosphate kinase activity. *E. coli* contains a polyphosphate kinase which incorporates [γ - ^{32}P]ATP into TCA-precipitable material, this reaction being stimulated by histone or protamine (Li & Brown, 1973). However, the stimulation by histone occurs only in phosphate buffer, and in the absence of phosphate ions, histone is inhibitory; the polyphosphate kinase activity is also inhibited by NaCl and NaF (Li & Brown, 1973). Since our protein kinase assays were performed in the presence of NaF and NaCl and in the absence of phosphate, it is unlikely that polyphosphate kinase activity would be reflected in our assay. More definitive evidence that our assay for PK I and PK II was not detecting polyphosphate kinase activity was our finding that when the radiolabelled products of the kinase assay were subjected to SDS-PAGE and Sephadex G-100 chromatography, all of the radioactivity coincided precisely with the histone peaks (data not shown).

L. micdadei PK I is cyclic-nucleotide-independent, whereas PK II is cAMP- and cGMP-dependent. In this respect, PK I is similar to certain protein kinases in other pathogens including *Leishmania donovani* (Das *et al.*, 1986), *Plasmodium berghei* and *Plasmodium chabudi* (Wiser *et al.*, 1983). These organisms also contain protein kinases that are cAMP-independent and which phosphorylate serine residues of certain proteins.

Polyamines affect the activity of several protein kinases (Glover *et al.*, 1983; Tabor & Tabor, 1976). Like a protein kinase of *Candida albicans*, *Legionella micdadei* PK I was markedly inhibited by the polyamines spermine and spermidine (Gupta Roy & Datta, 1986).

We have not yet determined the subcellular location of the protein kinases of *L. micdadei*. Nevertheless, it is possible that these enzymes may play some role in disarming host cells by interfering with antimicrobial processes. For example, it has been shown that phosphorylation of tubulin, the principal protein of microtubules, inhibits the ability of this cytoskeletal protein to bind to microtubule-associated proteins or to polymerize (Wandosell *et al.*, 1986). It remains to be seen if host cell proteins such as tubulin are phosphorylated by the *L. micdadei* protein kinases. Further studies are needed to elucidate the role of these bacterial protein kinases and to identify endogenous (bacterial) or exogenous (host cellular) substrates that might function as phosphate acceptors for these enzymes.

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