Detection of protein kinase substrates in extracts of "Onchocerca volvulus"

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Detection of protein kinase substrates in extracts of *Onchocerca volvulus*

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Summary

Extracts of *Onchocerca volvulus* were phosphorylated in the presence of $(\gamma\,^{32}P)ATP$ and Mg^{2+} by endogenous protein kinase activity and exogenous rabbit muscle catalytic sub-unit of the adenosine 3'5' monophosphate dependent protein kinase (E.C. 2.7.1.37). Sodium dodecylsulfate polyacrylamide gel electrophoretic analysis of the ^{32}P-labelled extracts revealed at least seven (^{32}P)-phosphoproteins with apparent Mr of 92,000; 86,000; 40,000; 27,000; 26,000; 23,000 and 17,000. The phosphorylation of the components with apparent Mr of 23,000 and 17,000 was catalysed by both endogenous and exogenous protein kinases, whereas the other components required exogenous protein kinase for their phosphorylation. The endogenous protein kinase activity was inhibited by suramin and the heat-stable protein inhibitor of the adenosine 3'5' monophosphate dependent protein kinase. The (^{32}P)phosphoproteins identified in this investigation are probably candidate regulatory molecules in *O. volvulus;* though their physiological functions remain to be determined.

Key words: *Onchocerca volvulus;* filaria; phosphoproteins; protein kinase; phosphorylation; suramin.

Introduction

The parasitic nematode *Onchocerca volvulus* is a leading cause of blindness and a major public health hazard in tropical endemic countries (Nelson, 1970).

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Currently, there is no satisfactory method of controlling the disease. No preventive vaccine exists against onchocerciasis; filaricidal drugs like suramin and diethylcarbamazine are known to provoke dangerous reactions in patients (Goodwin, 1984). There is thus an urgent need to investigate the metabolism of the parasite more fundamentally in order to identify potential targets for drug development (WHO 1984).

Recently Walter and Schulz-Key (1980) showed that suramin inhibited a cyclic-AMP independent protein kinase from *O. volvulus,* but the endogenous substrates of this and other protein kinases were not identified. Such protein kinase substrates may themselves be important in the regulation of key metabolic processes within the cell (Engström et al., 1984). The aims of this investigation were to identify the protein kinase substrates in extracts of *O. volvulus* and to determine the effects of suramin on their phosphorylation.

Materials and Methods

Reagents. (γ ^{32}P)ATP was purchased from the New England Nuclear Corporation. The catalytic sub-unit of rabbit muscle cyclic-AMP dependent protein kinase (protein kinase A) was prepared according to the method of Bechtel et al. (1977). The heat-stable protein inhibitor of the protein kinase A (Walsh et al., 1971) was kindly donated by Dr. Pia Ekman. Suramin from FBA, West Germany, was kindly provided by Dr. Heldin. All other reagents were of the highest purity available from Sigma, Merck and Serva.

Parasites. Nodules were extracted from onchocerciasis patients in a dispensary at Batchenga, Republic of Cameroon, under aseptic conditions and transported to the laboratory. Viable female worms were then isolated from the nodules as previously described (Engelbrecht and Schulz-Key, 1984). The worms were freed of adherent nodular tissue, washed with phosphate buffered saline and transported frozen in a thermos flask to the University of Uppsala, Sweden, where they were kept at $-70\,^{\circ}$C until thawed once and used in the experiments.

Homogenates. These were prepared essentially as previously described (Titanji et al., 1985) except: (i) 1 mM diisoproprylfluorophosphate was included in all buffers as a proteolytic inhibitor; (ii) buffer "b" contained 1.5% Triton X-100 as an additive; (iii) 1 mM dithiothreitol (DTT) was added to all buffers to stabilize endogenous protein kinases; (iv) all centrifugations were done at 30,000×g for 10 min. The starting material was 2.5 g wet weight of worms and 2 volumes of the respective buffers (Titanji et al., 1985) were used at each extraction step. The pooled extracts were desalted at 4 °C by chromatography on a Sephadex G-50 column (2.4×30 cm) equilibrated with 10 mM phosphate buffer, pH 7.1, containing 30% (W/V) glycerol and 0.5 mM DTT. Fractions of 5 ml were collected at a flow rate of 30 ml/h. The fractions in the void volume containing *O. volvulus* proteins, were pooled, concentrated in a collodion bag (Sartorius) to 3.5 mg protein per ml and stored at $-70\,^{\circ}$C in 0.5 ml aliquots until used.

Analytical procedures

Protein phosphorylation. This was done as previously described (Engström et al., 1984), except that the final reaction mixture (40–100 μl) contained 25 mM morpholinoethane sulfonic acid (Mes), pH 8.0, 2 mM ethylene glycol tetraacetic acid (EGTA), 1 mM DTT, 12.5 mM MgCl$_2$ 3.1 mM (γ ^{32}P)ATP (15–20 cpm/pmol) and 170 μg *O. volvulus* proteins. The reaction was started by the addition of (γ ^{32}P)ATP and stopped by spotting 25 μl of the incubation mixture on Whatman n° 3 filter paper strips and washing the latter in trichloroacetic acid. In some experiments mixed calf histone (Sigma) were phosphorylated by protein kinase A separately at a final concentration of 1.25 mg per ml. When used, protein kinase A was present at 0.5–1.0 μg per test. The effects of adenosine 3′5′

monophosphate (cyclic-AMP) and guanosine 3'5' monophosphate (cyclic-GMP) on the phosphorylation of *O. volvulus* proteins were tested at a final concentration of 10^{-5} M. All reactions were run in duplicate.

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to identify the (^{32}P)phosphoproteins, the phosphorylation reaction was stopped by boiling the incubation mixture for 3 min in electrophoresis sample buffer at a final concentration of 62.5 mM Tris-HCl, pH 6.8, with 2% SDS, 10% glycerol and 0.01% bromophenol blue. Samples containing (100–150) 10^3 cpm were then applied to a 10–20% polyacrylamide gradient slab gel and electrophoresis was carried out according to the method of Laemmli (1970). The gel was stained, dried and placed on Kodak X-omat S films for autoradiography at –70°C. The following proteins, obtained from Pharmacia and Biorad, were used as molecular weight markers: myosin, 200,000; beta-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 67,000; carbonic anhydrase, 30,000; trypsin inhibitor, 21,000 and alpha-lactoglobulin, 14,400.

Protein concentration was determined according to the method of Read and Northcote (1981). Radioactivity was determined by counting samples in a Packard scintillation counter.

Results

Phosphorylation of O. volvulus proteins

When *O. volvulus* homogenates were incubated in the presence of (γ ^{32}P)ATP and Mg^{2+}, (^{32}P)orthophosphate was incorporated into a trichloro-

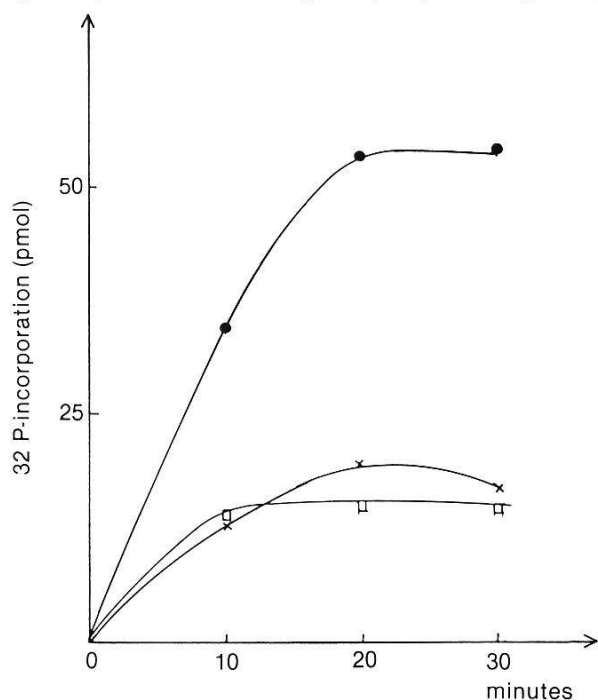
Fig. 1   Fig. 2
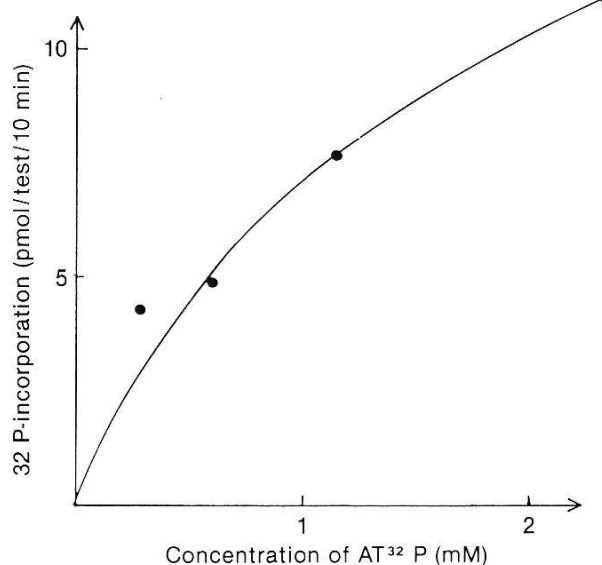
Fig. 1. Dependence of (^{32}P)orthophosphate incorporation into *O. volvulus* proteins on time. The incubation mixture contained 170 μg *O. volvulus* proteins of which 53.7 μg were analysed for (^{32}P)orthophosphate incorporation. ●———● = *O. volvulus* proteins + protein kinase A; X———X = *O. volvulus* proteins alone; ■———■ = *O. volvulus* proteins + cyclic AMP or cyclic-GMP. Other details are given in Materials and Methods and in the text.

Fig. 2. Effect of AT ^{32}P concentration on the rate of (^{32}P) incorporation. The reaction mixture contained 170 μg *O. volvulus* proteins. No exogenous protein kinase was added to the reaction mixture.

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acetic acid insoluble fraction in a time-dependent reaction (Fig. 1). The addition of cyclic-AMP or cyclic-GMP to the incubation mixture had no effect on the reaction. However, the catalytic sub-unit of protein kinase A stimulated the rate of ^{32}P incorporation 2.5 fold (Fig. 1). This increase in the rate of phosphorylation was not due to the self-phosphorylation of protein kinase A because background phosphorylation of the latter was subtracted from the corresponding values reported on Fig. 1. The rather moderate rate of (^{32}P)incorporation observed in the absence of exogenous protein kinase could not be explained by the sequestering of (γ ^{32}P)ATP since the latter was nearly saturating under the conditions used (Fig. 2).

In the presence of 10^{-5} M suramin, a macrofilaricide, more than 70% of the endogenous protein kinase activity was inhibited in *O. volvulus* homogenates. However, the same concentration of suramin was only slightly inhibitory when tested on mammalian protein kinase A, thereby confirming the specificity of action of suramin on filarial protein kinase activity. In other experiments 80% of
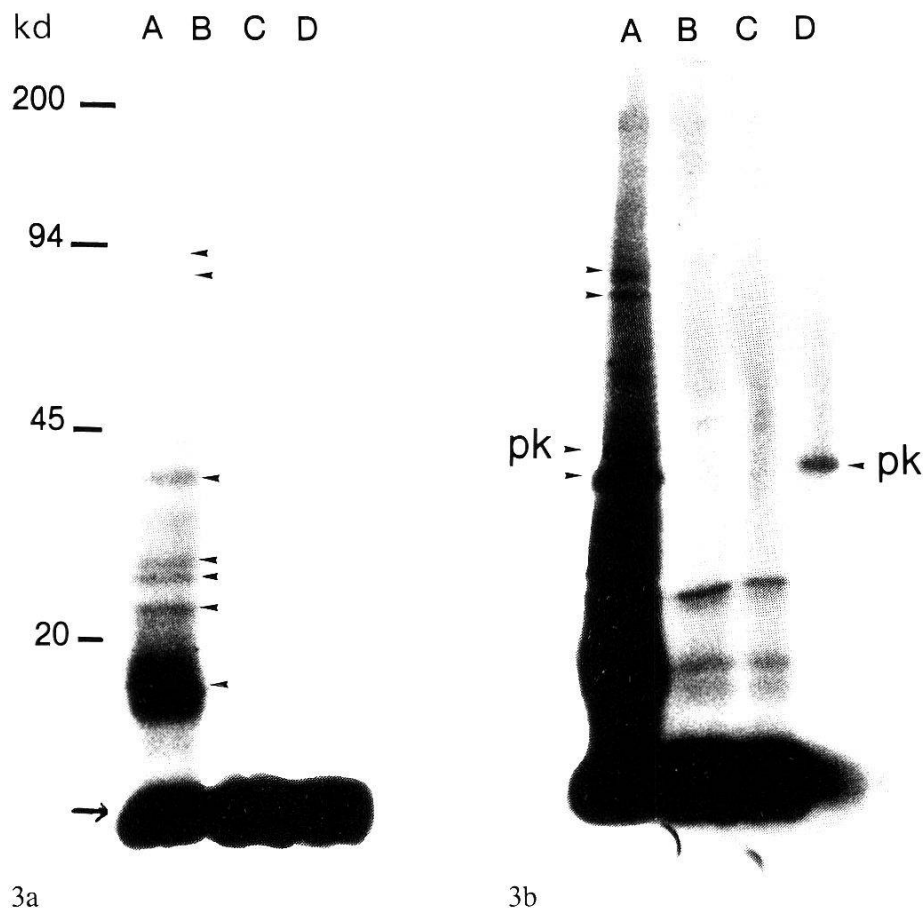

Fig. 3. Gel patterns of (^{32}P)phosphoproteins in extracts of *O. volvulus*. The standard phosphorylation mixture was used. After heating the reaction mixture in electrophoresis buffer, an amount corresponding to 85 μg *O. volvulus* proteins (O.V.P.) was applied to the gel and electrophoresis performed as described under Materials and Methods. Kd = Kilodaltons; A = O.V.P. + protein kinase A; B = O.V.P. alone; C = O.V.P. + cyclic AMP; D = protein kinase A (PK) alone. a) Autoradiograph after 6 h of exposure; b) after 18 h of exposure. Arrows indicate the positions of phosphoproteins described in the text.

the endogenous protein kinase activity in *O. volvulus* homogenates was inhibited by the heat-stable inhibitor tested at a final concentration of 100 μg per ml or by 5 mM ethylene diamine tetraacetic acid (results not illustrated). These observations strongly suggested that the susceptible protein kinase activity was of the type that normally depends on cyclic-AMP.

SDS-PAGE of the (^{32}P)phosphoproteins

Fig. 3 shows the autoradiograph of a sodium dodecyl sulfate polyacrylamide gel of *O. volvulus* proteins phosphorylated by endogenous and exogenous protein kinases. It can be seen that the addition of protein kinase A to the incubation mixture resulted in the phosphorylation of at least seven components with apparent molecular weights of 92,000; 86,000; 40,000; 27,000; 26,000 and 17,000 daltons. The radioactivity signal from the 17,000 component was particularly strong. The component with Mr of 40,000 migrated differently from protein kinase with a Mr 41,500 (Bechtel et al., 1977). When the *O. volvulus* homogenates were phosphorylated in the absence of exogenous protein kinase A, only two components with Mr of 23,000 and 17,000 were labelled.

Discussion

It is now generally accepted that the phosphorylation and dephosphorylation of key proteins catalysed by specific protein kinases and phosphoprotein phosphatases, respectively, is a principal mechanism for the control of cellular function (Nishizuka, 1984). Consequently considerable effort has been deployed towards the identification of phosphoproteins and their physiological functions (Engström et al., 1984). In this investigation at least seven (^{32}P)phosphoproteins were identified in the extracts of *O. volvulus* (Fig. 3). Two of the phosphoproteins were rapidly phosphorylated by endogenous protein kinases (Figs. 1 and 3). The inhibition of the endogenous phosphorylation by the heat-stable inhibitor (Walsh et al., 1971) confirmed the involvement of the cyclic-AMP dependent protein kinase in catalysing the reaction. The apparent lack of stimulation of the endogenous protein kinase activity by cyclic-AMP remains, however, to be explained.

In agreement with the results of Walter and Schulz-Key (1980) suramin was found to be a potent inhibitor of the endogenous protein kinase activity in *O. volvulus* extracts. However, Walter and Schulz-Key (1980) found the cyclic-AMP dependent protein kinase activity to be less susceptible to suramin inhibition than the cyclic-AMP independent protein kinases. The use of endogenous substrates in this study is a possible explanation for the apparent discrepancy between this study and the results of Walter and Schulz-Key. The type of substrate used often influences the response of protein kinases to modulators (Berglund et al., 1977). In conclusion the (^{32}P)phosphoproteins identified in this investigation are candidate regulatory molecules in *O. volvulus*. Further inquiry into their physiologic functions is clearly indicated.

Acknowledgments

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