Modulation of protein kinases and phosphoprotein phosphatases by a small acidic protein from bovine brains

W.N. Kuo, L.P. Liu and M.A. Rahmani

Division of Science and Mathematics, Bethune-Cookman College, Daytona Beach (Florida 32015, USA), 29 March 1984

Summary. A small, acidic and heat-stable protein was purified from bovine brains by column chromatography on DEAE-cellulose, Bio-Gel HTP, Affi-Gel phenothiazine and Sephadex G-75. This protein stimulates megamodulin-dependent protein kinase I from brains and phosphoprotein phosphatases from either brain or yeast. However, it inhibits cyclic AMP-dependent protein kinases from skeletal muscle.

Key words. Protein kinase; phosphoprotein phosphatases; acidic modulator protein.

The occurrence of two classes of acidic and heat-stable modulator proteins have been reported: The first one being calmodulin[23] which has molecular weight (Mr) of 15,000 and functions mainly as Ca^{2+}-mediator to regulate a broad spectrum of cellular activities; the second one, megamodulin[4], named originally as stimulatory protein kinase modulator[5] which has Mr of 69,000 and functions in conjunction with Mg^{2+} or Mn^{2+} to regulate several enzymes[4-6]. Despite these differences, both modulins were shown to have not only strong intraction with basic proteins[7,8] but also great affinity with phenothiazine[9,10]. In this paper, we will describe yet another acidic and heat-stable protein factor purified primarily by excluding the above two modulins from brain homogenates.

Materials and methods. Arginine-rich histone (HA) was purchased from Worthington. Other histones, DEAE-cellulose, trypsin and muscle cyclic AMP-dependent protein kinases (peak I and II) were obtained from Sigma. Sephadex G-75 was from Pharmacia. [γ-^{32}P]ATP was supplied by New England Nuclear. Bio-Gel HTP (DNA grade) and Affi-Gel phenothiazine were products from Bio-Rad.

The preparation of phosphoproteins was basically the same as that described by others[11] except with some modifications[6]. The preparations and assays of phosphoprotein phosphatases[6] and megamodulin-dependent protein kinase I[4] from rabbit brain or baker's yeast were essentially the same as reported in our previous studies. However, the assay of cyclic AMP-dependent protein kinase was performed with 40 µg of arginine-rich histone as exogenous substrate protein and in the presence of 40 µM cyclic AMP[5]. The reactions for all enzymes were carried out for 10 min.

Throughout the entire process of purification, the detection of the presence of acidic protein(s) in all fractions of column eluates was monitored by the strong interaction[7,8] of acidic protein(s) with arginine-rich histones to form turbidity measured by the absorbance at 360 nm. Crude acidic protein was prepared by the steps of boiling and tricholoroacetic acid precipitation[5,12] of tissue homogenates from 850 g of bovine brains. Crude acidic protein, thus obtained, was applied to a DEAE-cellulose column (1.5 × 16 cm) previously washed and equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The chromatography was developed by a linear gradient (50 to 500 mM, total volume 240 ml) of potassium phosphate buffer. The fraction size was 5 ml. The pooled active fractions were dialyzed overnight against deionized water, and then concentrated to 5 ml. The subsequent purification was performed with column chromatography on Bio-Gel HTP (1.5 × 16 cm) developed as the same linear gradient of phosphate buffer (5 ml/fraction), Affi-Gel phenothiazine (0.5 × 3.5 cm) eluted with deionized water (1 ml/fraction) and Sephadex G-75 (1 × 56 cm) eluted with deionized water (1 ml/fraction).

Electrophoresis on both the small acidic protein from Sephadex G-75 and other standard proteins was performed with SDS-polyacrylamide gel (10 and 20%) by the method of Weber and Osborn[13]. In addition, the pI of the purified acidic protein was determined by isoelectric focusing on LKB Amphaline PAG-plate, pH 3.5–9.5 in LKB multiphor system[14].

The changes of ultraviolet (UV) spectra (from 200 through 340 nm) of the purified acidic protein was observed in a final volume of 1 ml of Tris-HCl buffer, 3 mM, pH 7.4; with small acidic protein from Sephadex G-75, 60 µg; with or without cation (Mg^{2+} or Mn^{2+}), 3 mM.

Results and discussion. One of the characteristics of this small acidic protein is that it exhibits strong interaction with basic proteins, such as arginine-rich histone. Such characteristic has greatly facilitated its purification. This protein was purified to apparent homogeneity after the step of Sephadex G-75 as judged by one single protein band in SDS-polyacrylamide gel electrophoresis. This protein has Mr of 10,000 estimated by a comparison of its mobility[13] with those of other standard proteins. Its small molecular weight was also confirmed by late-eluted protein peak around fraction 39 of Sephadex G-75 gel filtration. The acidic nature of this protein from Sephadex G-75 was further proven by its isoelectric point between pH 3.5 to 4.0 with 7 protein bands. Such appearance of multibands may be due to the different aggregated (or dissociated) forms of this protein and may partially account for its possible multifunctions. Some of these functions are discussed in the following paragraph.

The effects of small acidic protein on phosphoprotein phosphatases and protein kinases were compared (table). The activities of phosphoprotein phosphatases, either from rabbit brain or baker's yeast, were augmented to greater than 3-fold in the presence of Mn^{2+}. Nevertheless, when Mn^{2+} was omitted from the reaction mixture, less potent but undesirable activation of phosphoprotein phosphatase was observed. This may be due to minute contaminant of Mn^{2+} in the prepared enzymes. In addition, 1.8-fold stimulation of brain megamodulin-dependent protein kinase I in the presence of Mg^{2+} but ab-

Comparison of the effects of small acidic protein (SAP) on phosphoprotein phosphatases and protein kinases

Enzyme and source	Cation	Relative enzyme activity: −SAP	+SAP
Phosphoprotein phosphatases			
a) brain (12 µg):	None	100 ± 3	203 ± 12
	Mn^{2+} (2 mM)	106 ± 6	335 ± 15
b) yeast (12 µg):	None	100 ± 2	157 ± 8
	Mn^{2+} (0.5 mM)	118 ± 9	359 ± 26
Megamodulin-dependent protein kinase I, brain (21 µg):	None	100 ± 4	103 ± 4
	Mg^{2+} (10 mM)	211 ± 14	376 ± 23
Cyclic AMP-dependent protein kinase, muscle, a) peak I (2 µg):	None	100 ± 7	64 ± 3
	Mg^{2+} (10 mM)	597 ± 51	321 ± 17
b) peak II (2 µg):	None	100 ± 8	62 ± 5
	Mg^{2+} (10 mM)	613 ± 45	330 ± 19

Each value shown represents the mean ± (SEM) from 4 samples. Controls, expressed by 100, were 1168, 701, 508, 1951 and 1024 cpm for the relative activities of brain phosphatases, yeast phosphatases, brain megamodulin protein kinase I, muscle cyclic AMP-dependent protein kinase I and II, respectively. The amounts of the acidic protein (from Sephadex G-75) tested for above enzymes were 8, 8, 17, 8 and 8 µg, respectively.

sence of exogenous substrate protein (such as histone) was noted. In contrast, about 36% inhibition in the absence of Mg^{2+} and 47% inhibition in the presence of Mg^{2+} on cyclic AMP-dependent protein kinases by this acidic protein was observed when arginine-rich histone was added to reaction mixture as a substrate. Such inhibition may be partially due to the strong interaction between histone and this acidic protein which may deprive a part of histone from phosphorylation by these kinases. Cyclic AMP-dependent protein kinases have been found in many mammalian tissues[15], including brain. Therefore, the results of this study suggest that this acidic protein may also play an important regulatory role on this type of kinases in brains. It is of great interest that the small acid protein stimulates both enzymes, phosphoprotein phosphatases and megamodulin-dependent protein kinase I, which in general carry out opposite functions. Therefore, the time sequence for the activation of both enzyme in vivo may be different to avoid antagonism.

This acidic protein is a heat-stable factor since it retained original stimulatory effect on the same enzymes after being boiled at 100 °C for 30 min. However, its stimulatory activity was destroyed after being treated with trypsin at 37 °C for 2 h, indicating that it indeed is a protein. Moreover, altered UV spectra of this acidic protein in the presence of Mg^{2+} or Mn^{2+} were noted, suggesting that the concomitant conformational transition[16] of this protein may occur through its binding with the cation to convert into its active forms, acidic protein-cation complexes, which may thereby modulate enzymes.

1 Acknowledgments. This work was supported by a grant (RR-08229) from the National Institutes of Health, USA. W.N. Kuo is a recipient of a Distinguished Faculty Scholar Award from United Negro College Fund, Inc., USA.
2 Lin, Y.M., Molec. cell. Biochem. *45* (1982) 101.
3 Lin, Y.M., Liu, Y.P., and Cheung, W.Y., J. biol. Chem. *249* (1974) 4943.
4 Kuo, W.N., Cytobiose *36* (1983) 175.
5 Shoji, M., Brackett, N.L., Tse, J., Shapira, M., and Kuo, J.F., J. biol. Chem. *253* (1978) 3427.
6 Kuo, W.N., Biochem. biophys. Res. Commun. *114* (1983) 403.
7 Itano, T., Itano, R., and Penniston, J.T., J. Biochem. *189* (1980) 455.
8 Kuo, W.N., Experientia *39* (1983) 60.
9 Jamieson, G.A., Jr, and Vanaman, T.C., Biochem. biophys. Res. Commun. *90* (1979) 1048.
10 Kuo, W.N., unpublished observation.
11 Maeno, H., and Greengard, P., J. biol. Chem. *247* (1972) 3269.
12 Appleman, M.M., Birnbaumer, L., and Terres, H.N., J. biol. Chem. *116* (1966) 39.
13 Weber, K., and Osborn, M., J. biol. Chem. *244* (1969) 4406.
14 Winter, A., Ed, K., and Anderson, U.B., LKB Application Note 250 (1977).
15 Pasternak, Th., Cyclic AMP. Eds Robinson, Butcher, and Sutherland (1971) 127.

0014-4754/85/050622-02$1.50 + 0.20/0

Effects of diflubenzuron and tunicamycin on N-acetylglucosaminyl transferases in prepupae of the stable fly *(Stomoxys calcitrans)*

R.T. Mayer and A.C. Chen

Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research Service, US Department of Agriculture, P.O. Drawer GE, College Station (Texas 77841, USA), 16 March 1984

Summary. Tunicamycin, an antibiotic, and diflubenzuron, an insect growth regulator, were tested to determine their effects on N-acetylglucosaminyl transferase from *S. calcitrans* prepupae. Diflubenzuron had no effect, but tunicamycin inhibited the transfer of GlcNAc-1-P from UDP-GlcNAc to dolicholmonophosphate with an I_{50} of 1.5–4 ng/ml.
Key words. Stable fly; *Stomoxys calcitrans;* diflubenzuron; tunicamycin; N-acetylglucosaminyl transferase; prepupae.

Chitin synthesis inhibitors (CSI) such as diflubenzuron have been under investigation for a number of years now. Reports concerning the molecular mode of action of CSI are often conflicting and contradictory[1]. Initially, CSI were thought to inhibit chitin synthesis in insects by acting directly on chitin synthase[2–5]. There are now several reports that show that CSI do not operate on chitin synthase when studied in cell-free enzyme preparations[1,6,7].

However, chitin synthesis is inhibited by diflubenzuron (DFB) and other CSI when in vitro organ culture or in vivo assays are used[2–5,8,9], and a buildup of UDP-N-acetylglucosamine (UDP-GlcNAc) is observed in tissues or insects that have been treated with CSI. In addition, Meola and Mayer[9] and DeLoach et al.[10] showed that DFB acted as a cytostatic agent when topically applied to pupae of the stable fly. Consequently, it was suggested that the inhibition of chitin synthesis and buildup of UDP-GlcNAc in DFB-treated tissues and the cytostatic action of DFB might be explained by an effect on cell membrane permeability[1,10]. Indeed, recent studies with Harding-Passey melanoma cells showed that DFB significantly inhibited the uptake of certain nucleosides into those cells, which indicated that a membrane effect was involved[11]. It has been suggested that DFB might inhibit the N-acetylglucosamine-1-P (GlcNAc-1-P) transferases which transfer GlcNAc-1-P from UDP-GlcNAc to form GlcNAc-pyrophosphoryl-dolichol (Dol·PP-GlcNAc), and which are involved in membrane synthesis[1]. Inhibition of this enzyme system could alter membrane permeability and cause a buildup of UDP-GlcNAc.

We have investigated the GlcNAc-1-P transferases in prepupae of stable fly, and report here the effects of DFB on these enzymes. The present studies were done in conjunction with studies on the antibiotic tunicamycin, which has been reported to inhibit chitin[12] and glycoprotein[13,14] synthesis in insects. Our results are the first demonstration that tunicamycin inhibits insect GlcNAc-1-P transferases to prevent the formation of Dol·PP-GlcNAc, as it does in other organisms.

Materials and methods. Chemicals. UDP-[glucosamine-6-^{3}H]GlcNAc (24 Ci/mmole) and UDP-[glucosamine-1-^{14}C]GlcNAc (35 mCi/mmole) were purchased, respectively, from New England Nuclear, Boston, MA and ICN Pharmaceuticals, Inc., Irvine, CA. Betafluor scintillation cocktail was purchased from National Diagnostics, Somerville, N.J. Dolichol monophosphate (Dol·P), 80–90% pure based on phosphorus content, was purchased from Sigma Chemical Co., St. Louis, MO. A purified sample of tunicamycin was obtained from Dr J.D. Douros, Developmental Therapeutics Program, Chemotherapy, NCI, Bethesda, MD. Diflubenzuron (Dimi-