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PHOSPHOPROTEIN PHOSPHATASE IN THE CENTRAL NERVOUS SYSTEM OF *MANDUCA SEXTA*

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Summary

The existence and some enzymological properties of phosphoprotein phosphatase (EC 3.1.3.16) have been established in the larval central nervous system of the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). A simple, sensitive and reproducible assay employing ^{32}P-labeled protamine as a phosphoprotein substrate was employed to measure phosphatase activity in both soluble and particulate fractions of the insect nerve cord. The specific activity of soluble phosphatase in the *Manduca sexta* central nervous system is of the same order of magnitude as that in mammalian brain. Nerve cord phosphoprotamine phosphatase activity may be stimulated by a variety of monovalent salts, the optimal concentration of NaCl or KCl being 0.2 molar. Activity does not appear to be dependent on bivalent metals and is stimulated by EDTA. A reduced sulfhydryl group is obligatory for maximum activity. Phosphatase could be greatly inhibited by sodium fluoride, ATP and GTP. Cyclic AMP and cyclic GMP are without effect on enzyme activity. Although most of the phosphatase activity in the insect nerve cord appears to be of cytosolic origin, much latent activity can be unmasked by incubating membranous fractions with Triton X-100. In contrast to soluble phosphatase, the detergent-solubilized activity is moderately stimulated by Mn^{2+}.

Introduction

The activities of numerous enzymes are correlated with their state of phosphorylation [1] and the rate of phosphate turnover in these enzymes and

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in other cellular phosphoproteins is presumably the resultant of the relative activities of specific protein kinases and phosphoprotein phosphatases. The latter class of enzymes have been characterized in several tissues [2,6], but in comparison to protein kinases relatively little is known of their enzymological and regulatory properties. In another paper we report [7] the presence of high levels of cyclic AMP-stimulable and cyclic GMP-stimulable protein kinases in the central nervous system of *Manduca sexta.*

Phosphoprotein phosphatases may serve a very important biochemical function by catalyzing the removal of key phosphoprotein phosphate groups and thus effectively regulate the actions of cyclic AMP and cyclic GMP by balancing the protein kinase stimulating effects of these cyclic nucleotides. Mammalian brain tissue appears to be unique because both their kinase and phosphatase activities are particularly abundant relative to those in non-neural tissues [5,8,9]. Evidence has accumulated [10,11] that alterations in the excitability of the nerve cell plasma membrane result from changes in the state of phosphorylation of membrane proteins and that phosphorylation—dephosphorylation sequences may be involved in the regulation of synaptic transmission.

Materials and Methods

Preparation of [^{32}P] phosphoprotamine

[^{32}P] Phosphoprotamine was prepared by the enzymatic phosphorylation of protamine. The procedure was a modification of those utilized by Meisler and Langan [3] and by Maeno and Greegard [5]. The reaction mixture consisted of 200 μmoles of Tris—HCl, 20 mg of salmon protamine sulfate (Grade I, histone-free), 1.0 mg of cyclic AMP-dependent bovine heart protein kinase (Sigma), 9 μmoles of theophylline, 40 μmoles of [γ-^{32}P] ATP [9] (approximately 10^8 dpm, specific activity $4.8 \cdot 10^{11}$ dpm per mmole); total volume, 4 ml, pH 7.0. The reaction vessel was flushed with nitrogen, sealed and incubated at 37° C for 2 h. Then an additional 40 pmoles of cyclic AMP and 0.42 mole of [γ-^{32}P] ATP were added since the bovine heart kinase preparation contained phosphodiesterase and ATPase activities. After another 4 h of incubation, 10% (w/v) trichloroacetic acid was added dropwise with continuous mixing to a final concentration of 2%. The mixture was centrifuged (10 000 × *g*, 10 min), and phosphorylated protamine was precipitated from the supernatant by the addition of 5 ml of trichloroacetic acid. The precipitate was collected by centrifugation, washed twice in 10 ml of 10% trichloroacetic acid, recentrifuged, dissolved in 4 ml of 10 mM Tris—HCl (pH 7.0) by stirring for 1 h at 40° C, and precipitated again with an equal volume of 40% trichloroacetic acid. Phosphoprotamine was once again dissolved in 10 mM Tris buffer and precipitated with 40% trichloroacetic acid, then dissolved in 5 ml of warm 50 mM Tris—HCl (pH 7.0) and dialyzed at 4° C for 15 h against 8 l of distilled water. The phosphoprotamine solution was lyophilized, and for the enzyme assay the powder was stirred for 1 h at 40° C in 10 ml of 50 mM Tris—HCl, pH 7.5 to form a clear solution of 2 mg phosphoprotamine/ml as determined by the method of Lowry et al. [14] using protamine as a standard. Recovery of protamine carried through the phosphorylation reaction and numerous washing

steps was 100%. Incorporation of ^{32}P from [γ-^{32}P]ATP into protamine was 38% and amounted to 15 nmoles of phosphate per mg of protamine.

Enzyme preparation

The complete central nervous systems of fifth-instar *M. sexta* larvae were dissected as described previously [12,13]. 20—30 nerve cords were homogenized in all-glass tissue grinders in 1.0—1.5 ml of ice-cold 50 mM Tris—HCL (pH 7.0) which contained, where specified, 1 mM EDTA and 1 mM dithiothreitol. Centrifugation was at 4°C in screw-capped tubes filled with nitrogen gas. Since the majority of the protamine phosphatase activity was soluble, either the 34 000 × *g* (30 min) or the 105 000 × *g* (2 h) supernatants were employed as the enzyme source. Since the activity in 105 000 × *g* supernatants was moderately unstable to freezing and thawing, these samples were assayed immediately wherever possible.

Phosphoprotein phosphatase assay

Phosphatase activity was assayed by measuring the enzyme-catalyzed hydrolysis of radioactive orthophosphate from ^{32}P-labeled phosphoprotamine. The reaction mixture (0.10 ml total volume, pH 7.0) contained 5 μmoles of Tris—HCl, 100 μg of [^{32}P]phosphoprotamine ($3 \cdot 10^3$ dpm per μg), unless noted otherwise, and ordinarily less than 25 μg of enzyme protein. The reaction was initiated by the addition of enzyme, incubated in a shaking water bath at 30°C for 10 min, and terminated with 1.0 ml of 20% trichloroacetic acid. After the addition of 0.10 ml of 0.5% (w/v) bovine serum albumin carrier, the suspension was thoroughly mixed and allowed to remain at room temperature for 5 to 10 min. Precipitated protein was removed by centrifugation at 10 000 × *g* for 10 min. The deproteinized supernatant was mixed with carrier phosphorus (0.05 ml of 10^{-2} M KH_2PO_4), followed by the addition of 1.0 ml of 5% ammonium molybdate in 3.0 M H_2SO_4. This solution was periodically agitated over a 10 min period, and then the ^{32}P-containing phosphomolybdate complex was extracted once with 2.0 ml of water-saturated isobutyl alcohol—benzene (1 : 10, v/v). A single extraction removed > 99% of the total [^{32}P]orthophosphate. A 1.0 ml aliquot of the organic phase was mixed with 10 ml of fluor (Aquasol, New England Nuclear) and counted in a Packard 2425 liquid scintillation spectrometer. With 100 μg of phosphoprotamine present in the standard assay, the initial concentration of hydrolyzable ^{32}P was $1.6 \cdot 10^{-5}$ M. One unit of protamine phosphatase is defined as that amount which hydrolyzes one pmole (10^{-12} mole) of phosphate from 100 μg of phosphoprotamine in 10 min under the assay conditions employed. Replicate samples agreed to within 3%.

Results

Time, enzyme and substrate dependences of reaction velocity

With 100 μg of [^{32}P]protamine substrate the rate of dephosphorylation was linear in the presence of 1—25 μg of 105 000 × *g* supernatant for at least 10 min. Given sufficient time, the radioactive phosphate esterified to phosphoprotamine could be quantitatively hydrolyzed by soluble *M. sexta* phosphatase. As is indicated in Fig. 1, a double reciprocal plot of the relationship between

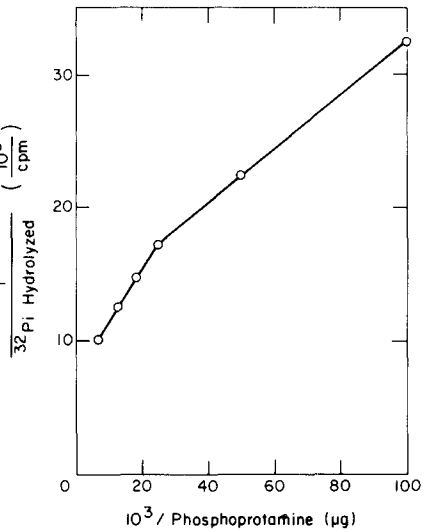

Fig. 1. Double-reciprocal representation of soluble protein phosphatase activity as a function of phosphoprotamine concentration. Source of enzyme was a 34 000 × *g* supernatant containing 1 mM EDTA and 1 mM dithiothreitol. Each reaction mixture contained 30 μg of enzyme protein and 10—160 μg of [^{32}P] protamine in a total volume of 0.10 ml. Assays were conducted as described in the text.

reaction velocity and substrate concentration revealed non-ideal Michaelis—Menten behavior. Two K_m values (20 μg and 40 μg of phosphoprotamine per 100 μl) were obtained by extrapolation of the linear sections of the plot. This kinetic behavior may be indicative of heterogeneity or cooperativity.

Sulfhydryl sensitivity

An appreciable loss in protamine phosphatase activity was noted in supernatants derived from homogenates prepared in Tris buffer containing no sulfhydryl protective reagents. An increase in activity of about 50% could be effected in such supernatants by including 1 mM dithiothreitol in the assay buffer but 10 mM dithiothreitol was less effective. Cysteine was also capable of stimulating activity (18% at 1 mM) and *p*-hydroxymercuribenzoate at 10^{-4} M resulted in a 94% inhibition of soluble activity.

Stimulation of activity by EDTA and effects of bivalent metals

EDTA promoted a considerable activation of phosphatase activity in 105 000 × *g* supernatants (Table I). If EDTA was absent during homogenization addition of 25 mM EDTA in the assay buffer resulted in a 4—5 fold increase in activity. In samples homogenized in the presence of 1 mM EDTA, activation was still possible by including additional EDTA in the reaction medium. The activation of phosphatase by EDTA is presumably due to removal of inhibitory metals from the enzyme. This is supported by the observation that addition of 1 mM 1,10-phenanthroline, a polyvalent metal ion complexer [17] resulted in a 20% stimulation. Larval nerve cord homogenates were prepared in the presence or absence of EDTA and dithiothreitol and centrifuged at either 34 000 × *g* or 105 000 × *g* (Table II). Highest activities were observed with both of these compounds present. The specific enzyme activity found for the

TABLE I

STIMULATION OF PROTAMINE PHOSPHATASE ACTIVITY BY EDTA

All assays were conducted in 50 mM Tris—HCl buffer as described in Methods. Concentrations of EDTA greater than 25 mM were not examined due to interference of this compound with phosphomolybdate complex formation. N.D., not determined.

EDTA* added to reaction mixture (mM)	Specific phosphatase activity of a 150000 × *g* supernatant prepared in:	
	Tris** (units/mg protein·10^{-3})	Tris—diothiothreitol—EDTA*** (units/mg protein·10^{-3})
0	3.4	7.3
1	5.7	9.7
5	8.4	N.D.
10	12.3	14.5
25	15.5	15.0

* A stock solution of EDTA (ethylenediaminetetraacetic acid) was prepared by neutralizing the fully protonated form with Tris.

** 30 *M. sexta* larval nerve cords were homogenized in neutral 50 mM Tris—HCl, and the 105000 × *g* supernatant was rapidly frozen in liquid nitrogen, followed by 2 days of storage before assay.

*** 30 nerve cords were homogenized in neutral 50 mM Tris—HCl—1 mM dithiothreitol—1 mM EDTA. An aliquot of the supernatant was assayed immediately and contributed 0.2 mM each of EDTA and dithiothreitol to the reaction mixture.

homogenate prepared in buffer containing EDTA and dithiothreitol is slightly higher than that reported by Maeno and Greengard [5] for a rat cerebral cortex homogenate.

The influence of various bivalent metals on soluble phosphatase activity was examined. At a concentration of 1 mM, neither Mg^{2+}, Ca^{2+}, nor Mn^{2+} exerted any significant effect, but at 10—50 mM they were increasingly stimulatory. Cu^{2+}, Zn^{2+} and Fe^{3+} strongly inhibited enzyme activity, possibly through reaction with sensitive sulfhydryl groups. The effects of these metals

TABLE II

PROTAMINE PHOSPHATASE ACTIVITY IN THE LARVAL *M SEXTA* NERVE CORD

Larval nerve cords were homogenized in 50 mM Tris—HCl (pH 7.0) which contained, where indicated, 1 mM dithiothreitol and 1 mM EDTA. All samples were assayed immediately after preparation. The reaction mixture contained one-tenth volume (10 μl) of enzyme protein, 100 μg of [^{32}P]phosphoprotamine, and no dithiothreitol or EDTA other than that contributed by the enzyme sample.

Expt	No. of nerve cords	Enzyme source	Homogenizing buffer	Specific activity (units/mg protein ·10^{-3})	Activity per nerve cord (units/central nervous system)
1a	20	Homogenate	Tris	0.86	390
1b	20	34000 × *g* supernatant	Tris	2.6	340
2	20	105000 × *g* supernatant	Tris	5.8	390
3	15	34000 × *g* supernatant	Tris—dithiothreitol	4.9	620
4a	30	Homogenate	Tris—dithiothreitol—EDTA	5.0	1540
4b	30	34000 × *g* supernatant	Tris—dithiothreitol—EDTA	11.0	1400

on *M. sexta* central nervous system phosphatase are qualitatively similar to those reported for soluble protamine phosphatase from the rat cerebral cortex [5] with the exception that the activity of rat brain phosphatase was nearly doubled in the presence of 2.5 mM $MnCl_2$. The stimulatory effects of EDTA and 1,10-phenanthroline together with the relative ineffectiveness of the bivalent metals tested strongly suggests that insect nerve cord phosphatase is not dependent on bivalent metals for activity.

Effects of salts and ionic strength

Soluble protamine phosphatase activity was appreciably stimulated in the presence of several monovalent salts (NaCl, KCl, $NaC_2H_3O_2$, $KC_2H_3O_2$, LiCl and $(CH_3)_4NCl$). All salts tested were approximately equipotent and at a concentration of 0.1 M resulted in about a 50% increase in the rate of [^{32}P]-protamine dephosphorylation. KCl was slightly more stimulatory than NaCl at concentrations greater than 75 mM and their effects were additive. Activity was optimal in the presence of 0.2—0.4 M salt and declined at higher concentrations. These effects appeared to be independent of the anion. Low concentrations of NaF strongly inhibited activity, an effect which has been reported in rat brain and liver preparations [5]. A simple explanation for the observed effects of univalent salts on *M. sexta* central nervous system phosphatase is that conditions of high ionic strength allow a more favorable interaction between the phosphatase and its positively charged substrate.

Effects of temperature and pH

In Fig. 2 is shown the dependence of protamine phosphatase activity on pH in sodium acetate, Tris—HCl, and Tris—maleate buffers. The fact that activ-

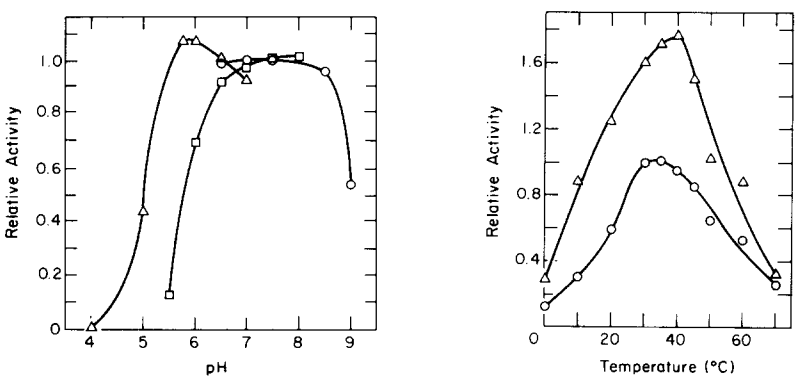

Fig. 2. Protamine phosphatase activity as a function of pH. Each reaction mixture was first preincubated in 33.1 μg of a 34 000 × *g* larval central nervous system supernatant prepared in Tris buffer containing dithiothreitol and EDTA for 5 min at 30°C in the indicated buffer and then initiating the reaction by the addition of 20 μg of [^{32}P]protamine Activities are expressed relative to that noted for Tris—HCl, pH 7.0 ($3.5 \cdot 10^3$ units/mg protein). Buffers (each 80 mM): ○———○, Tris—HCl; □———□, Tris—maleate; △———△, sodium acetate.

Fig. 3. Protamine phosphatase activity in the presence and absence of 0.2 M NaCl as a function of temperature. Each reaction mixture contained 20 μg of [^{32}P]protamine substrate and 33.1 μg of a 34 000 × *g* supernatant prepared in Tris buffer containing dithiothreitol and EDTA. Activities are expressed relative to that measured at 30°C in the absence of salt. ○———○, minus salt; △———△, 200 mM NaCl.

ity was relatively constant over a wide range of pH values suggests the presence of multiple soluble enzymes. Optimal activities in Tris buffers were at neutral pH values, and pH profiles similar to those shown were noted in Tris—HCl and acetate buffers prepared at constant ionic strength. At pH 7.0, activities in potassium phosphate and β-glycerophosphate buffers were 41% and 46%, respectively, of that observed in Tris—HCl.

The effect of temperature on phosphatase activity in the presence and absence of salt is shown in Fig. 3. With no NaCl present, optimal activity occurred at about 30°C. In contrast, activity in the presence of 0.2 M NaCl steadily increased until approximately 40°C, beyond which temperature activity sharply declined.

Effects of phosphate-containing compounds

At a concentration of 1 mM, ATP caused a 60% inhibition and GTP an 85% inhibition of soluble protamine phosphatase activity. The effects of these compounds may represent a physiologically significant control mechanism in that a partial inhibition of phosphoprotein phosphatase in vivo would result in an apparent enhancement of endogenous protein kinase activity. At a concentration of 1 mM the following compounds caused little or no inhibition: adenosine, cyclic AMP, cyclic GMP, phosphoserine, phosphothreonine, glucose 1-phosphate, glucose 6-phosphate, β-glycerophosphate and potassium phosphate.

Particulate phosphoprotein phosphatase

As shown earlier (Table II) equal volumes of a homogenate prepared in hypotonic Tris buffer and of the high speed supernatant derived therefrom contained very nearly the same total quantity of protamine phosphatase activity, suggesting that most of the activity of the *M. sexta* nerve cord was soluble. However, later experiments demonstrated that a considerable amount of phosphatase activity in the larval central nervous system is present in particulate fractions and is masked.

These experiments consisted of treating particulate fractions with Triton and finding an increase in activity. This released activity was stimulated by Mn^{2+} to a much greater extent (220% at 10 mM) than the soluble enzyme suggesting the presence of a discrete soluble and particulate phosphoprotein phosphatase. The anionic detergent, deoxycholate completely inhibits (at 0.01%) the homogenate activity.

Discussion

Protamine was chosen as a substrate for the present research principally because it is known to be readily phosphorylated and dephosphorylated in vivo and in vitro [3,16]. Protamines are exceptionally basic, low molecular weight proteins having high isoelectric pH values (p$I \approx 12$). They typically consist of approximately 70% arginine and therefore, for values of pH less than pI, they are highly positively charged. Because of this great charge at physiological pH values necessary for assaying phosphatase, it was anticipated that enzyme activity might be strongly dependent upon the ionic strength of the assay medium.

"Ionic effects" were, in fact, observed in the present study. It will be necessary to conduct assays with less highly charged substrates such as histone or casein in order to ascertain if the observed variations of enzyme activity with ionic strength are properties of phosphatase per se, or if they reflect primarily the highly-charged nature of the phosphoprotamine substrate.

The observed stimulation of phosphoprotein phosphatase activity in the presence of univalent salts may possibly have physiological significance. We have previously reported [7] that the activity of cyclic nucleotide-stimulable protein kinase of the *M. sexta* central nervous system is diminished as the ionic strength of the medium is increased. Therefore, it is apparent that conditions of elevated ionic strength would favor the net dephosphorylation of cellular proteins in the insect nerve cord.

In a particularly comprehensive study of phosphoprotein phosphatase in mammalian brain tissue, Maeno and Greengard [5] found that multiple enzymes were present in the rat cerebral cortex and that a membrane-associated enzyme could be distinguished from cytosolic phosphatases by its kinetic properties and its activation by Mn^{2+}. Several of the observations reported in this paper support the idea that there may also be present multiple phosphatases in the central nervous system of the insect.

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