RESEARCH COMMUNICATION

Phosphohistidine and phospholysine phosphatase activities in the rat: potential protein-lysine and protein-histidine phosphatases?

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We have detected phosphohistidine and phospholysine phosphatase activities in rat tissue extracts using partially phosphorylated, high-molecular-mass (> 10 kDa) polymers of histidine and lysine as substrates. Multiple phosphohistidine- and phospholysine-specific phosphatases were present in these extracts based on observed differences in heat stability, sensitivity to bivalent

metal ions and thiol modifying reagents, and/or elution from DE-52 cellulose. The properties of these phosphohistidine and phospholysine phosphatases were distinct from those of the phosphomonoester-specific protein phosphatases or the N-P phosphohydrolases that act on the free phosphoamino acids phosphoarginine, 3-phosphohistidine or phospholysine.

INTRODUCTION

In eukaryotic cells, several proteins undergo covalent modification via the phosphorylation of nitrogen atoms in the side chains of the N-amino acids histidine, lysine and arginine, including histones H1 and H4 [1,2], the plasma membrane proteins p38 [3] and p36 [4], and myelin basic protein [5]. In rats, the quantities of these N-phosphoamino acids in proteins are comparable with those of the O-phosphoamino acids phosphoserine and phosphothreonine, and far outstrip the level of phosphotyrosine [2,6–8]. Relatively little is known concerning the biochemistry or function of N-phosphorylation. Various groups have reported the detection of histidine- [9–12], lysine- [9,10], or arginine-specific [13–15] protein kinases, one of which, a protein-histidine kinase, has been purified to homogeneity [16]. However, it is not known whether phosphatases exist that target phosphohistidine and/or phospholysine residues in proteins. In this paper we describe the identification of phosphatase activities that act on phosphohistidine and phospholysine residues in high-molecular-mass (> 10 kDa) homopolymers of histidine and homo- and heteropolymers of lysine. These enzymes appear to have great potential as protein-histidine and protein-lysine phosphatases.

EXPERIMENTAL

Materials

Polylysine hydrochloride, polyhistidine hydrochloride, poly(Lys₁,Ala₁), poly(Lys₅,Ala₈,Glu₂,Tyr₁), purine nucleoside phosphorylase and 2-amino-6-chloropurine ribonucleoside were purchased from Sigma, St. Louis, MO, U.S.A. All other materials were from previously listed sources [17].

Preparation of soluble extracts

Male Sprague–Dawley rats were asphyxiated with CO₂, exsanguinated, and tissues were excised and washed in ice-cold saline. Tissues were homogenized in 3 vol. of 20 mM Mops, pH 7.5, containing 0.5 mM dithiothreitol, 0.5 mM EGTA, 0.2 mM phenylmethanesulphonyl fluoride and 5 µg/ml leupeptin (homogenization buffer) using a motor-driven glass–Teflon

homogenizer. The homogenate was centrifuged at 12000 *g* for 20 min and then at 100000 *g* for 60 min. The supernatant liquid was desalted on a column of Sephadex G-25 (Fine) equilibrated in homogenization buffer and then stored at –70 °C.

Separation of DE-52 peaks I and II

Soluble extract (25 ml) was applied to a 2.5 cm × 16 cm column of DE-52 cellulose equilibrated in homogenization buffer. The column was washed with 160 ml of homogenization buffer and developed with a linear gradient consisting of 140 ml each of homogenization buffer and of homogenization buffer containing 400 mM NaCl. Fractions (10 ml each) were collected and assayed for protein and activity.

Standard assay for N-phosphatase activity

Polyhistidine, polylysine, poly(Ala₁,Lys₁) and poly(Ala₈,Lys₅,Glu₂,Tyr₁) were phosphorylated to a stoichiometry of 1 mol of phosphohistidine or phospholysine per 3–7 mol of total histidine or lysine by a modification of the procedure of Wei and Matthews [16]. Briefly, commercially available amino acid homo- and hetero-polymers were treated with phosphorous oxychloride at basic pH (9.5–12.0) using triethylamine as buffer. Unreacted phosphorous oxychloride (etc.) was then removed by dialysis. The phosphopolyhistidine thus obtained contained a mixture of 1- and 3-phosphohistidine in which the latter predominated (85% of the total [16]). Portions (32.5 µl) of column fractions or other sources of N-phosphatase activity were incubated at 25 °C in a volume of 50 µl of 20 mM Mops, pH 7.5, containing 1 mM dithiothreitol, 10 mM MgCl₂, and 1–5 mM polymer-bound phosphate. The reaction was terminated, typically after a period of 1 h, by adding 150 µl of distilled water and immediately centrifuging in a Centricon-10 centrifugal concentrator/filtrator for 5 min at 5000 *g*. A 50 µl portion of the filtrate was analysed for P_i using Malachite Green [18]. For assay of phospholysine phosphatase activity, phosphopolylysine was utilized as substrate unless otherwise indicated. Hydrolysis of polymer-bound phosphate generally ranged from 10 to 45% and was dependent upon both time and the quantity of extract or column fraction used as source of N-phosphatase activity.

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Hydrolysis of polymer-bound phosphate by non-enzymic means occurred at a rate of $\leq 3\%/h$.

RESULTS

Incubation of soluble extracts from rat brain, heart, kidney, liver, lung, skeletal muscle and spleen with phosphopolylysine or phosphopolyhistidine resulted in the appearance of material in the filtrate of a Centricon-10 centrifugal ultrafiltration device that gave a positive reaction using the Malachite Green assay for P_i (Table 1). The identity of the reaction product was verified to be P_i by measuring the enzyme-catalysed phospholysis of a synthetic purine nucleoside, 2-amino-6-mercapto-7-methylpurine ribonucleoside [19] (Table 2). No material reactive with either ninhydrin or the Pauly reagent for imidazoles was detectable in the filtrates.

Phosphatase activity towards each of these substrates was sensitive to heating in the presence of SDS and was retained by

Table 1 Tissue distribution of phosphopolyhistidine and phosphopolylysine phosphatase activities

Soluble extracts (100 μg) from the rat tissues listed were assayed for phosphopolyhistidine and phosphopolylysine phosphatase activities as described in the Experimental section. Shown is the estimated specific activity towards each substrate for each tissue.

Tissue	Phosphatase activity (nmol of P_i /min per mg) towards:	
	<i>P</i> His	<i>P</i> Lys
Brain	1.2	5.0
Heart	1.4	3.1
Kidney	1.4	3.5
Liver	1.5	2.5
Lung	1.6	2.2
Skeletal muscle	1.0	2.2
Spleen	1.4	4.3

Table 2 Identification of the reaction product as P_i

Samples of DE-52 peak I (see Figure 1) (117 μg) and DE-52 peak II (59 μg) from rat liver were assayed in triplicate for phosphopolylysine and phosphopolyhistidine phosphatase activities as described in the Experimental section, which involves phosphate analysis of a 50 μl aliquot of the filtrate from a centrifugal concentrator using Malachite Green. In addition, a second 50 μl aliquot of filtrate was analysed for phosphate using the 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG)-coupled enzyme assay of Webb [19]. Shown is the total phosphate (mol) produced during the assay as determined using each procedure. Results are means $\pm$ S.E.M. ($n = 3$).

Sample	Substrate	Phosphate released (nmol)	
		Malachite Green	MESG
DE-52 peak I	<i>P</i> His	7.0 $\pm$ 0.2	6.1 $\pm$ 0.3
DE-52 peak I	<i>P</i> Lys	18.1 $\pm$ 0.2	14.8 $\pm$ 0.3
DE-52 peak II	<i>P</i> His	12.1 $\pm$ 0.3	11.4 $\pm$ 0.6
DE-52 peak II	<i>P</i> Lys	41.6 $\pm$ 0.5	41.3 $\pm$ 0.4

Figure 1 Fractionation of N-phosphatase activities in rat brain extracts by ion-exchange chromatography

Soluble extract from rat brain was fractionated on a column of DE-52 cellulose using a linear gradient of 0–400 mM NaCl in homogenization buffer as described in the Experimental section. Fractions were collected and assayed for 3-phosphopolyhistidine phosphatase activity ($\blacktriangle$) and phosphopolylysine phosphatase activity ($\triangle$), for protein by measuring absorbance at 280 nm (+), and for salt concentration by conductivity (O). DE-52 peaks I and II are marked by arrows.

the 10 kDa cut-off membrane of a Centricon-10 centrifugal ultrafiltration device, suggesting that the causative agent(s) was a large biomolecule such as a protein. At pH 7.5 both phosphopolylysine and phosphopolyhistidine phosphatase activities bound to DE-52 cellulose, but not to CM-trisacryl. Elution from DE-52 cellulose with a linear salt gradient revealed the presence of two major, roughly coincident peaks of phosphatase activity towards phosphopolylysine and phosphopolyhistidine in extracts from each of the aforementioned tissues (Figure 1). These peaks were designated DE-52 peak I and DE-52 peak II. In addition to acting on phosphopolylysine itself, DE-52 peaks I and II displayed phosphatase activity towards phospholysine residues present in a 1:1 random copolymer of lysine and alanine, or in a 5:6:2:1 random copolymer of lysine, alanine, glutamic acid and tyrosine.

The phosphopolyhistidine and phosphopolylysine phosphatase activities in DE-52 peak I appear to reside on independent proteins. Treatment of DE-52 peak I with *N*-ethylmaleimide or 5,5'-dithiobis-2-nitrobenzoic acid resulted in a dramatic loss of phosphopolylysine phosphatase activity, with little or no effect on the phosphopolyhistidine phosphatase activity in the same fraction (Table 3). Moreover, activity towards phosphopolyhistidine was moderately stimulated by Mg^{2+} , but not by Mn^{2+} , while activity toward phosphopolylysine exhibited the opposite pattern (Table 4). Similarly, DE-52 peak II appears to be composed of distinct phosphohistidine- and phospholysine-specific activities. Activity towards phosphopolylysine was noticeably more heat-labile than was activity towards phosphopolyhistidine (Figure 2), while both *N*-ethylmaleimide and 5,5'-dithiobis-2-nitrobenzoic acid were observed to dramatically attenuate phosphopolylysine phosphatase activity, with little or no effect on phosphopolyhistidine phosphatase activity (Table 3). DE-52 peak II phosphopolyhistidine phosphatase activity, but not phospholysine phosphatase activity, was inhibited by Co^{2+} and Mn^{2+} (Table 3).

Table 3 Effect of thiol modifying reagents on N-phosphatase activities

This Table summarizes the results of several experiments. DE-52 peak I (13–91 μg) and DE-52 peak II (23–36 μg) from rat liver were incubated with the reagents listed (3.2 mM), then assayed for phosphopolyhistidine and phosphopolylysine phosphatase activities as described in the Experimental section. All results are reported as a percentage of the activity measured in controls from which the modifying reagent was omitted. All results represents the means $\pm$ S.E.M. of n determinations, where n is listed in parentheses.

Reagent	Activity (% of control)			
	DE-52 peak I		DE-52 peak II	
	PHis	P.Lys	PHis	P.Lys
None	100	100	100	100
Iodoacetamide	131 $\pm$ 16 (3)	82 $\pm$ 5 (2)	125 $\pm$ 2 (2)	82 $\pm$ 7 (4)
N-Ethylmaleimide	74 $\pm$ 24 (5)	25 $\pm$ 3 (2)	108 $\pm$ 1 (4)	< 3 (4)
5,5'-Dithiobis-2-nitrobenzoic acid	109 $\pm$ 43 (5)	13 $\pm$ 8 (2)	110 $\pm$ 7 (4)	< 3 (4)
GSH	109 $\pm$ 8 (3)	120 $\pm$ 1 (2)	114 $\pm$ 8 (2)	95 $\pm$ 7 (4)

Table 4 Effect of bivalent metal ions on N-phosphatase activities

Samples of DE-52 peaks I and II from rat liver were dialysed against 20 mM Mops, pH 7.5, containing 0.1 M NaCl and 0.1 mM EGTA to remove dithiothreitol. DE-52 peak I (75–124 μg), and DE-52 peak II (13–33 μg) were then assayed for phosphopolyhistidine and phosphopolylysine phosphatase activities as described in the Experimental section, with the exceptions that dithiothreitol was omitted and the compounds listed were substituted for MgCl_2 at the indicated final concentrations. All results are reported as the amount of activity relative to that measured in the presence of 1 mM EDTA. $n = 3$, except for CaCl_2 + calmodulin, where $n = 2$. All results are means $\pm$ S.E.M.

Addition(s)	Activity (%)			
	DE-52 peak I		DE-52 peak II	
	PHis	P.Lys	PHis	P.Lys
EDTA (1 mM)	100	100	100	100
MgCl_2 (5 mM)	260 $\pm$ 21	104 $\pm$ 11	119 $\pm$ 13	138 $\pm$ 9
MnCl_2 (5 mM)	122 $\pm$ 21	206 $\pm$ 6	42 $\pm$ 8	106 $\pm$ 5
CoCl_2 (5 mM)	130 $\pm$ 1	95 $\pm$ 8	5 $\pm$ 3	97 $\pm$ 4
CaCl_2 (5 mM)	73 $\pm$ 12	112 $\pm$ 21	102 $\pm$ 7	134 $\pm$ 5
CaCl_2 (5 mM) + calmodulin (10 μM)	115 $\pm$ 20	116 $\pm$ 16	105 $\pm$ 7	130 $\pm$ 4

All N-phosphatase activities were insensitive to second messengers such as Ca^{2+} , Ca^{2+} /calmodulin (Table 4), cyclic nucleotides and inositol 1,4,5-trisphosphate. Addition of ATP, ADP or AMP had no effect. Likewise, the various N-phosphatase activities showed little or no sensitivity to the acid phosphatase inhibitor tartrate, to the alkaline phosphatase inhibitors tetramisole and EDTA, or to micromolar concentrations of the very potent inhibitors of the type 1 and 2A protein-serine/threonine phosphatases okadaic acid, calyculin A and microcystin-LR. In addition, sodium orthovanadate proved ineffective at a concentration (0.1 mM) that is effective at inhibiting protein-tyrosine phosphatases. NaF had no effect at a concentration of 5 mM, but at 50 mM it completely inhibited the phosphopolyhistidine phosphatase activity and partially inhibited the phosphopolylysine phosphatase activity in DE-52 peak I.

Figure 2 Differential heat stabilities of the phosphopolyhistidine and phosphopolylysine phosphatase activities in DE-52 peak II

DE-52 peak II (1.1 mg/ml protein) from rat liver was heated at 100 °C for the indicated times, cooled on ice, then centrifuged for 3 min at 10 000 g . The supernatant liquid was assayed for phosphopolyhistidine (●) and phosphopolylysine (○) phosphatase activities. Shown is the proportion of initial activity remaining as a function of heating time.

DISCUSSION

The study of protein phosphorylation events has frequently witnessed the employment of artificial substrates such as physiologically irrelevant phosphoproteins, synthetic peptides and polymers of amino acids such as poly(Glu₄, Tyr₁) for the detection and assay of protein kinases and protein phosphatases. In our search for potential protein-histidine and protein-lysine phosphatases in mammalian cells we have turned to chemically phosphorylated homopolymers of histidine or lysine as substrates.

We have detected phosphopolyhistidine and phosphopolylysine phosphatase activities in soluble extracts from a number of rat tissues. Based on the behaviour of these activities on DE-52 ion-exchange columns and differences in their susceptibility to heat, thiol modifying reagents, bivalent metal ions and various potential inhibitors, there appear to be multiple N-phosphatases present in these tissues. The simplest, but certainly not the only, interpretation of our data is that these enzymes totalled four in number: two chromatographically distinct phosphopolyhistidine phosphatases, the first of which (DE-52 peak I) was stimulated 2.5–3.5-fold by Mg^{2+} , and two chromatographically distinct phosphopolylysine phosphatases, the first of which (DE-52 peak I) was stimulated approx. 2-fold by Mn^{2+} .

Rat tissues contain a myriad of enzymes capable of acting as protein-serine, -threonine or -tyrosine phosphatases *in vitro*, as well as phosphohydrolases able to act upon N–P bonds in free phosphoamino acids or other small molecules. From the available evidence, it seems unlikely that the N-phosphatase activities identified in this study can be accounted for by any of these previously characterized enzymes. Phosphoramidase itself, which hydrolyses phosphoramidate to phosphate and ammonium ion, is sensitive to concentrations of fluoride ion (IC_{50} approx. 1 mM) [20] that produced no appreciable effect on any of the N-phosphatases described herein. That enzyme also is associated with microsomes [21], while the present phosphopolyhistidine

and phosphopolylysine phosphatase activities were soluble. The phosphatases that act on the free phosphoamino acids phospho-arginine, 3-phosphohistidine and phospholysine all bind strongly to CM-type, but not to DEAE-type, ion-exchange resins [22,23]. By contrast, the phosphopolyhistidine and phosphopolylysine phosphatases described in the present study displayed the opposite chromatographic behaviour, binding to DE-52 cellulose but not to CM-trisacryl. Thus the N-phosphatases identified herein would appear to be quite distinct from these N-amino acid phosphatases. While this paper was under review, a report appeared indicating that protein phosphatases 1, 2A and 2C each have histidine phosphatase activity *in vitro* towards histone H4 that contains 1-phosphohistidine [24]. However, their inhibitory signatures and other properties suggest that none of the phosphopolyhistidine or phosphopolylysine phosphatase activities reported herein can be accounted for by previously described protein-serine, protein-threonine or protein-tyrosine phosphatases; or by either alkaline or acid phosphatases.

More important than the issue of their identity, or lack thereof, with previously encountered enzymes is the question of whether any or all of the phosphopolyhistidine or phosphopolylysine phosphatase activities described herein represent potential protein N-phosphatases. The feature that distinguishes protein phosphatases from other phosphohydrolases is not the chemical nature of the bonds they cleave (phosphoramidate or phosphomonoester), but the context in which these bonds are found. At its most basic a protein phosphatase can be defined as an enzyme that hydrolyses phosphoryl groups bound to the side chains of amino acids present in polypeptides of high molecular mass (i.e. proteins). The phosphopolyhistidine and phosphopolylysine phosphatases described in this study acted on phosphohistidine or phospholysine residues present in the context of a protein-sized polypeptide chain of ≥ 120 amino acids. While these homopolymers do not possess the variety of amino acids found in naturally occurring proteins, it should be noted that the phospholysine phosphatase activities in DE-52 peaks I and II were each fully active toward a polymer that contained a mixture of neutral aliphatic (Ala) and aromatic (Tyr), basic (Lys) and acidic (Glu) amino acids. Further work is needed to ascertain whether these enzymes act on N-phosphorylated proteins, with all their subtleties of secondary and tertiary structure, etc., and

not just on polymers of limited compositional and spatial complexity. However, their ability to meet the basic definition outlined above suggests that the enzymes described herein possess the attributes necessary to function as protein-histidine and protein-lysine phosphatases.

This work was supported by Grant GM45368 from the National Institutes of Health and a John Le Pratt Animal Nutrition Senior Research Scholarship awarded to B.F.

REFERENCES

- 1 Chen, C. C., Smith, D. L., Bruegger, B. B., Halpern, R. M. and Smith, R. A. (1974) *Biochemistry* **13**, 3785–3789
- 2 Chen, C. C., Bruegger, B. B., Kern, C. W., Lin, Y. C., Halpern, R. M. and Smith, R. A. (1977) *Biochemistry* **16**, 4852–4855
- 3 Hegde, A. N. and Das, M. R. (1987) *FEBS Lett.* **217**, 74–80
- 4 Motojima, K. and Goto, S. (1993) *FEBS Lett.* **319**, 75–79
- 5 Smith, D. L., Kern, C. W., Halpern, R. M. and Smith, R. A. (1976) *Biochem. Biophys. Res. Commun.* **71**, 459–465
- 6 Zetterqvist, O. (1967) *Biochim. Biophys. Acta* **141**, 540–546
- 7 Zetterqvist, O. (1967) *Biochim. Biophys. Acta* **141**, 533–539
- 8 Mattoo, R. L., Khandelwal, R. L. and Waygood, B. F. (1984) *Anal. Biochem.* **139**, 1–16
- 9 Smith, D. L., Bruegger, B. B., Halpern, R. M. and Smith, R. A. (1973) *Nature (London)* **246**, 103–104
- 10 Smith, D. L., Chen, C. C., Bruegger, B. B., Holtz, S. L., Halpern, R. M. and Smith, R. A. (1974) *Biochemistry* **13**, 3780–3785
- 11 Huebner, V. and Matthews, H. R. (1985) *J. Biol. Chem.* **260**, 16106–16113
- 12 Huang, J., Wei, Y.-F., Kim, Y., Osterberg, L. and Matthews, H. R. (1991) *J. Biol. Chem.* **266**, 9023–9031
- 13 Wilson, M. E. and Consigli, R. A. (1985) *Virology* **143**, 516–525
- 14 Levy-Favatier, F., Delpech, M. and Kruh, J. (1987) *Eur. J. Biochem.* **166**, 617–621
- 15 Wakim, B. T., Pickens, M. M. and DeLange, R. J. (1990) *Biochem. Biophys. Res. Commun.* **171**, 84–90
- 16 Wei, Y.-F. and Matthews, H. R. (1991) *Methods Enzymol.* **200**, 389–414
- 17 Kennelly, P. J., Oxenrider, K. A., Leng, J., Cantwell, J. S. and Zhao, N. (1993) *J. Biol. Chem.* **268**, 6505–6510
- 18 Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. and Candia, O. A. (1979) *Anal. Biochem.* **100**, 95–97
- 19 Webb, M. B. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4884–4887
- 20 Singer, M. F. and Fruton, J. S. (1957) *J. Biol. Chem.* **229**, 111–119
- 21 Parvin, R. and Smith, R. A. (1969) *Biochemistry* **8**, 1748–1755
- 22 Kuba, M., Ohmori, H. and Kumon, A. (1992) *Eur. J. Biochem.* **208**, 747–752
- 23 Ohmori, H., Kuba, M. and Kumon, A. (1993) *J. Biol. Chem.* **268**, 7625–7627
- 24 Kim, Y., Huang, J., Cohen, P. and Matthews, H. R. (1993) *J. Biol. Chem.* **268**, 18513–18518