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Removal of phosphate from phosphohistidine in proteins

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Abstract

Kinetic constants of $K_M = 0.8$ μM, 3 μM and 1.6 μM, and $k_{cat} = 9\ s^{-1}$, $7\ s^{-1}$ or $9\ s^{-1}$ were determined for histidine dephosphorylation by protein phosphatases 1, 2A and 2C respectively. IC_{50} values were determined for the inhibition of protein phosphatase 1 by inhibitor 1 ($IC_{50} = 1$ nM), inhibitor-2 ($IC_{50} = 3$ nM) and okadaic acid ($IC_{50} = 30$ nM) and for the inhibition of protein phosphatase 2A by okadaic acid ($IC_{50} = 0.02$ nM) and microcystin-LR ($IC_{50} = 1$ nM). Inhibitor-1 ($K_i = 0.7$ nM) and okadaic acid ($K_i = 32$ nM) are noncompetitive with protein phosphatase 1. Some of the IC_{50} values were low enough to violate the assumptions of the usual inhibition equations and a more general approach to the analysis of the data was used. On the basis of these kinetic parameters and the presence of phosphohistidine, the major cellular protein serine/threonine phosphatases are likely to act as protein histidine phosphatases in the cell.

Keywords: Protein phosphatase; Phosphohistidine; Tight-binding inhibitor

1. Introduction

Protein phosphatases are key components of signal transduction and other regulatory events in eukaryotic cells [1–5]. In some circumstances, the role of protein phosphatases in regulation has been clearly defined, such as in the regulation of glycogen metabolism by the cyclic AMP-dependent protein kinase [1]. In other circumstances, the critical nature of the role of protein phosphatases is shown by the effects of specific inhibitors, particularly okadaic acid acting as a tumour promoter [6] or microcystin acting as a potent toxin. In these cases, however, the nature of the protein substrates involved is still under intensive investigation.

Histidine phosphorylation in eukaryotic proteins [7] was reported by Boyer and his colleagues in the 1960s [8,9]. The phosphate is linked to histidine through a phosphoramidate (P-N) bond which is stable at alkaline pH but unstable at acidic pH [10]. Although phosphohistidine as an amino acid is relatively unstable at neutral pH (especially the 1-isomer), the phosphohistidine in histone H4 is relatively stable at pH 7.5, possibly due to the absence of the α-amino group which has been implicated in the instability of 1-phosphohistidine [10,11]. Phosphohistidine is completely degraded by the acid hydrolysis conditions used for phosphoamino acid analysis [10,12,13] and is thus not seen in most studies of protein phosphorylation. Histidine phosphorylation has been reported on histone H4 in proliferating rat tissues [14,15] and on a rat plasma membrane protein [16–19] whose expression is negatively correlated with cell division. None of these reports address the question of how much phosphohistidine is present in cellular proteins. There are several estimates of alkali-stable phosphate in eukaryotic proteins (reviewed in Ref. [7]) but this does not solely represent phosphohistidine. Current data from our laboratory (Pesis and Matthews, unpublished) indicate that 5% to 7% of the phosphate in nuclear proteins from the slime mould, *Physarum polycephalum*, is in phosphohistidine. This is approximately equivalent to the amount of phosphothreonine, much less than phosphoserine, but much higher than phosphotyrosine which is less than 1% of protein phosphate.

Recently, two eukaryotic genes were discovered with deduced protein domains homologous to bacterial histidine kinase domains. One of these genes is involved in ethylene receptor action and may code for the receptor itself [20];

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the other is involved in regulation of protein degradation in budding yeast [21] and regulates the osmosensing MAP kinase cascade [22]. Two mitochondrial protein serine kinases homologous to the bacterial histidine kineses have been described [23,24]. Yeast cells (*Saccharomyces cerevisiae*) contain a specific protein histidine kinase [25] and protein histidine kinase activity has been found in several rat tissues [26], in the slime mold *Physarum polycephalum* [27], and in rat cell lines [28] (H.R.M., unpublished).

In the bacterial two-component regulatory system, the histidine phosphate produced by the kinase domain is transferred to an aspartate residue in an effector protein [29]. In eukaryotes, the catalytic subunits of protein phosphatases 1, 2A and 2C were shown to act on phosphohistidine in histone H4 but the data are largely qualitative [30]. Protein lysine phosphatases and protein histidine phosphatases have been inferred from data with phosphorylated polylysine and polyhistidine [31] and phosphatases that act on free phosphohistidine or phospholysine have been reported [32]. Motojima and Goto [19] deduced the presence of a histidine phosphatase activity with several of the properties of protein phosphatase 2C in rat liver. We now report kinetic characterization of the protein histidine phosphatase activities of protein phosphatases 1, 2A and 2C.

2. Materials and Methods

2.1. Materials

Histone H4 was purified from total calf thymus histone (Sigma Chemicals) by chromatography on a Biogel P-10 gel filtration column (5 cm diameter × 1 m long) eluted with 10 mM HCl. Protein histidine kinase was purified from *Saccharomyces cerevisiae* and stored in liquid nitrogen [13]. [^{32}P]H4 was prepared by incubating histone H4 with protein histidine kinase and [γ-^{32}P]ATP. After mild alkaline hydrolysis, [^{32}P]H4 was recovered, unincorporated ATP was removed [33], and the [^{32}P]H4 was dialysed into phosphatase buffer A (50 mM Tris-Cl [pH 7.5], 0.1 mM EGTA, 0.1% β-mercaptoethanol) and frozen in aliquots. This substrate is stored in phosphatase buffer A at −20°C or −70°C in aliquots and no degradation is found over the 4 weeks or so that it is used, although freezing and thawing must be avoided. If a sample of [^{32}P]H4 is allowed to stand at room temperature in phosphatase buffer A, phosphate is released at the rate of 5 to 6% per day so that under these conditions (pH 7.6) the phosphohistidine in histone H4 is reasonably stable.

Protein phosphatases were gifts from Dr. Philip Cohen and his colleagues. The catalytic subunits of PP1 and PP2A (Dr. D. Schelling) [34] and inhibitor-1 (Dr. M. Hubbard) [35] were purified from rabbit skeletal muscle at the University of Dundee by the investigators indicated in parentheses. PP2C, also from rabbit skeletal muscle, was partially purified up to the Sephadex G-100 step [36] and provided by Miss J. Corton and Dr. D.G. Hardie, University of Dundee. Inhibitor-2 expressed in *E. coli* was a gift to Dr. Philip Cohen from Dr. Anna De Paoli-Roach, University of Indiana, Indianapolis. Okadaic acid was a gift to Dr. Philip Cohen from Dr. Y. Tsukitani, Fujisawa Pharmaceutical Company, Tokyo, Japan. Microcystin-LR was provided by Dr. Carol MacKintosh, University of Dundee. The product of the CL100 gene was provided by Dr. D. Alessi and the phosphatase encoded by phage λ was provided by Dr. J. Dixon.

Protein phosphatase assays were carried out [33] in a final volume of 30 μl. Protein phosphatases 1 and 2A were diluted with 1 mg bovine serum albumin/ml in phosphatase buffer A just prior to assay [34]. An aliquot, 10 μl, of the diluted enzyme was mixed with 10 μl of 0.03% (w/v) Brij-35 in phosphatase buffer A on ice. The reaction was initiated by adding 10 μl of [^{32}P]H4. After incubation at 30°C, the reaction was stopped by adding ice-cold 50 mM Na phosphate (pH 8.5), 0.1 mM EGTA to a final volume of 400 μl. Released [^{32}P]phosphate was separated by ultrafiltration [34]. The assay for PP2C con-

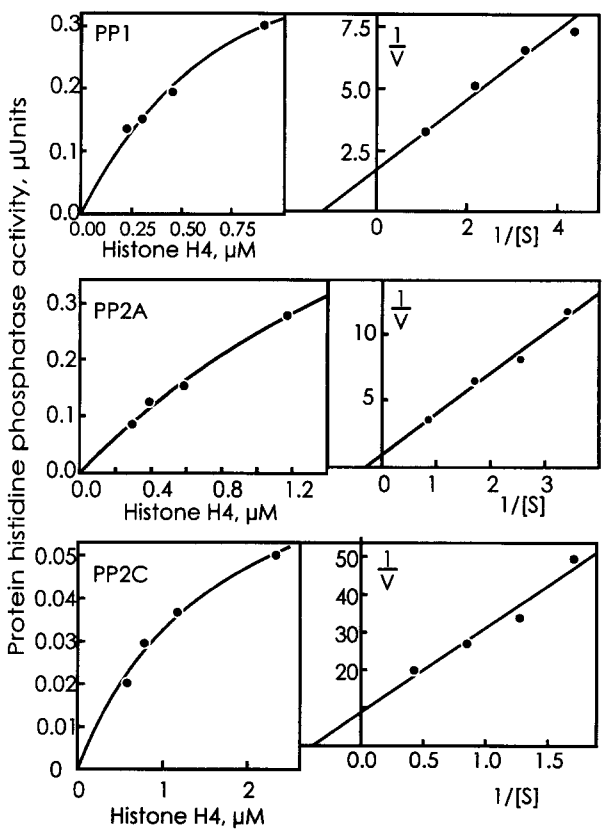

Fig. 1. Initial rate kinetics for protein histidine phosphatase activity of PP1, PP2A and PP2C. The left-hand panels show histidine phosphatase activity as a function of substrate concentration; the points are experimental data; the lines are the best fit to the Michaelis-Menten equation (parameters in Table 1). The right-hand panels show the double reciprocal plots; the points are experimental data (the same as the left-hand panel data) and the lines are derived from the fit shown in the left-hand panel. The substrate is 1-phosphohistidine at residue 75 in histone H4.

tained 10 mM Mg^{2+}. One Unit of phosphatase corresponds to the release of 1 μmol of phosphate per minute. Although prolonged incubation or high enzyme concentrations will remove at least 90% of the [^{32}P] from [^{32}P]H4, the kinetic data reported here were all obtained with less than 20% of the [^{32}P] removed.

Inhibitors were diluted in 0.03% (w/v) Brij-35 in phosphatase buffer A. Diluted protein phosphatase, 10 μl, was preincubated with 10 μl of diluted inhibitor for 2 min at 30°C before starting the reaction [35].

2.2. Kinetic equations

The Michaelis-Menten equation was used as: $V = V_{max} \cdot [S]/(K_M + [S])$ and the best fit values of K_M and V_{max} were determined by calculating V as a function of [S] for estimated values of K_M and V_{max} and determining the 'error' between the calculated and experimental values. The sum of squared errors was minimized by varying K_M and V_{max} using the default non-linear minimization routine in the computer program, Microsoft Excel (version 4.0), and the values of K_M and V_{max} that gave the minimum sum of squared errors were taken as the best fit values.

The same procedure was used to fit the equations for inhibition and to determine inhibition constants. The following equations were used when inhibitor binding was small compared with its total concentration: competitive inhibition:

$$V = V_{max} \frac{[S]/Ks}{1 + [S]/Ks + [I]/Ki}$$

noncompetitive inhibition:

$$V = V_{max} \frac{[S]/Ks}{1 + [S]/Ks + [I]/Ki + [S].[I]/(Ks.Ki)}$$

uncompetitive inhibition:

$$V = V_{max} \frac{[S]/Ks}{1 + [S]/Ks + [S].[I]/(Ks.Ki)}$$

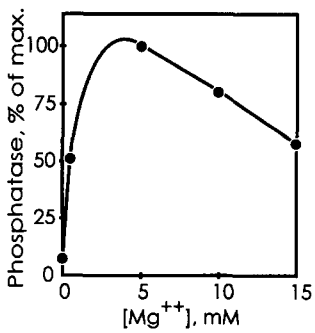

Fig. 2. Mg^{2+} requirement for protein histidine phosphatase activity of PP2C. PP2C was at 0.67 mU/ml and [histone H4] was 0.65 μM.

When this approximation was not valid, the following equations were derived from Henderson's analysis [37]:-

$$V = Vo \frac{Et - Kh - [I] + \sqrt{(Kh - Et + [I])^2 + 4.Et.Kh}}{2.Et}$$

where Kh is given by the following expressions, specific to the type of inhibition: noncompetitive inhibition

$$Kh = Ki$$

mixed noncompetitive inhibition

$$Kh = \frac{[S] + Ks}{(Ks/Kis) + ([S]/Kii)}$$

and competitive inhibition

$$Kh = Ki \frac{[S] + Ks}{Ks}$$

In these equations, Ks, Ki, Kis, Kii and V_{max} are the kinetic constants, [S] and [I] are the substrate and inhibitor concentrations, respectively; V is the initial reaction velocity; Vo is the reaction velocity in the absence of inhibitor; and Et is the total enzyme concentration.

The latter approach was used for the fits shown as the solid lines in Fig. 3, Fig. 4A,B, Fig. 5 and Fig. 6. The best

Table 1
Comparison of the activity of protein phosphatases against phosphohistidine in histone H4 and phosphoserine in phosphorylase a (PP1, PP2A) or myosin P-light chain (PP2C)

Substrate:	Phosphohistidine				Phosphoserine			
Phosphatase:	K_M μM	V_{max} U/mg	K_{cat} s^{-1}	K_{cat}/K_M $\mu M^{-1}s^{-1}$	K_M μM	V_{max} U/mg	K_{cat} s^{-1}	K_{cat}/K_M $\mu M^{-1}s^{-1}$
PP1	0.8	16	9	11	18 [a]	40–50 [b]	~ 20	~ 1
PP2A	3	11	7	2	5 [c]	3 [d]	2	0.4
PP2C	1.6	12	9	5	8 [e]	50 [e]	35	4

[a] Values for phosphorylase a: a value of 20 μM was quoted in [70] and 18 μM in [71].
[b] Given in [43] for the 33 kDa form of PP1.
[c] Values for phosphorylase a [72].
[d] Given in [43] for the 36 kDa catalytic subunit of PP2A.
[e] Values for myosin P-light chain [73]; phosphorylase a is a very poor substrate for PP2C [42]

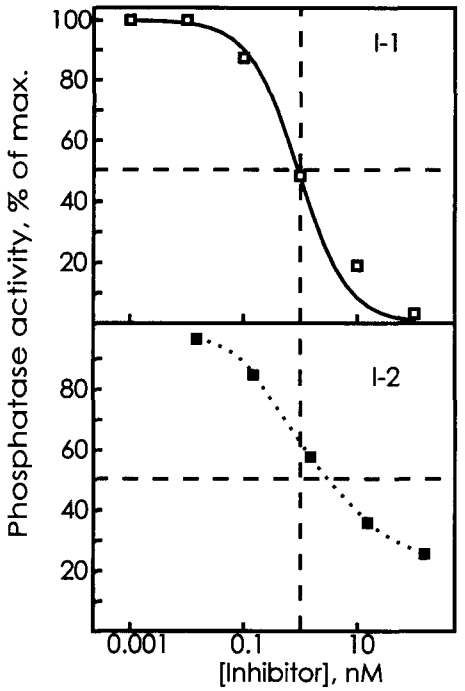

Fig. 3. Inhibition of protein histidine phosphatase activity of PP1 by inhibitor-1 and inhibitor-2. Inhibitor concentration is shown on a logarithmic scale. The vertical broken line is at 1 nM okadaic acid and the horizontal broken lines are at 50% inhibition. The upper panel shows data for inhibitor-1 and the lower panel for inhibitor-2. The inhibitor-1 data was fit to the equation for noncompetitive inhibition (given in Section 2) and the best fit is shown as the solid line. For inhibitor-1, PP1 was at 42 μU/ml and [^{32}P]histone H4 was 0.3 μM; for inhibitor-2, PP1 was at 160 μU/ml and [^{32}P]histone H4 was 0.3 μM.

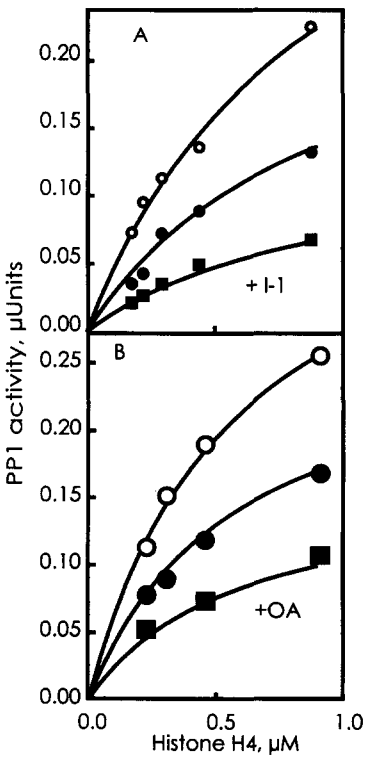

Fig. 4. Noncompetitive inhibition kinetics for protein histidine phosphatase activity of PP1. The solid lines show the best fit to the Henderson equation for noncompetitive inhibition (the equation is given in Section 2 and the best fit parameters are listed in Table 2). (A) Inhibitor-1; the concentrations shown were 0, 1.3 and 5.8 nM. Data were also obtained at 0.25 nM but are not shown, for clarity. (B) Okadaic acid; the concentrations shown were 0, 16 and 50 nM.

fit parameters for Fig. 5A,B are given in Table 2 and these were used to draw the solid lines in Fig. 4 and Fig. 5. For Fig. 6 the best fits to noncompetitive inhibition are shown. The following inhibition constants were used to draw the lines in the figures: $K_i = 0.2$ nM (Fig. 5); $K_i = 15$ nM (Fig. 6, PP1); $K_{ii} = 0.02$ nM, $K_{is} = 0.01$ nM (Fig. 6, PP2A).

3. Results

Protein histidine phosphatase activity was determined using a substrate labeled specifically on histidine [33]. Initial rates of reaction were used to confirm agreement with the Michaelis-Menten equation, Fig. 1. Table 1 gives the best-fit values of K_M and V_{max}. The Mg^{2+} requirement for PP2C showed a maximum at 4–5 mM and a significant reduction in activity at higher [Mg^{2+}] (Fig. 2). Inhibition of protein histidine phosphatase activity of PP1 by inhibitor-1 and inhibitor-2 was indistinguishable, by IC_{50}, from inhibition of the protein serine phosphatase activity (Fig. 3). Inhibitor-1 was non-competitive (Fig. 4) with $K_i = 0.7$ nM.

The protein serine phosphatase activity of PP1 and PP2A is very specifically inhibited by several natural products, including okadaic acid [6] and microcystin [38]. Microcystin-LR inhibits the histidine phosphatase activity of PP2A with an IC_{50} of 0.4 nM (Fig. 5). Okadaic acid

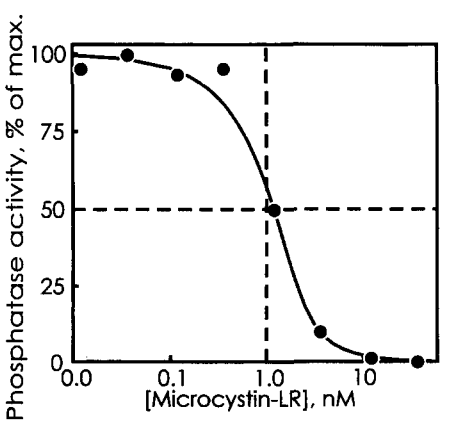

Fig. 5. Inhibition of protein histidine phosphatase activity of PP2A by microcystin-LR. Microcystin concentration is shown on a logarithmic scale. The vertical broken line is at 1 nM and the horizontal broken line is at 50% inhibition. PP2A was at 100 μU/ml and [^{32}P]histone H4 was 1 μM.

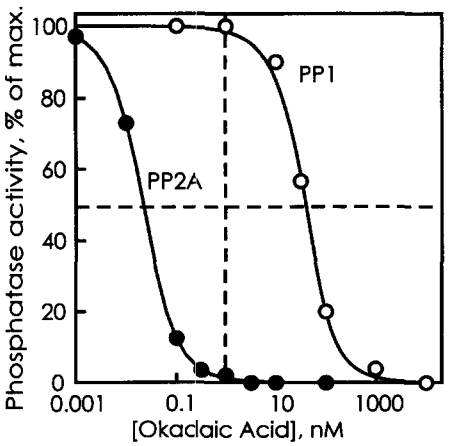

Fig. 6. Inhibition of protein histidine phosphatase activity of PP1 and PP2A by okadaic acid. Okadaic acid concentration is shown on a logarithmic scale. The vertical broken line is at 1 nM okadaic acid and the horizontal broken line is at 50% inhibition. The data were fitted to the equation for noncompetitive inhibition (given in Section 2) and the best fits are shown as the solid lines. PP1 was at 42 μU/ml, PP2A was at 6.3 μU/ml and [^{32}P]histone H4 was 0.3 μM (PP1 curve) or 0.7 μM (PP2A curve).

distinguishes between PP1 and PP2A, being much more potent with PP2A (Fig. 6). As okadaic acid is a tight-binding inhibitor, the apparent IC_{50} varies with enzyme concentration. A set of equations, given in Section 2, was derived from Henderson's equations [37] and used to analyse the kinetic data without making the conventional assumption of low inhibitor binding relative to inhibitor concentration.

The mechanism of inhibition of PP1 by okadaic acid was found to be non-competitive by varying the substrate concentration at different okadaic acid concentrations. The best fit kinetic constants are given in Table 2.

Two protein phosphatases not previously tested with a substrate containing phosphohistidine were incubated with [^{32}P]H4. The λ phage-encoded phosphatase [39] was highly active in releasing phosphate from phosphohistidine in histone H4 under conditions similar to those used for PP1

Table 2
Inhibition of protein histidine phosphatase

Inhibitor	Phosphatase	IC_{50} nM	K_i nM	K_s μM	$[E]_t / K_i$
Inhibitor-1	PP1	1	0.9	0.5	0.9
Inhibitor-2	PP1	3	–	–	–
Okadaic acid	PP1	30	32	0.6	0.013
Okadaic acid	PP2A	0.02	–	–	–
Microcystin-LR	PP2A	1	–	–	–

K_i and K_s values were determined as described in Section 2 for simple non-competitive inhibition using data relating reaction rate to substrate concentration at several inhibitor concentrations. The equations used did not rely on the assumption of relatively low inhibitor binding since this fails in the case of inhibitor-1 [73,74].

and PP2A (1 μg/ml of λ phosphatase contained more than 4 mU/ml of protein histidine phosphatase activity under the standard assay conditions [33]). In contrast, a phosphatase (the product of the human CL100 gene) that dephosphorylates MAP kinase [40] was completely inactive against [^{32}P]H4.

4. Discussion

Protein phosphatases 1 and 2A comprise most of the divalent ion-independent protein serine/threonine phosphatase activity in eukaryotic cells [1,41,42]. PP1 and PP2A are related but distinct proteins [43–45]. Each has more than one isoform [46,47] and several other members of this family of protein phosphatases have been isolated through DNA sequence homology [48]. The other two families of serine/threonine protein phosphatases are divalent ion-dependent: PP2B requiring Ca^{2+}; and PP2C requiring Mg^{2+}. Under physiological conditions these enzymes do not attack phosphotyrosine. The other major phosphorylated amino acid that has been found in cells is phosphohistidine in which the phosphate is bound to the amino acid residue through a phosphoramidate (P-N) bond rather than the phosphoester (P-O) bond found in phosphoserine and phosphothreonine. Surprisingly, it was recently found that PP1, PP2A and PP2C do attack phosphohistidine [30] although primarily qualitative data was collected on this hitherto unknown activity. PP2B, and the protein tyrosine phosphatase, PTP-1B, did not attack phosphohistidine. In this paper, two dual-specificity protein phosphatases have been tested. The list of protein phosphatases that work with phosphohistidine in histone H4 now comprises PP1, PP2A, PP2C and the λ-encoded protein phosphatase; the list of protein phosphatases that do not work with this substrate includes PTP-1B, PP2B and the product of the human CL100 gene. The phosphohistidine phosphatase activity of PP1, PP2A and PP2C has now been systematically characterized, allowing a careful assessment of the significance of the newly discovered activity.

The protein histidine phosphatase activity of PP1 is described well by the Michaelis-Menten equation with a K_M for the [^{32}P]H4 substrate of 0.8 μM. PP1 appears to be present in chromatin at high concentration [1,49] and histone H4 has been reported to be phosphorylated on histidine [14,15]. The histone H4 concentration in nuclei is much higher than 0.8 μM but the concentration of phosphorylated histone H4 is not known, although overall nuclear proteins in *Physarum* have 5–7% of their phosphate in phosphohistidine (Pesis and Matthews, unpublished). Thus, the kinetic parameters for PP1 are fully consistent with it acting as a protein histidine phosphatase in vivo.

In the cell, PP1 is mostly, if not entirely, complexed with other proteins so that it is found predominantly in the

particulate fraction of homogenized cells [1]. The PP1 in the cytoplasm is complexed with an inhibitor protein, inhibitor-2, to form PP1I which does not show serine/threonine phosphatase activity [50–53]. Inhibitor-2 acts as a molecular chaperone to fold PP1 into its active conformation [54]. Inhibitor-2 inhibits the protein histidine phosphatase activity of PP1 with an IC_{50} in the nanomolar range. Thus, PP1I is expected to be inactive towards phosphohistidine-containing substrates as it is towards phosphoserine or phosphothreonine-containing substrates.

Cells contain another specific protein inhibitor of PP1, inhibitor-1 [55,56], which is closely related to the dopamine-regulated neuronal phosphoprotein, DARPP-32 [57]. Inhibitor-1 inhibits the protein histidine phosphatase activity of PP1 with an IC_{50} in the nanomolar range (Table 2), comparable with the inhibition of the protein serine phosphatase activity. The mechanism of inhibition is non-competitive with an inhibition constant, K_i, for [^{32}P]H4 of only 0.9 nM. The low K_i made it necessary to use more general equations for analysis of the kinetic data since the conventional approximation of low binding relative to inhibitor concentration no longer holds.

The protein histidine phosphatase activity of PP2A is described well by the Michaelis-Menten equation with a K_M for the [^{32}P]H4 substrate of 3 μM. PP2A is also found in chromatin [1,49] and thus, like PP1, has the potential to dephosphorylate histone H4 in vivo.

Many studies of protein phosphorylation are carried out using specific enzyme inhibitors. For example, genistein is used as a tyrosine kinase inhibitor and may have potential as an anti-tumour agent [58]. Genistein also inhibits protein histidine kinase [59]. In the case of protein phosphatases, a number of inhibitors, including okadaic acid, are used to infer the involvement of protein serine/threonine phosphatases in cellular events. In fact, okadaic acid is a potent tumour promoter with multiple effects on cells [6,60–65]. Okadaic acid has potent effects on the protein histidine phosphatase activity of PP2A (IC_{50} = 20 pM) and also inhibits the same activity of PP1 (IC_{50} = 30 nM). Inhibition of PP1 is noncompetitive with an inhibition constant, K_i, of 32 nM; the inhibition mechanism for PP2A is also probably noncompetitive (data not shown). Since the effect on the histidine phosphatase activity is essentially indistinguishable from the effect on the serine phosphatase activity, the cellular effects of okadaic acid may be due to effects on either histidine phosphorylation or serine/threonine phosphorylation, or both. This needs to be taken into account when interpreting the results of experiments with okadaic acid.

PP2C has a relatively low K_M (1.6 μM) for the [^{32}P]H4 substrate. PP2C requires Mg^{2+} for its protein histidine phosphatase activity but, unlike the serine phosphatase activity, the histidine phosphatase activity is inhibited by [Mg^{2+}] above 5 mM. This may be due to aggregation of the substrate rather than an intrinsic property of PP2C. Thus, the [Mg^{2+}] required for apparently half maximal activation of the histidine phosphatase activity of PP2C is about 1 mM while the corresponding value for the serine phosphatase activity is 5 mM [36].

Comparison of the K_M values for phosphorylase a and histone H4 shows that histone H4 has the lower K_M in all cases suggesting that PP1 and, to a lesser extent, PP2A have a higher affinity for the histidine site on histone H4 than for the serine site on phosphorylase a. The histidine site on histone H4 (residue 75) is highly charged and very hydrophilic: -Thr-Tyr-Thr-Glu-His-Ala-Lys-Arg-Lys-Thr-. Basic proteins and other molecules such as heparin or polyamines affect the activity of PP1 and PP2A [1]. With phosphorylase a, PP1 is inhibited by basic proteins [1]. The low K_M for phosphorylated histone H4 suggests that the active site of PP1 may bind basic molecules strongly. If so, this suggestion predicts that the inhibition of PP1 by basic proteins and other molecules may be competitive. The K_M for histone H4 and PP2A is not as low as for PP1, suggesting that the active site of PP2A may be less acidic. This is supported by the fact that basic proteins inhibit PP2A only at high concentrations. At low concentrations, basic proteins activate PP2A. The histone H4 concentrations used in the current study were generally less than 1 μM and are thus in the range that would activate PP2A [1]. This may account for the higher k_{cat} of PP2A for histone H4 than for phosphorylase a.

Although various criteria may be used to determine the substrate preference of an enzyme, the ratio k_{cat}/K_M is a good way to take into account the effects of both K_M and V_{max}. By this criterion, histidine in histone H4 is preferred to serine in phosphorylase a by both PP1 and PP2A; PP2C uses histone H4 equally with myosin P-light chain. Given the wide range of k_{cat}/K_M values for different enzymes, all the k_{cat}/K_M values are reasonably close and, in ranging up to 10 or 11 $\mu M^{-1}s^{-1}$, show the phosphatases working with high efficiency with either substrate, since a diffusion-controlled reaction would have a k_{cat}/K_M of about 100 to 1000 $\mu M^{-1}s^{-1}$ [66].

In prokaryotes, the two-component system–which involves histidine phosphorylation–plays a central role in a major group of signal transduction mechanisms [67,68] and similar roles may occur in eukaryotes [20–22]. The study of the enzymes in eukaryotes capable of phosphorylating proteins on histidine has produced a novel protein kinase that appears to be specific for histidine phosphorylation and differs from the two-component system in phosphorylating an exogenous substrate [25]. The role of this protein histidine phosphorylation in eukaryotes has been speculated to involve an anti-proliferation or pro-differentiation signal [7,69]. We now demonstrate that three major cellular protein serine/threonine phosphatases are as active with phosphohistidine as with phosphoserine. Thus, eukaryotic cells have the enzymatic machinery to regulate protein phosphorylation on histidine. It remains to identify the cellular proteins that are so phosphorylated and to determine their roles in eukaryotes.

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