Further evidence that inhibitor-2 acts like a chaperone to fold PP1 into its native conformation

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Abstract **The γ_1-isoform of protein phosphatase-1 expressed in *Escherichia coli* (PP1γ) and the native PP1 catalytic subunit (PP1C) isolated from skeletal muscle dephosphorylated Ser-14 of glycogen phosphorylase at comparable rates. In contrast, PP1γ dephosphorylated several tyrosine-phosphorylated proteins at similar rates to authentic protein tyrosine phosphatases (PTPases), but native PP1C was almost inactive towards these substrates. The phosphorylase phosphatase (PhP) and PTPase activities of PP1γ were inhibited by vanadate with IC_{50} values (30–100 μM) comparable to authentic PTPases, whereas the PhP activity of native PP1C was insensitive to vanadate. PP1γ lost its PTPase activity, and its PhP activity became insensitive to vanadate, after interaction with inhibitor-2, followed by the reversible phosphorylation of inhibitor-2 at Thr-72. These findings support and extend the hypothesis that inhibitor-2 functions like a chaperone to fold PP1 into its native conformation, and suggest that the correct folding of PP1 may be critical to prevent the uncontrolled dephosphorylation of cellular phosphotyrosine residues.**

Key words: Protein phosphatase-1; Inhibitor-2; Chaperone; Protein tyrosine phosphatase

1. Introduction

Protein phosphatase-1 (PP1), one of the major serine/threonine-specific protein phosphatases in eukaryotic cells, is inhibited specifically by two small thermostable proteins termed inhibitor-1 and inhibitor-2 [1]. Inhibitor-1 only becomes a PP1 inhibitor after it has been phosphorylated by cyclic AMP-dependent protein kinase and is likely to play a role in the regulation of PP1 by agonists that modulate the cellular concentration of cyclic AMP. In contrast, the phosphorylation of inhibitor-2 (at Thr-72), which is catalysed by glycogen synthase kinase-3 (GSK3) and several other kinases in vitro, is not required to inhibit PP1 but essential to initiate reactivation of the PP1–inhibitor-2 complex (hereafter termed PP1I) [2].

A new hypothesis for the role of PP1I emerged while studying the properties of PP1 expressed in *E. coli* [3]. Four isoforms of the PP1 catalytic subunit have been identified in mammalian cells, termed PP1α, PP1β and PP1γ_1 and PP1γ_2. When expressed in *E. coli*, several of the properties of PP1α, PP1β and PP1γ_1 were found to resemble those of the 'native' PP1 catalytic subunit extracted from mammalian tissues, while others were quite different. For example, 'expressed' and 'native' forms of PP1 had a similar activity towards glycogen phosphorylase and similar sensitivity to inhibitor-2, but the activity of 'expressed' PP1 towards histone H1 (phosphorylated at Ser/Thr-Pro motifs by a cyclin-dependent protein kinase) was far higher. Moreover, 'expressed' forms of PP1 were 100–1000-fold less sensitive to inhibitor-1, PP1α and PP1γ_1 were unable to interact with the M-subunits that target PP1 to myosin and PP1β was less sensitive to inhibition by naturally occurring toxins, such as okadaic acid and microcystin-LR [3]. However, when 'expressed' forms of PP1 were combined with inhibitor-2 and Thr-72 was reversibly phosphorylated, the properties reverted to those of 'native' PP1. Taken together, these observations suggested that inhibitor-2 might play an important role in the correct folding of PP1 in vivo.

The concept that inhibitor-2 may function in a manner analogous to a 'chaperone' or 'matchmaker' has been questioned [4,5] but, in this paper, we present further evidence in support of this hypothesis. We show that while 'native' PP1 is devoid of protein tyrosine phosphatase (PTPase) activity, PP1γ expressed in *E. coli* is an extremely active PTPase. Moreover, the PTPase activity is lost when 'expressed' PP1 is combined with inhibitor-2 and Thr-72 reversibly phosphorylated. We suggest that the reversible phosphorylation of inhibitor-2 is not only essential to fold PP1 correctly, but is likely to be critical to prevent the uncontrolled dephosphorylation of cellular phosphotyrosine residues.

2. Materials and methods

2.1. Materials

PP1C and the glycogen-bound form of PP1 (PP1G) were purified from rabbit skeletal muscle [6]. Human PP1γ_1 [7], PP1α and PP1β were expressed in *E. coli* and purified [3] by Drs. D. Alessi and A. Street (MRC Protein Phosphorylation Unit, Dundee). Inhibitor-2 was purified from rabbit skeletal muscle by Dr. M. Hubbard (MRC Protein Phosphorylation Unit, Dundee) [8]. PTP-PEST was purified as described [9]. The c-Fgr protein tyrosine kinase (TPK-III) and the synthetic peptide AFLEDFFTSTEPQYQPGENL corresponding to residues 514–533 of p60src (except for Phe in place of Tyr at residue 6) [10] (termed src-tide) were gifts from Professor L. Pinna and Dr. Maria Ruzzene (University of Padua, Italy).

2.2. Preparation of ^{32}P-labelled substrates and protein phosphatase assays

^{32}P-labelled glycogen phosphorylase, phosphorylated at Ser-14 by phosphorylase kinase [6], and ^{32}P-labelled casein, phosphorylated at serine residues by cyclic AMP-dependent protein kinase [11], were prepared as described previously. The src-tide peptide (see Section 2.1) was phosphorylated at the tyrosine residue by cFgr protein tyrosine kinase in the presence of polylysine [10], and myelin basic protein (MBP) and reduced-carboxymethylated lysozyme (RCML) were

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^{32}P-labelled on tyrosine using the β-subunit of the insulin receptor as the tyrosine kinase [12].

All PP1 preparations were assayed at 30°C in the absence of divalent cations [6,11]. The final substrate concentrations in terms of ^{32}P-radioactivity were: phosphorylase (10 μM), casein (6 μM), src-tide (0.8 μM), MBP (1 μM) and RCML (1 μM). The *para*-nitrophenyl phosphatase (*p*NPP) concentration was 5 mM [6]. Caffeine (5 mM) was included when the substrate was glycogen phosphorylase [6]. One unit of activity (U) was that amount which released 1 μmol of phosphate from each substrate in 1 min.

3. Results

3.1. PP1γ expressed in E. coli has high PTPase activity

While investigating the possibility of developing a non-radioactive assay for PP1, we noticed that the *para*-nitrophenyl phosphatase (pNPP) activity of bacterially expressed PP1γ was 30-fold higher than that of the native catalytic subunit of PP1 (PP1C) purified from rabbit skeletal muscle and about 1000-fold higher than that of the glycogen-associated form of PP1 (PP1G) isolated from the same tissue (Table 1). Since the dephosphorylation of a phenolic residue is akin to the dephosphorylation of a tyrosyl residue, we examined the activity of different PP1 preparations towards several substrates phosphorylated on tyrosine residues. These experiments revealed that the PTPase activity of PP1γ was also remarkably high, the rate of dephosphorylation of MBP being slightly higher than that of one authentic PTPase (PTP-PEST) towards the same substrate (Fig. 1). As observed with pNPP as substrate, PP1γ dephosphorylated a tyrosine-phosphorylated peptide substrate (src-tide) 20-fold more rapidly than PP1C and 1000-fold more rapidly than PP1G (Table 1).

3.2. Inhibition of bacterially expressed PP1γ by vanadate and okadaic acid

It is well established that the dephosphorylation of tyrosine residues by authentic PTPases is inhibited by vanadate, whereas the dephosphorylation of serine and threonine residues by native PP1C is not (Fig. 2). Surprisingly, however, we found that both the phosphorylase phosphatase (PhP) (Fig. 2) and PTPase activities (Fig. 1) of PP1γ were sensitive to vanadate with IC_{50} values (0.1 mM for the PhP activity and 0.03 mM for the src-tide-PTPase activity) similar to those of authentic PTPases. Both the PhP [3] and PTPase activities of PP1γ were inhibited by okadaic acid, whereas PTP-PEST was completely insensitive to this toxin (Fig. 1).

3.3. Conversion of bacterially expressed PP1γ to a serine/threonine-specific, vanadate-insensitive enzyme

PP1γ was incubated with inhibitor-2 and the PP1I thus generated was incubated with MgATP and GSK3 to trigger the reversible phosphorylation of inhibitor-2 at Thr-72. This procedure abolished the PTPase and pNPP activities of PP1γ (Table 1) without affecting the PhP activity [3]. Moreover, the PhP activity was now insensitive to vanadate (not shown), like native PP1C (Fig. 2).

3.4. The dephosphorylation of casein by bacterially expressed PP1γ

In addition to its high activity towards tyrosine phosphorylated substrates, bacterially expressed PP1γ also dephosphorylated casein (phosphorylated at serine residues by cyclic AMP-dependent protein kinase) at a 30–40-fold higher rate than native PP1C. This high casein phosphatase activity was also lost when PP1γ was incubated with inhibitor-2 and then incubated with GSK3 and MgATP (Table 1).

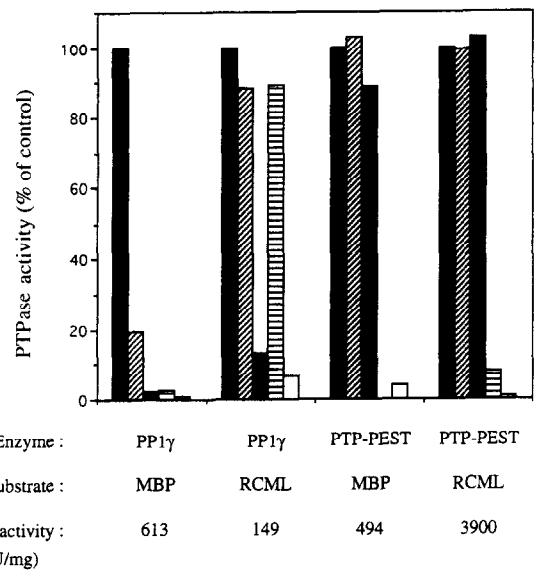

Fig. 1. Comparison of protein tyrosine phosphatase activity and inhibitor sensitivities of PP1γ and PTP-PEST. Enzymes were assayed in the absence (filled bars) or presence of 1 μM okadaic acid (hatched bars), 10 μM okadaic acid (stippled), 1 mM vanadate (horizontal lines) or 10 mM vanadate (open), using MBP or RCML phosphorylated on tyrosine as substrate. Results are expressed as a percentage of the 'control activity' (mU/mg) measured in the absence of inhibitor, represent the means of duplicate assays and are typical of several experiments.

We have also noticed that the properties of 'native' PP1C gradually revert to those of 'expressed' PP1γ during prolonged storage at −20°C or after brief exposure to 50 mM NaF. These preparations can also be converted to 'native' PP1C by incubation with inhibitor-2, GSK3 and MgATP (data not shown).

4. Discussion

The data presented here and in [3] (see also Section 1) have revealed remarkable differences between bacterially 'expressed' PP1γ and 'native' forms of PP1 extracted from mammalian tissues. Although 'expressed' and 'native' PP1 have a similar specific activity towards glycogen phosphorylase and are inhibited by the same concentration of inhibitor-2 [3], 'expressed' PP1 has a much broader specificity, not only dephosphorylating tyrosine residues as high rates (Table 1, Fig. 1) but also some serine and threonine residues such as those in histone H1 (phosphorylated at Ser/Thr-Pro motifs) [3] and casein (Table 1) that are dephosphorylated extremely poorly by 'native' PP1. The 'expressed' PP1α and PP1β isoforms also had high activity towards all substrates tested (not shown). Unlike 'native' PP1, 'expressed' PP1 was inhibited by vanadate (Fig. 2), a relatively specific inhibitor of 'authentic' PTPases. It should be emphasised that the experiments performed in this paper were carried out in the absence of divalent cations, although essentially the same differences between 'expressed' and 'native' PP1 were observed in the presence of 0.5 mM $MnCl_2$ (data not shown). The differences reported here do not result from 'native' PP1 being assayed in the

absence and 'expressed' PP1 in the presence of Mn^{2+}, as suggested in [5].

The data are consistent with a model in which the catalytic subunit of PP1 exists in at least two conformations, termed 'native' and 'expressed'. The 'native' conformation is the form which exists in vivo, while the 'expressed' conformation is adopted when PP1 is expressed in *E. coli*. 'Expressed' PP1 can be converted to 'native' PP1 in vitro by combination with inhibitor-2 followed by the reversible phosphorylation [3] of inhibitor-2 at Thr-72 (Table 1). No other PP1-binding protein so far tested [3] can mimic the effect of inhibitor-2, suggesting that the ability to convert 'expressed' to 'native' PP1 is likely to be one of the physiological roles of inhibitor-2. The correct folding of PP1 is clearly critical to prevent it from dephosphorylating many proteins that are not physiological substrates, including many phosphotyrosyl-containing proteins, and to enhance or permit interaction with important regulators, such as inhibitor-1 and targetting subunits [3]. The pleiotropic effects of loss-of-function mutations in Glc8p (the putative homologue of inhibitor-2 in *S. cerevisiae*) are consistent with such a role [13]. It will be interesting to examine whether PP1 extracted from yeast that lack Glc8 has a high PTPase activity.

A possible explanation for the more restricted specificity of native PP1 compared with expressed PP1 is a conformational change in the vicinity of the catalytic site that allows access of the phosphoseryl and phosphothreonyl residues but sterically prevents binding of the more bulky phosphotyrosine side chains. This would also explain the loss of sensitivity towards vanadate of the native form as the large ionic radius of vanadate may exclude its binding to the native form, but not to the more open catalytic site of expressed PP1.

The catalytic (C) subunit of PP2A (a serine/threonine-specific phosphatase related to PP1) is complexed to an A-subunit in vivo, while the A-subunit interacts with a variety of 'B-type' subunits (reviewed in [14]). The AC complex (but not ABC complexes) is transformed into a very active PTPase upon incubation with MgATP and a protein termed PTPA ('protein tyrosine phosphatase activator') through an unknown mechanism that does not involve phosphorylation of either the A or the C-subunit [15]. This enhancement of the PTPase activity of PP2A has been suggested as a potential mechanism for regulating (as yet unidentified) phosphotyrosine containing proteins, but it should be recalled that the effect of PTPA can be mimicked by NaF [16] which also converts 'native' PP1 to 'expressed' PP1. An alternative hypothesis, suggested by the present results, is that PTPA converts 'native' PP2A to a conformation resembling that of 'expressed' PP1, and that one role for the B-type subunits in vivo is to protect PP2A against the 'deforming' effects of PTPA, whose real physiological role might be unrelated to effects on PP2A. It would clearly be interesting to know whether, like PP1, PP2A is initially synthesised in the 'expressed' conformation and whether a protein analogous to inhibitor-2 is required to induce the 'native' conformation.

In summary, the present study not only supports and extends the hypothesis that inhibitor-2 acts as a 'chaperone' or 'matchmaker' to ensure the correct folding of PP1, but has emphasised two further points that are of technical relevance for the study of this important enzyme. Firstly, when expressed in *E. coli*, PP1 is not a serine/threonine-specific phosphatase, and investigators who are purchasing this enzyme from a commercial source need to be aware of its high PTPase activity and the abnormally high rates at which it dephosphorylates other substrates. Secondly, the three dimensional structures of PP1 that have been reported so far [17,18] describe the 'expressed' and not the 'native' conformation, and to understand why 'native' PP1 is unable to dephosphorylate

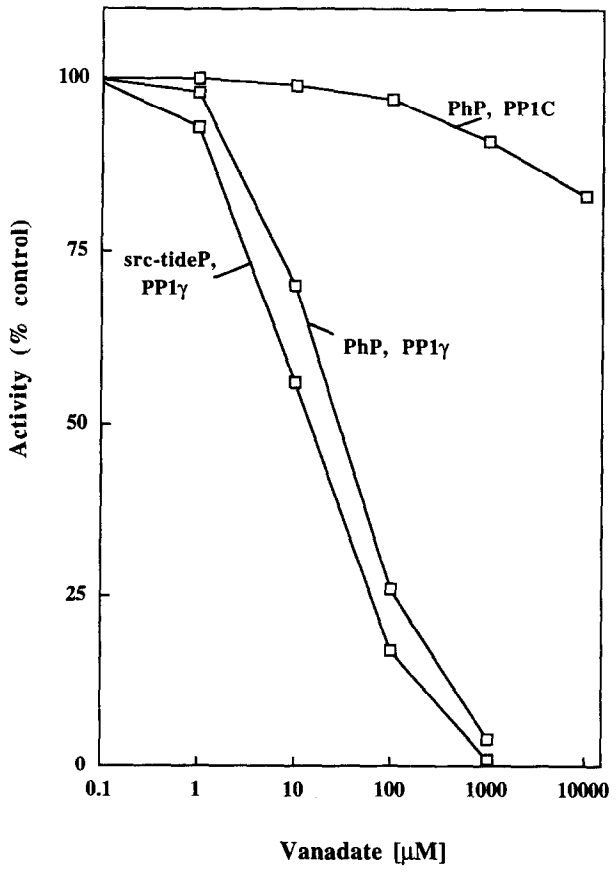

Fig. 2. Inhibition of PP1γ and PP1C by vanadate. PP1C and PP1γ were assayed for phosphorylase phosphatase (PhP) or src-tide phosphatase (src-tideP), and in the presence of the indicated concentrations of vanadate.

Table 1
Specific activities of forms of PP1 towards different substrates

Condition	Form of PP1	Specific activities (mU/mg) towards			
		Ph	casein	pNPP	src-tide
I	PP1C	36000	108	72	126
	PP1G	8000	40	<4	<4
	PP1γ	15000	2690	2150	2710
II	PP1C	40000	28	<1	4
	PP1γ	40000	116	<1	<1

Assays were performed in the absence of Mn^{2+} ions using phosphorylase *a* (Ph), casein, *para*-nitrophenylphosphate (pNPP), or src-tide as substrate. In I, PP1C and PP1G (the glycogen-bound form of PP1) were from rabbit skeletal muscle, and $PP1\gamma_1$ was expressed in *E. coli* and purified according to Alessi et al. [3]. In II, the enzymes were first converted to PP1I by preincubation with inhibitor-2 and the inactive complexes purified by gel filtration. PP1I was then activated by preincubation with MgATP and GSK3 prior to assay (see Section 2). Because the protein concentrations were too low to measure accurately after gel filtration, the tabulated PhP activities for condition II are quoted from [3] and were close to our estimates based on the recovery of activity.

tyrosine residues, it will be important, therefore, to solve the structure of 'native' PP1.

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