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Short communication

Metals in the active site of native protein phosphatase-1

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ABSTRACT

Protein phosphatase-1 (PP1) is a major protein Ser/Thr phosphatase in eukaryotic cells. Its activity depends on two metal ions in the catalytic site, which were identified as manganese in the bacterially expressed phosphatase. However, the identity of the metal ions in native PP1 is unknown. In this study, total reflection X-ray fluorescence (TXRF) was used to detect iron and zinc in PP1 that was purified from rabbit skeletal muscle. Metal exchange experiments confirmed that the distinct substrate specificity of recombinant and native PP1 is determined by the nature of their associated metals. We also found that the iron level associated with native PP1 is decreased by incubation with inhibitor-2, consistent with a function of inhibitor-2 as a PP1 chaperone.

Protein phosphatase-1 (PP1) is one of seven members of the phosphoprotein phosphatase (PPP) superfamily of protein Ser/Thr phosphatases [1,2]. It is expressed in all eukaryotic cells and regulates numerous cellular processes through dephosphorylation of key regulatory proteins. The activity and substrate specificity of PP1 is tightly regulated by ~200 PP1-interacting proteins (PIPs). Most eukaryotic cells express several isoforms of PP1 (α, β and γ in mammals), which are associated with distinct subsets of regulatory PIPs but have an identical catalytic core and cannot be distinguished by their enzymatic properties. The catalytic activity of PP1 is critically dependent on two metal ions in the active site [3,4]. These metals are only separated by 3.3 Å due to the bridging Asp92 (numbering of mammalian PP1α) and two water molecules. One of these water molecules is believed to be replaced by the phosphate group of an incoming substrate. The metal ion at site 1 is additionally bound by a third water molecule, Asp64 and His66, whereas the metal ion at site 2 is further coordinated by Asn124, His173 and His248. The active site metals fulfill a dual role during catalysis: they generate a hydroxide ion for nucleophilic attack on the phosphorus atom and bind the negatively charged oxygens of the phosphate group, thereby positioning the phosphate group for nucleophilic attack and increasing the electrophilicity of the phosphorus atom [5–7].

All structural data on PP1 have been obtained with bacterially expressed enzyme, which, however, has catalytic properties that are strikingly different from those of native PP1 [8,9]. In our hands, freshly prepared native PP1, i.e., a mixture of PP1α, β and γ purified from rabbit skeletal muscle [10], was a much better glycogen–phosphorylase phosphatase than was recombinant His-PP1α, whereas the opposite was true for the dephosphorylation of the phospho-tyrosine mimicking substrate *p*-nitrophenylphosphate (pNPP) (Fig. 1).[1] This is consistent with previous data showing that only recombinant PP1 expresses a tyrosine phosphatase activity [8]. We also noted that the metal chelator EDTA inhibited recombinant His-PP1α but barely affected native PP1, hinting at the presence of distinct metals in their catalytic site (Fig. 2).[2] Consistent with this notion, bacterially expressed PP1α has been reported to contain 2 manganese ions in its catalytic site

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[1] Recombinant His-PP1α was purified as described [11]. The RP1B-PP1 plasmid was a kind gift of Dr. Wolfgang Peti.

[2] The structurally related PP2A is insensitive to EDTA [12], possibly related to the fact that it was expressed in eukaryotic cells, resulting in a more native-like folding.

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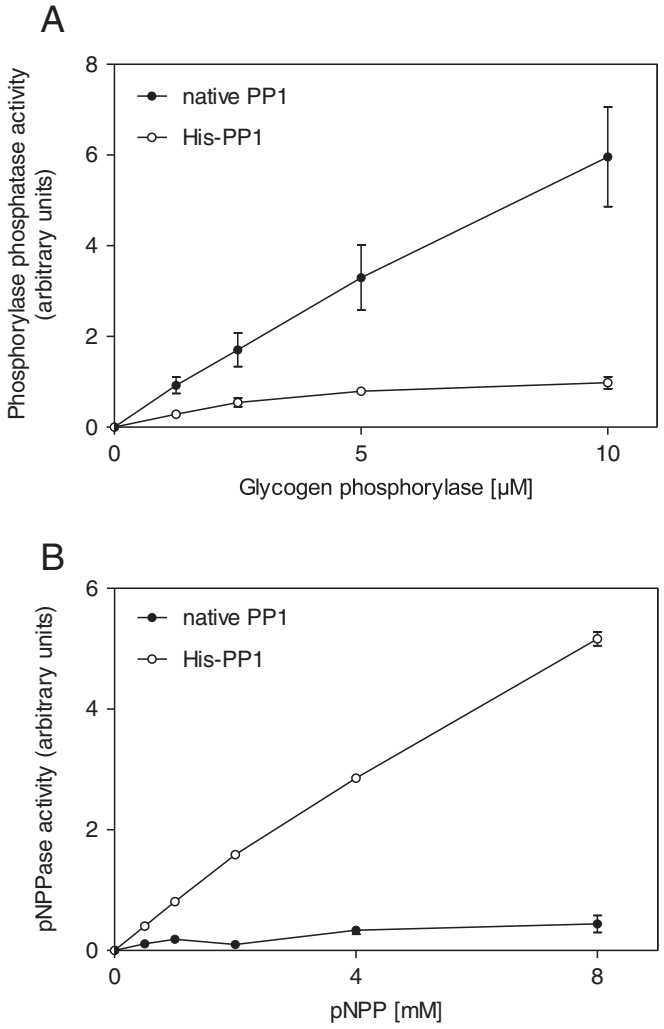

Fig. 1. Recombinant and native PP1 have distinct substrate specificities. Bacterially expressed His-PP1α (recombinant PP1) and PP1 from rabbit skeletal muscle (native PP1) were purified and assayed with the indicated concentrations of glycogen–phosphorylase *a* (A) and pNPP (B) as substrates [11,30]. The results are shown as means ± SEM ($n = 3$).

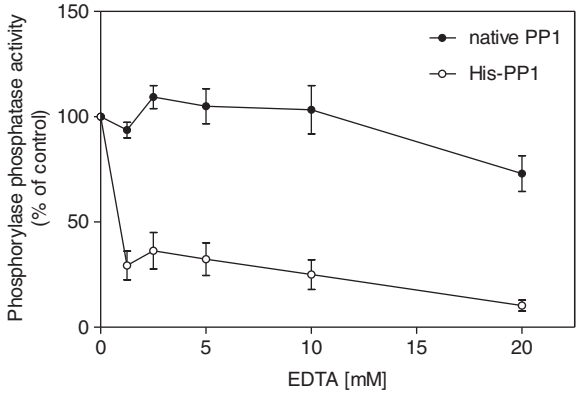

Fig. 2. Native PP1 is not inhibited by metal chelation. The glycogen–phosphorylase phosphatase activity of recombinant and native PP1 was assayed in the presence of the indicated concentrations of EDTA. The results are shown as means ± SEM ($n = 3$).

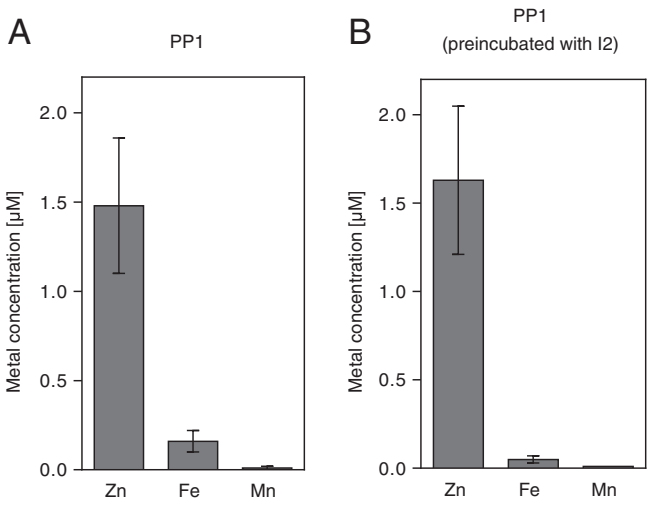

Fig. 3. TXRF analysis of the metals associated with native PP1 (A) and PP1 that was preincubated with inhibitor-2 (B). Results are shown as means ± [the propagation error of 1σ acquisition error + SEM ($n = 6$)].

[13], whereas native forms of the related phosphatases PP2A and PP2B contain iron and zinc [12,14,15]. The more distantly related purple acid phosphatases (PAPs) have Fe^{3+} and M^{2+} in their active site, where M^{2+} is Fe^{2+} (mammalian PAPs), Zn^{2+} (e.g. kidney bean PAP) or Mn^{2+} (e.g. sweet potato PAP) [16–19]. However, the metals in native PP1 have not yet been identified.

Here, we have used TXRF to identify the metals that are associated with active PP1 purified from rabbit skeletal muscle [20].[3] Since the metal concentrations are underestimated when the total atomic concentration exceeds the linear concentration range, the spike recovery rate for nickel in each sample was calculated as $(C_1 - C_0) / C_S \times 100\%$, where C_1 refers to the detected concentration in the spiked sample, C_0 refers to the detected concentration in the unspiked sample and C_S refers to the spike concentration [21]. This recovery rate was used as a correction factor for further quantifications, assuming that all other examined elements in the same sample have the same recovery rate. The concentrations of zinc, iron and manganese were 1.48 ± 0.38 μM, 0.16 ± 0.06 μM and 0.01 ± 0.01 μM, respectively (Fig. 3A), whereas the concentration of purified PP1 was 2 μM as determined by Bradford [22]. Associated potassium, calcium, vanadium, cobalt, nickel and copper were below the detection limit (not shown), but the TXRF measurements did not allow quantitative magnesium assays. Hence, iron and zinc appear to be the metals that are associated with active native PP1 (binding of manganese is almost negligible), but the presence

[3] The purification of the native catalytic subunit of PP1 from rabbit skeletal muscle involves an ethanol precipitation and consecutive ion-exchange chromatography on DEAE-Sepharose, Heparin-Sepharose and Polylysine-Sepharose [10]. No metals were added during the purification procedure. Prior to the TXRF measurements, a buffer exchange was applied to all samples using ultrafiltration and a 1000-fold dilution with 10 mM Tris–HCl at pH 7. All materials were pre-cleaned with 5% HNO_3 and rinsed with ultra-pure water. Activity of the final samples was verified using a phosphorylase phosphatase assay. TXRF measurements were performed in a class 1000 clean room. For each sample, six 5-μl aliquots were pipetted on a 200 mm silicon wafer. For the spike recovery test, three aliquots of each sample were spiked with a 5-μl droplet containing nickel at a final concentration of 10 pg/μl. Sample aliquots were completely dried by exposure to an infrared lamp. TXRF analysis was applied to each residue with an acquisition time of 1000 sec and an X-ray incident angle of 2.3 mrad, which corresponds to 70% of the critical incident angle for the silicon wafer surface. TXRF assays were performed with an Atomika 8300 W, equipped with a sealed tungsten X-ray tube and configured in a W-L_β excitation mode. When required, the X-ray fluorescence was attenuated by using a Zr20 filter. Final quantification was based on the sensitivity of a certified nickel standard and a specific relative sensitivity factor for each of the quantified elements.