FEBS Letters 350 (1994) 328–332

FEBS 14418

Aspartic-129 is an essential residue in the catalytic mechanism of the low M_r phosphotyrosine protein phosphatase

Niccolò Taddei, Paola Chiarugi, Paolo Cirri, Tania Fiaschi, Massimo Stefani*, Guido Camici, Giovanni Raugei, Giampietro Ramponi

Department of Biochemical Sciences, University of Florence, Viale Morgagni 50, 50134 Florence, Italy

Received 30 June 1994

Abstract

The crystal structure of the bovine liver low M_r phosphotyrosine protein phosphatase suggests the involvement of aspartic acid-129 in enzyme catalysis. The Asp-129 to alanine mutant has been prepared by oligonucleotide-directed mutagenesis of a synthetic gene coding for the enzyme. The purified mutant elicited an highly reduced specific activity (about 0.04% of the activity of the wild-type) and a native-like fold, as judged by ^{1}H NMR spectroscopy. The kinetic analysis revealed that the mutant is able to bind the substrate and a competitive inhibitor, such as inorganic phosphate. Moreover, trapping experiments demonstrated it maintains the ability to form the E-P covalent complex. The Asp-129 to alanine mutant shows extremely reduced enzyme phosphorylation (k_2) and dephosphorylation (k_3) kinetic constant values as compared to the wild-type enzyme. The data reported indicate that aspartic acid-129 is likely to be involved both in the first step and in the rate-limiting step of the catalytic mechanism, i.e. the nucleophilic attack of the phosphorylated intermediate.

Key words: Low M_r PTPase; Phosphotyrosine protein phosphatase; Low M_r PTPase mutagenesis; Low M_r PTPase catalytic site

1. Introduction

The low M_r phosphotyrosine protein phosphatase (PTPase, recently reviewed in [1]) is a small enzyme present in many vertebrate tissues as two isoforms (type 1 and 2) whose amino acid sequences differ from each other in the 40–73 region. These isoforms have been sequenced from varying sources [2–4], and the crystal structure of the bovine liver enzyme (belonging to type 2) has been recently determined [25]. The low M_r PTPase catalyses the hydrolysis of arylphosphates [5], including phosphotyrosine, as well as of phosphotyrosine containing peptides and proteins [6,7] of great biological interest. The low M_r PTPase does not elicit any sequence homology to other members of the PTPase family; however, it displays the active-site signature motif CXXXXXR common to all PTPases [8]. The catalytic mechanism of the enzyme has been extensively investigated by chemical modification and oligonucleotide-directed mutagenesis [8–13]. The substrate hydrolysis proceeds in a two-steps reaction mechanism via the formation of a cysteinyl-phosphate intermediate [14] as in the case of all the members of the PTPase family [15] (Scheme 1). The cysteinyl-phosphate intermediate involves the cysteine residue of the active-site motif, corresponding to Cys-12 in the low M_r PTPase [8]. Other residues, in particular Arg-18 and Cys-17, have been demonstrated to participate to the catalytic mechanism [8]. The three-dimensional structure of the bovine liver enzyme has confirmed most of the previously reported data. Furthermore, it has shown the presence of a novel residue, Asp-129, in a position suitable for its involvement in the enzyme catalysis. It has been proposed that Asp-129 could act as proton shuttle: in its indissociated form, this residue would donate the proton for the release of the tyrosine-OH leaving group and, subsequently, it would perform an activation of a water molecule generating the nucleophile responsible for the hydrolysis of the cysteinyl-phosphate intermediate [25]. In the light of this intriguing hypothesis, we have performed an oligonucleotide-directed mutagenesis of the synthetic gene coding for bovine liver low M_r PTPase [16] by replacing aspartate-129 with an alanine residue. The mutated protein has been purified and studied with regard to kinetic behaviour and structural features.

2. Materials and methods

2.1. Materials

Enzymes: Klenow fragment of DNA polymerase I of *E. coli*, T4 DNA ligase, Taq DNA polymerase and restriction enzymes were obtained from Promega; Sequenase from USBC and bovine thrombin from Sigma.

$$E + S \underset{k_1}{\overset{k_{-1}}{\leftrightarrows}} ES \underset{k_2}{\overset{k_{-2}}{\leftrightarrows}} E\text{-}Pi \underset{k_3}{\overset{k_{-3}}{\leftrightarrows}} E + Pi$$

Scheme 1.

*Corresponding author. Fax: (39) (55) 422-2725.

Abbreviations: PTPase, phosphotyrosine protein phosphatase; PNPP, *p*-nitrophenylphosphate; D129A, aspartate-129 to alanine mutant; IPTG, isopropylthiogalattoside; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; EDTA, ethylenediaminotetraacetic acid; UV, ultraviolet; NMR, nuclear magnetic resonance.

SSDI 0014-5793(94)00805-1

Vectors: pMALcPTP is a derivative of pMALc (New England Biolabs), harbouring a chemically synthesized gene coding for bovine liver low M_r PTPase [16]; pGEX-KT [17] was a gift of J.E. Dixon.

Bacterial strains: DH5α *E. coli* (BRL) was used for mutagenesis and TB1 *E. coli* (New England Biolabs) was used for expression of recombinant fusion protein.

Oligonucleotides and U.S.E. mutagenesis kit were obtained from Pharmacia. Glutathione, glutathione-linked agarose beads, and *p*-nitrophenylphosphate (PNPP) were from Sigma. Coomassie brilliant blue was from BioRad. [α-^{32}P]ATP (800 Ci/mmol) was obtained fron NEN. 99.8% deuterium oxide and 1,4-dioxane were purchased from Merck. All other reagents were analytical grade or the best commercially available. Gel radioactivity measurements were made by using a Storage Phosphor screen and a Phosphor Imager (Molecular Dynamics).

2.2. Methods

Construction of pGEX-KTPTP plasmid: Two synthetic oligonucleotides (5′-CGCTGAGCAGGTGACCAAGTC-3′ and the '-40 forward primer' present on the vector) were used in a PCR experiment in the presence of pMALcPTP as a template. The resulting fragment, encompassing the entire coding sequence of the bovine liver low M_r PTPase, was inserted into the unique restriction site BamHI of the pGEX-KT vector, downstream and in frame with the glutathione-*S*-transferase coding sequence.

Oligonucleotide directed mutagenesis: The unique restriction method for site directed mutagenesis [18] with minor modifications, was performed directly on the pGEX-KTPTP construct. The GAC codon for Asp-129 was replaced with the GCC codon for alanine, using a synthetic mutation target oligonucleotide (5′-TGATCATTGAGGCCCCATACTATGG-3′) and the *Apa*I restriction site eliminating oligonucleotide (5′-CGCTGTTAGCAGGTCCATTAAGTTC-3′). The presence of the mutation was confirmed by nucleotide sequence analysis.

Expression and purification of recombinant and D129A mutated proteins: 1 litre of LB broth containing 100 mg/l ampicillin was added to 10 ml of an overnight culture of a bacterial strain harbouring the pGEX-KTPTP or the pGEX-KTPTP-D129A vectors. The culture was incubated at 37°C under continuous shaking until reaching an OD at 600 nm of 0.4. IPTG was added up to 0.3 mM final concentration, and the culture was grown for 2 h. The fusion proteins were then purified by affinity chromatography using a glutathione-linked agarose resin, equilibrated with a 20 mM 3,3-dimethylglutarate buffer, pH 7.0, containing 200 mM NaCl, 10 mM 2-mercaptoethanol and 1 mM EDTA. The resin was subsequently washed with the equilibrating buffer and with a 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The elution was performed using the latter buffer containing 5 mM reduced glutathione.

Thrombin cleavage: Fusion proteins were incubated with 1:500 (w/w) bovine thrombin in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 2.5 mM $CaCl_2$ and 10 mM 2-mercaptoethanol, for 1 h at room temperature. Cleaved PTPase was purified from glutathione-*S*-transferase and from uncleaved fusion protein by a second affinity chromatography on the glutathione-linked resin.

Equilibrium dialysis experiments: Equilibrium dialysis was performed using the apparatus described by Reinard and Jacobsen [19] according to the procedure previously reported [8] in the presence of [^{32}P]inorganic phosphate concentrations ranging from 0.3 mM to 2.0 mM (specific radioactivity 170 dpm/pmol) and 0.24 mM low M_r PTPase. The radioactivity data were subjected to Scatchard analysis.

Phosphoenzyme trapping. The trapping experiments were performed according to Guan and Dixon [15] and to Pot et al. [20]. The methods consists in a rapid mixing of 2 μl (10 μg) of enzyme with 18 μl of 20 mM ^{32}P-labeled benzoylphosphate (specific radioactivity 9450 dpm/pmol) synthesized according to Cirri et al. [8], followed by an addition of 20 μl of Laemmli SDS-PAGE sample buffer containing 4% SDS. The trapped sample was then analysed by SDS-PAGE according to Laemmli [21] without heating. ^{32}P-Labeled proteins were visualized by autoradiography.

Protein concentration determination: Protein concentration was determined by Coomassie brilliant blue assay.

Enzyme activity measurements: The low M_r PTPase activity was measured by following the *p*-nitrophenol release at 37°C in 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA, from PNPP as a substrate (ε_{400} = 18,000 $M^{-1}\cdot cm^{-1}$) in alkaline solution, according to Heinrikson [22]. The D129A mutant activity was measured at a final enzyme concentration of 0.025 mg/ml. All kinetic parameters reported in Table 1 were calculated as described in [8].

NMR analysis: Protein concentration of the sample used for ^{1}H NMR spectroscopy measurements was 0.5 mM. The samples were prepared by 5 concentration/dilution cycles in Centricon-10 microconcentrators (Amicon) in 150 mM d_3-acetate/D_2O buffer at pH 5.5. ^{1}H NMR one dimensional spectra were recorded at 600 MHz and 25°C on a Bruker AMX600 spectrometer, using a sweep width of 7463 Hz and 16k data points. The residual water resonance was suppressed by a selective pulse during the relaxation delay (2.0 s). Prior to Fourier transformation the free-induction decays were zero-filled to 32k data points, and a signal-to-noise ratio enhancing exponential multiplication (line broadening of 4 Hz) was applied. 1,4-dioxan was used as an internal shift reference at 3.74 ppm.

3. Results

A PCR amplification was performed on the pMALcPTP construct [9] in order to subclone in the pGEX-KT vector the entire polynucleotide sequence coding for the wild-type low M_r bovine liver PTPase. Clones with the correct insertion of the gene were screened by hybridisation and completely checked by sequencing with the Sanger method. The vector pGEX-KT was able to easily produce an heterologous polypeptide in *E. coli* as a fusion protein where the carboxyl-terminus of the glutathione-*S*-transferase was linked to the recombinant PTPase amino-terminus. A thrombin recognition site was present at the joining point of the chimeric protein. pGEX-KT contained a glycine kinker [17] just before the thrombin cleavage site: this insertion dramatically improved the fusion protein cleavage rate. In vitro oligonucleotide-directed mutagenesis was performed on the pGEX-KT-PTP construct in order to replace Asp-129 by an alanine residue (see under section 2). The presence of the mutation was confirmed by DNA sequencing according to Sanger.

Both the wild-type recombinant and the D129A mutant enzymes were cleaved in a yield of about 70% after 1 h treatment in the presence of thrombin. The subse-

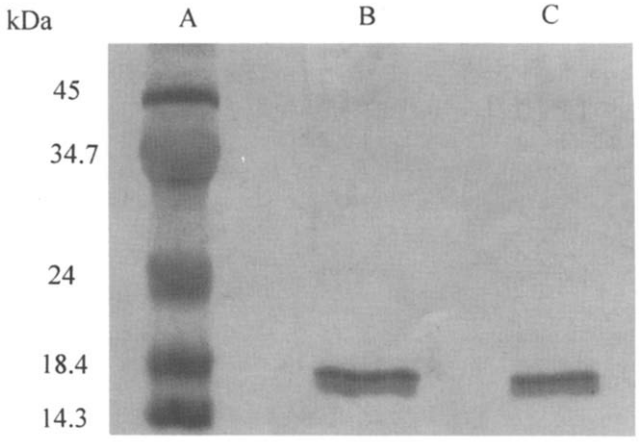

Fig. 1. SDS-PAGE of the purified recombinant low M_r PTPases. Lane A: M_r markers; lane B: wild-type enzyme; lane C: D129A mutant.

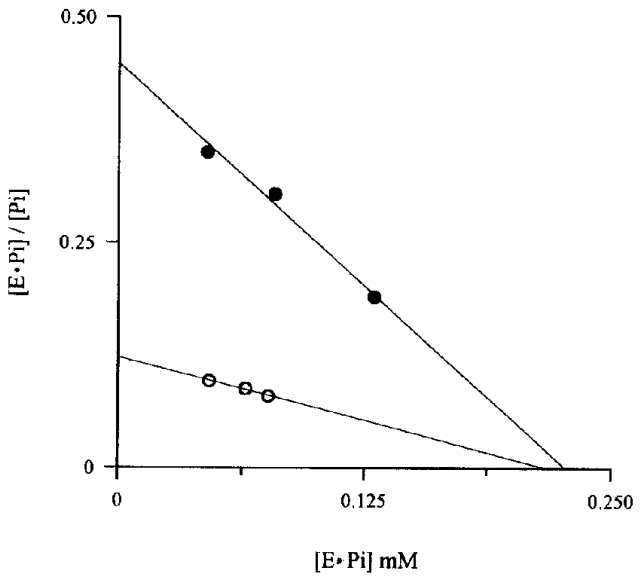

Fig. 2. Scatchard plot determined from the equilibrium dialysis experiment in the presence of (^{32}P) inorganic phosphate. ●, wild-type recombinant low M_r PTPase; ○, D129A mutant. The K_d values were 0.5 mM and 1.7 mM, respectively.

quent purification enabled us to obtain at least 8 mg of pure proteins per liter of culture. Fig. 1 shows the SDS-PAGE of the purified protein. It can be seen that the mutant was obtained with a good purity and a molecular weight corresponding to that of the wild-type recombinant enzyme. The purified mutant was subjected to kinetic analysis. The main kinetic parameters are summarized in Table 1. The D129A mutant exhibits an extremely reduced specific activity (less than 0.04%) as compared to the wild-type enzyme. For this reason the activity assays were performed by adding to the assay mixture an amount of enzyme over one hundred times higher than that usually used for the wild-type enzyme assay. In order to assess whether the reduced specific activity of the D129A mutant was due to the loss of the ability to bind the substrate or to an impaired catalytic mechanism, the affinity of the mutant enzyme for both PNPP and inorganic phosphate was determined. The apparent K_m of the D129A mutant using PNPP as a substrate was 66 μM, about three times lower as compared to that of the wild-type recombinant enzyme (190 μM). The substrate affinity of the mutant enzyme was also determined by a non-kinetic approach. An equilibrium dialysis experiment was therefore carried out using [^{32}P]inorganic phosphate, a well known competitive inhibitor of the enzyme. The radioactivity values obtained for both the D129A mutant and the wild-type recombinant enzyme were processed by Scatchard analysis. Fig. 2 reports the Scatchard plot so obtained, resulting in a K_d for inorganic phosphate of about 1.7 mM. This value is only three times higher than that calculated for the wild-type recombinant enzyme (0.5 mM), demonstrating that the mutant enzyme is still able to efficiently bind the substrate.

Table 1
Main kinetic parameters for recombinant wild-type and D129A mutated low M_r PTPase, as calculated in 100 mM acetate buffer, pH 5.5, in the presence of 1 mM EDTA at 37°C[a]

	K_m (mM)	k_{cat} (s^{-1})	K_s (mM)	k_2 (s^{-1})	k_3 (s^{-1})
Wild-type	0.19	30.0	5.0	789.5	31.2
D129A	0.066	0.011	1.7	0.294	0.012

[a] Using PNPP as a substrate.

The catalytic pathway for the D129A mutant enzyme was investigated particularly by looking for the possible presence of the cysteinylphosphate intermediate demonstrated for the wild-type enzyme. Trapping experiments were performed using [^{32}P]benzoylphosphate, one of the enzyme substrates which was previously found to be hydrolysed by a mechanism involving the cysteinylphosphate intermediate formation [8]. Fig. 3 shows the autoradiography of the SDS-PAGE after the trapping experiment carried out on the D129A mutant and the recombinant wild-type enzymes. The radioactivity of the spot relative to the D129A mutant was over 30-fold as compared to that corresponding to the recombinant wild-type enzyme. A compared kinetic analysis was carried out in order to assess the effectiveness of phosphointermediate accumulation and to calculate the K_s value for PNPP of both the D129A mutant and the recombinant wild-type enzyme. The kinetic analysis was performed according to the approach reported elsewhere [8] by investigating the transphosphorylation activity of the mutant and wild-type enzymes versus glycerol using PNPP as a substrate. Table 1 summarizes the results obtained. Both the enzymes elicit similar K_s values indicating that their true affinities for the substrate are nearly the same. The ratio of the k_{cat} values is about 3,000, in agreement

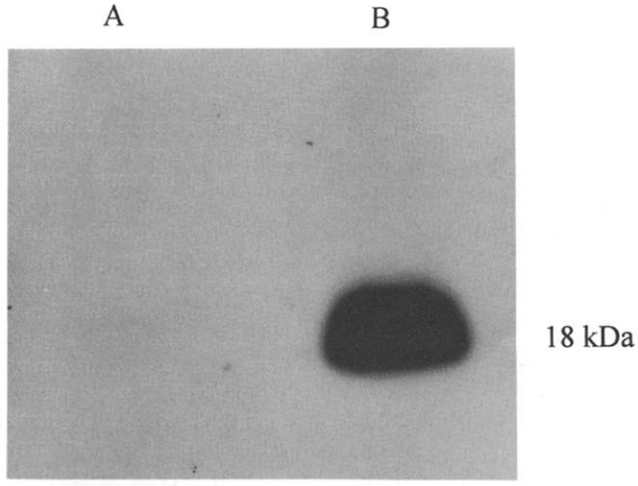

Fig. 3. Autoradiography of the SDS-PAGE relative to the phosphoenzyme trapping experiment. Lane A: wild-type recombinant low M_r PTPase; lane B: D129A mutant.

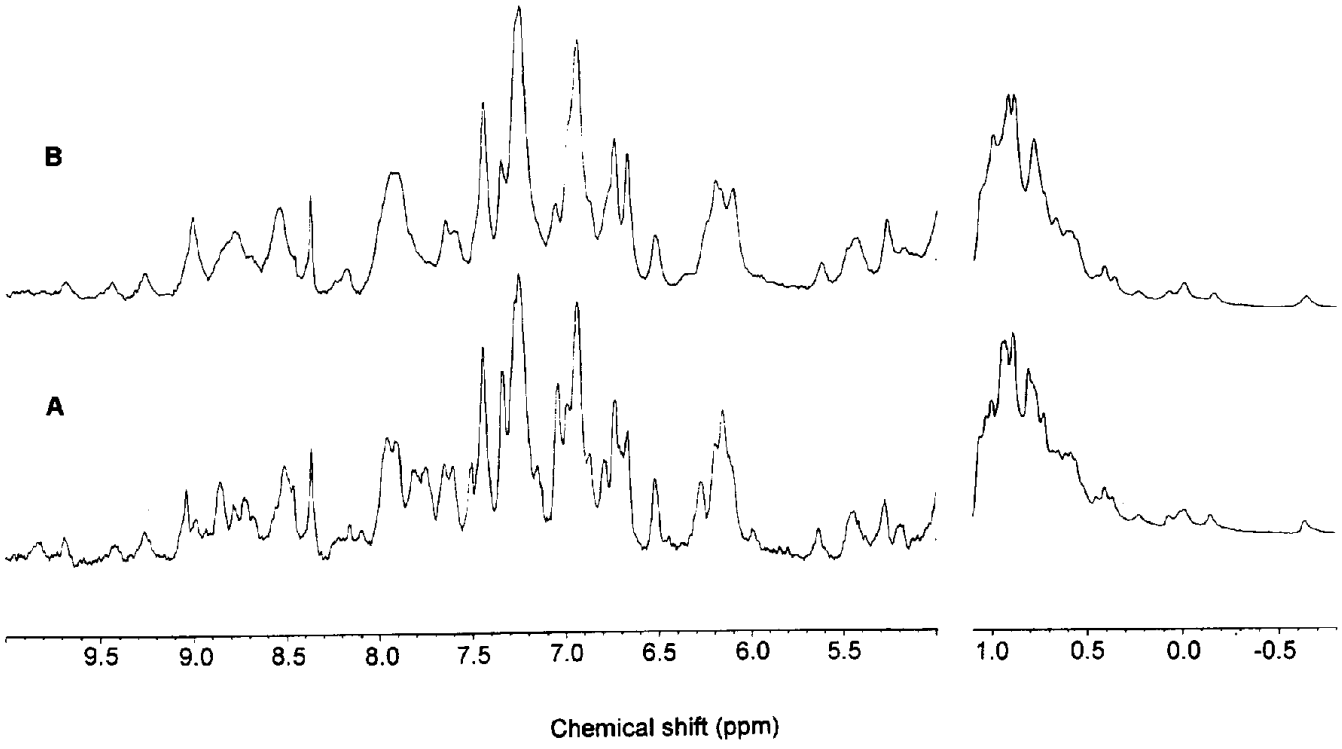

Fig. 4. Aromatic and aliphatic regions of the 600 MHz ^{1}H NMR spectra of the recombinant PTPase. A: wild-type enzyme; B: D129A mutant.

with the low specific activity of the D129A mutant. Furthermore, the D129A mutant elicits rate constants of the phosphointermediate formation (k_2) and hydrolysis (k_3) which are significantly lower with respect to those of the wild-type enzyme. In particular, the k_2 and k_3 ratios of the two enzymes are about 2,500 and 3,000, respectively, indicating that the lack of Asp-129 slows down both the formation and the hydrolysis of the phosphointermediate. Finally, the data reported in Table 1 indicate that the radioactivity spot found in the phosphoenzyme trapping experiments for the D129A mutant is likely to be due to its extremely reduced k_3 value. Under these experimental conditions, the time needed for SDS enzyme denaturation is not large enough to allow a significant nucleophilic attack of water to the phosphointermediate. It results that the fraction of E-P complex blocked by the trapping experiments is higher in the D129A mutant than in the wild-type enzyme, as it has been effectively found. This is further confirmed by the values of the k_2/k_3 ratios relative to the D129A mutant and the wild-type enzymes (24.7 and 25.3, respectively), indicating very similar steady-state levels of phosphointermediate.

To exclude the possibility that the sharp loss of activity of the D129A mutant was due to major conformational changes of the enzyme structure, an ^{1}H NMR spectroscopy study was done. Fig. 4 reports the 600 MHz ^{1}H NMR spectra of the D129A mutant and the recombinant wild-type enzymes. Both spectra are characterised by an extensive chemical shift dispersion in the aromatic as well as in the aliphatic proton regions. This finding is a consequence of persistent contacts in the enzyme three-dimensional structure, indicating the existence of a well-constrained fold typical of a native protein [23]. Furthermore, the ^{1}H NMR spectra are very similar to each other, except for some minor differences in the backbone amide proton region (~7.5–10.0 ppm). These differences, arising from a variable extent of proton exchange with the deuterated solvent during the sample preparation, do not account for a significant modification in the overall structure. The ^{1}H NMR spectrum of the wild-type recombinant protein is very similar to previously reported spectra of a corresponding protein from bovine heart [12,13].

4. Discussion

The possible involvement of Asp129 in the catalytic mechanism of the low M_r PTPase, suggested by the crystal structure of the enzyme, has been studied by oligonucleotide-directed mutagenesis. The kinetic, substrate affinity, and NMR data resulting from the experiments indicate that the D129A mutant elicits an extremely reduced activity, though maintaining a native-like structure and a good affinity for both inorganic phosphate, a competitive inhibitor, and PNPP, an enzyme substrate. On the other hand, the trapping experiments result shows that the D129A mutant apparently accumulates

the cysteinylphosphate intermediate formed in the reaction mechanism. The kinetic analysis indicates that the D129A mutant elicits very reduced k_2 and k_3 values, whereas the k_2/k_3 ratios of the two enzymes are nearly identical to each other, revealing a similar phosphointermediate steady-state level. This data indicates that the phosphoenzyme accumulation in the D129A mutant, as revealed by the trapping experiments, can be ascribed to the low k_3 value and hence to a reduced susceptibility of the phosphoenzyme to hydrolysis during the SDS denaturation. It has previously been demonstrated that the cysteinylphosphate hydrolysis is the rate-limiting step, for most substrates, in the overall catalytic process of the low M_r PTPase [5]. The dramatically reduced specific activity of the D129A mutant is consistent with the participation of Asp-129 to the catalysis rate-limiting step, supporting the involvement of such residue in the cysteinylphosphate nucleophilic attack suggested by the crystal structural data [25]. However, the kinetic analysis supports an involvement of Asp-129 in the phosphointermediate formation, as indicated by the value of the k_2, which is about 2500-fold lower in the D129A mutant as compared to the wild-type enzyme. The reduced k_2 value of the D129A mutant agrees with the hypothesis of an involvement of Asp-129 in its indissociated form as an electrophile in the release of the phenolic leaving group during the formation of E-P covalent complex. Such results confirms the hypothesis of a double role of Asp-129 in enzyme catalysis.

This conclusion is further supported by the ^{1}H NMR data, allowing to exclude the presence of any major conformational change in the D129A mutant, whose spectrum in almost identical to that of the recombinant wild-type enzyme. However, local structural changes at the active site due to the replacement of an acidic residue by an hydrophobic one, cannot be excluded. It follows that the slight differences in substrate affinity between the D129A mutant and the recombinant wild-type enzyme could be the consequence of such minor conformational changes involving the active site region. Nevertheless, this possibility does not contradict our conclusion about the double role of Asp-129 in enzyme catalysis. Further X-ray experiments on the D129A mutant are necessary to address the existence of such active site modifications and to further confirm the involvement of Asp-129 in the phosphointermediate formation. Recently, the presence of an Asp residue in the active site region of the *Yersinia enterocolitica* PTPase, a member of the soluble PTPases subfamily, has been suggested on the basis of a site-directed mutagenesis study [24]. These authors propose that this residue is likely to act as a general acid in the enzyme catalytic mechanism. Our data are in agreement with these findings further supporting the role of an Asp residue in the mechanism of the reaction catalysed by soluble PTPases.

Acknowledgements: This work was supported by grants from the Italian MURST (40% and 60%), from Italian CNR (Target project on Biotechnology and Bioinstrumentation) and from the Italian Association for Cancer Research (AIRC). The authors thank Dr. Egle Solito for densitometric measurements. The ^{1}H NMR experiments were carried out at the Laboratorio di Risonanze Magnetiche of the University of Florence. The authors are grateful to M_r; Massimo Lucci for his technical support. pGEX-KT was a generous gift from Prof. J.E. Dixon.

References

[1] Ramponi, G. (1994) Adv. Prot. Phosphatases (in press).
[2] Camici, G., Manao, G., Cappugi, G., Modesti, A., Stefani, M. and Ramponi, G. (1989) J. Biol. Chem. 264, 2560 2567.
[3] Dissing, J., Johnsen, A.H. and Sensabaugh, G.F. (1991) J. Biol. Chem. 266, 20619–20625.
[4] Manao, G., Pazzagli, L., Cirri, P., Caselli, A., Camici, G., Cappugi, G., Saeed, A. and Ramponi, G. (1992) J. Prot. Chem. 11, 333–345.
[5] Zhang, Z.-Y. and Van Etten, R.L. (1991) Biochemistry 30, 8954–8959.
[6] Ramponi, G., Manao, G., Camici, G., Cappugi, G., Ruggiero, M. and Bottaro, D.P. (1989) FEBS Lett. 250, 469–473.
[7] Stefani, M., Caselli, A., Bucciantini, M., Pazzagli, L., Dolfi, F., Camici, G., Manao, G. and Ramponi, G. (1993) FEBS Lett. 326, 131–134.
[8] Cirri, P., Chiarugi, P., Camici, G., Manao, G., Raugei, G., Cappugi, G. and Ramponi, G. (1993) Eur. J. Biochem. 214, 647–657.
[9] Chiarugi, P., Marzocchini, R., Raugei, G., Pazzagli, C., Berti, A., Camici, G., Manao, G., Cappugi, G. and Ramponi, G. (1992) FEBS Lett. 310, 9 12.
[10] Cirri, P., Chiarugi, P., Camici, G., Manao, G., Pazzagli, L., Caselli, A., Barghini, I., Cappugi, G., Raugei, G. and Ramponi, G. (1992) Biochim. Biophys. Acta 1161, 216–222.
[11] Chiarugi, P., Cirri, P., Camici, G., Manao, G., Fiaschi, T., Raugei, G., Cappugi, G. and Ramponi, G. (1994) Biochem. J. 298, 427–433.
[12] Davis, J.P., Zhou, M.-M. and Van Etten, R.L. (1994) Biochemistry 33, 1278–1286.
[13] Davis, J.P., Zhou, M.-M. and Van Etten, R.L. (1994) J. Biol. Chem. 269, 8734–8740.
[14] Wo, Y.Y.P., Zhou, M.-M., Stevis, P., Davis, J.P., Zhang, Z.-Y. and Van Etten, R.L. (1992) Biochemistry 31, 1712–1721.
[15] Guan, K.L. and Dixon, J.E. (1991) J. Biol. Chem. 266, 17026–17030.
[16] Raugei, G., Marzocchini, R., Modesti, A., Ratti, G., Cappugi, G., Camici, G., Manao, G. and Ramponi, G. (1991) Biochem. Int. 2, 317–326.
[17] Hakes, D.J. and Dixon, J.E. (1992) Anal. Biochem. 202, 293–298.
[18] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[19] Reinard, T. and Jacobsen, H.J. (1989) Anal. Biochem. 176, 157–160.
[20] Pot, D.A., Woodford, T.A., Remboutsika, E., Haun, R.S. and Dixon, J.E. (1991) J. Biol. Chem. 266, 19688–19696.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Heinrikson, R.L. (1969) J. Biol. Chem. 244, 299–307.
[23] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, Wiley, New York.
[24] Zhang, Z.Y., Wang, Y. and Dixon, J.E. (1994) Proc. Natl. Acad. Sci. USA 91, 1624–1627.
[25] Su, X.-D., Taddei, N., Stefani, M., Ramponi, U. and Nordlund, P. (1994) Nature, in press.