Journal of Enzyme Inhibition and Medicinal Chemistry, 2002 Vol. 17 (5), pp. 345–350

Inhibition of Bovine Kidney Low Molecular Mass Phosphotyrosine Protein Phosphatase by Uric Acid

JOSÉ MAURO GRANJEIRO[a,*], CARMEN VERISSIMA FERREIRA[b], PAULO AFONSO GRANJEIRO[b], CINTHIA CELESTINO DA SILVA[b], EULÁZIO MIKIO TAGA[c], PEDRO LUIZ ONOFRE VOLPE[a] and HIROSHI AOYAMA[b,†]

[a]*Departamento de Físico-Química, IQ;* [b]*Departamento de Bioquímica, Instituto de Biologia, UNICAMP, Cidade Universitária, CEP 13083-970 CAMPINAS, São Paulo, Brasil;* [c]*Departamento de Ciências Biológicas/Bioquímica, FOB-USP, Bauru (SP), CEP 17043-101 Brasil*

(Received 5 June 2002)

Uric acid inhibited 50% of the activity of bovine kidney low molecular mass phosphotyrosine protein phosphatase at concentrations of 1.0, 0.4, 1.3, and 0.2 mM, respectively for *p*-nitrophenyl phosphate (*p*-NPP), flavine mononucleotide, β-naphthyl phosphate and tyrosine phosphate (Tyr-P) as substrates. The mixed type inhibition of *p*-NPP hydrolysis was fully reversible, with K_{ic} and K_{iu} values of 0.4 and 1.1 mM, respectively; the inhibition by uric acid shifted the pH optimum from 5.0 to 6.5. When Tyr-P was the substrate, competitive inhibition was observed with a K_i value of 0.05 mM. Inhibition studies by uric acid in the presence of thiol compounds, and preincubation studies in the presence of inorganic phosphate suggest that the interaction of uric acid with the enzyme occurred at the active site, but did not involve SH residues, and that the mechanism of inhibition depended on the structure of the substrates.

Keywords: Low molecular mass phosphotyrosine protein phosphatase; Uric acid; Enzyme kinetics; Inhibition

INTRODUCTION

Low molecular mass phosphotyrosine protein phosphatases (E.C. 3.1.3.48) are basic and cytosolic proteins which, generally, consist of 157 amino acid residues, having very similar kinetic properties and a highly conserved primary structure, especially in the active site.[1–4] LM_r phosphotyrosyl protein phosphatases, originally known as LM_r acid phosphatases, are members of a PTP superfamily that act in conjunction with protein tyrosine kinases to regulate a variety of cellular functions by controlling the tyrosine phosphorylation state of different proteins.[5–8] The family of PTP also includes the specific tyrosine phosphatases, and dual specificity protein phosphatases VH1-like and cdc25. Despite significant diversity in their primary sequences, these proteins share a signature motif, (H/V)CxAGxxR(S/T)G, which includes catalytically essential cysteine and arginine residues.[8]

LM_r PTPs have an absolute requirement for free sulfhydryl groups for full enzymatic activity.[9,10] Zhang *et al.*[11] reported the crystal structure of bovine heart LM_r PTP in which they confirmed the position and role of Cys-12, Arg-18 and Asp-129 in the active site.

Two distinct isoforms of LM_r PTP (IF1 and IF2) have been identified in mammalian cells and differ in their sequence of amino acid residues in the region between 40–73, in their sensitivity to purine compounds,[12–15] to pyridoxal-5-phosphate,[16] and in their substrate specificity.[17] Mutagenic studies of IF2 have identified the molecular site that determines the kinetic characteristics of each isoform. Residues 49 and 50 are involved in the strong activation of IF2 LM_r PTP with residue 50 being the main determinant of substrate specificity.[15] Human PTP isoforms IF1 and IF2, expressed in *E. coli*, and purified, showed kinetic parameters very similar to those previously

*Present Address: Departamento de Ciências Biológicas/Bioquímica, FOB-USP, Bauru (SP), CEP 17043-101 Brasil.
†Corresponding author. Tel.: +55-19-3788-6141. Fax: +55-19-3788-6129. E-mail: aoyama@unicamp.br

Abbreviations: HM_r, relative high molecular mass; IM_r, relative intermediate molecular mass; IF1, isoform 1; IF2, isoform 2; LM_r, relative low molecular mass; Pi, inorganic phosphate; *p*-NPP, *p*-nitrophenyl phosphate; PTP, phosphotyrosine protein phosphatase; Tyr-P, tyrosine-phosphate

ISSN 1475-6366 print/ISSN 1475-6374 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/1475636021000013939

determined for the enzymes purified by classical procedures.[18]

The exact biological function of these small cytoplasmic PTPs is still unknown, although they readily hydrolyze phosphotyrosine residues present in autophosphorylated epidermal growth factor[19] and platelet derived growth factor[20] receptors. Flavine mononucleotide[21,22] and tyrosine-phosphorylated caveolin[23] are also efficiently dephosphorylated by LM_r PTP.

Ioshida and Tamiya[24] reported the inhibition of HM_r acid phosphatase activity by adenosine, guanosine and cytidine. On the other hand, LM_r PTP could be activated by purine compounds, such as guanosine, adenine, adenosine and hypoxanthine.[2,25,26] Distinct effects by purine compounds have been described, depending on the PTP IF1 and IF2 isoforms used. In avian pectoral muscle two distinct LM_r acid phosphatase isoforms differed in relation to the isoelectric point, specificity to substrates, and susceptibility to guanosine, where only one isoform was activated.[13]

At least two mechanisms for modulation of LM_r acid phosphatases by purine analogs have been proposed. One involving the binding of the purine to free enzyme,[12] and the other involving binding either to the free enzyme or to the enzyme-substrate complex.[14]

The metabolism of purine nucleotides, nucleosides, and bases involve a common pathway in which hypoxanthine, xanthine and uric acid are the most important products.

The effects of such compounds on the acid phosphatase activities are only scarcely related. Differential effects by hypoxanthine and adenine were observed depending on the human LM_r acid phosphatase isoforms used.[14] In contrast to the effect of hypoxanthine and xanthine on red cell acid phosphatases, uric acid inhibited these enzymes.[27]

In order to contribute to the mechanism of modulation by purine compounds, we studied in detail the effect of uric acid on bovine kidney LM_r PTP, including the influence of substrates and pH as well as inhibition constants determination, thermal stability and reversibility of inhibition.

MATERIAL AND METHODS

Enzyme and Other Reagents

Bovine kidneys were obtained from a local slaughterhouse. All the chemicals were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). The chromatographic medium was purchased from Pharmacia (Sweden). LM_r PTP was purified from bovine kidney as previously described.[28]

Methods

Phosphotyrosine Protein Phosphatase Assay

PTP activity was determined, as previously described,[29] in a final volume of 1 mL, containing 100 mM acetate buffer (pH 5.0) and 5 mM substrate, by measuring the *p*-nitrophenol (with *p*-NPP as substrate) or inorganic phosphate (with FMN as substrate) released.[22] The molar extinction coefficients used to determine the enzyme activities for the other substrates were: tyrosine, $\xi_{293} = 2,411\,M^{-1}\,cm^{-1}$; and β-naphthol, $\xi_{346} = 2,780\,M^{-1}\,cm^{-1}$ in alkaline solution.[30] One unit of activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per minute. All determinations were done at least in quadruplicate.

Effect of Uric Acid on the pH Profile

PTP activity was determined as described above using 5 mM *p*-NPP or Tyr-P as the substrate in 100 mM glycine (pH 2.5, 3.0 and 9.0), acetate (pH 3.5–5.5), Bis-Tris (pH 6.0 and 6.5), Hepes (pH 7.0 and 8.0) buffers. Enzyme activity was determined in the absence or presence of 1 mM uric acid.

Determination of Inhibition Constants

Lineweaver-Burk plots[31] of the inhibition of LM_r PTP were obtained for the hydrolysis of *p*-NPP or Tyr-P. The Dixon plot was used to determine the competitive inhibition constant. The competitive and uncompetitive inhibition constants (K_{ic} and K_{iu}, respectively) were calculated using the Cornish-Bowden plots.[32]

Effect of Uric Acid on the Stability of LM_r PTP

LM_r PTP (1.5 μg/mL) was preincubated without or with uric acid (0.1, 0.25, 0.5 or 1.0 mM) in 100 mM acetate buffer (pH 5.0), at 37°C. At different times, a 50 μL aliquot was withdrawn from this mixture and used to start the hydrolysis of *p*-NPP as described above.

Protective Effects of Inorganic Phosphate (Pi)

LM_r PTP (2.3 μg/mL) was preincubated at 45°C in 100 mM acetate buffer (pH 5.0), containing 5 mM uric acid, 10 mM Pi, 5 mM uric acid plus 10 mM Pi. At different times, a 500 μL aliquot was withdrawn from this mixture and stored on ice. The residual enzyme activity was determined as described above using *p*-NPP as substrate.

RESULTS AND DISCUSSION

Previous studies of the effects of purine analogs on the activity of LM_r PTP suggest that modulation by these compounds probably play an important role in the regulation of the two isoforms of these enzymes. Thus, guanosine (1 mM) increased the relative enzyme activity by 3-fold whereas the same concentration of uric acid and guanine inhibited this activity by 50 and 20%, respectively (Fig. 1). Activation by guanosine in the *p*-nitrophenol formation have been reported by Tanizaki *et al.*[12] and Cirri *et al.*[15]

We studied the inhibitory effect of uric acid in more detail. Besides *p*-NPP, the inhibition by this purine compound was dose-dependent with respect to FMN, β-naphthyl-P and Tyr-P as substrates (Fig. 2); the uric acid concentrations that produced 50% inhibition of activity (I_{50}) were 1.0, 0.4, 1.3 and 0.2 mM, respectively. The I_{50} values were lowest for physiological substrates, FMN and Tyr-P, when compared with the synthetic substrates *p*-NPP and β-naphthyl-P. The hydrolysis of Tyr-P was almost completely (90%) inhibited by 1 mM uric acid.

The variable region between residues 40–73 of PTP seems to be involved in the modulation of purine compounds and in determining substrate specificity.[15]

The inhibition of *p*-NPP hydrolysis by uric acid was pH-dependent as shown by the shift in optimum pH from 5.0 to 6.5 (Fig. 3). A comparison of the two curves in the absence or presence of uric acid showed that the inhibitory effect was greater below pH 7.0 with a maximal effect (greatest difference between the curves) at pH 5.0, near the pKa (5.4) of the hydroxyl group of uric acid. In contrast, uric acid did not affect the pH profile for Tyr-P hydrolysis. Zhang *et al.*[11] proposed that His-72 (pKa = 7.2) located near the active site, was part of a

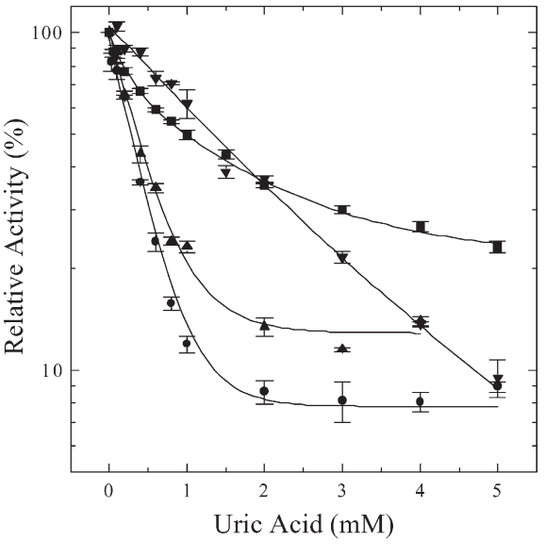


FIGURE 2 Effect of uric acid on bovine kidney LMr PTP activity using different substrates. The enzyme activity was determined as described in "Methods", using *p*-NPP (■), FMN (▲), Tyr-P (○), or β-naphthyl-P (▼). 100% corresponds to the activity in the absence of uric acid. Bars represent the s.e. of quadruplicate determinations.

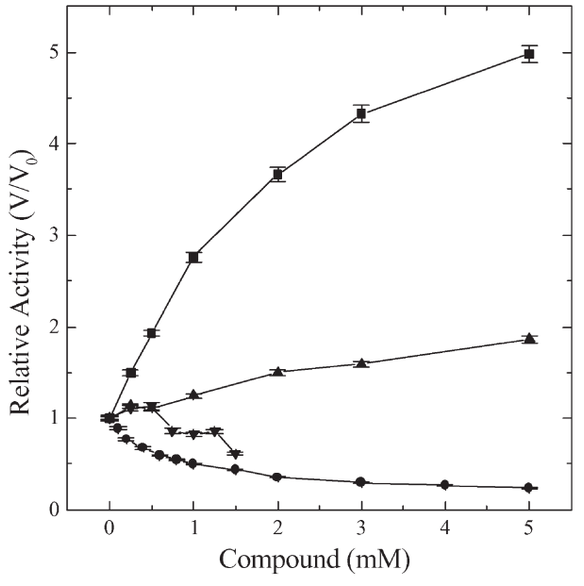


FIGURE 1 Effect of purine compounds on the hydrolysis of *p*-NPP by bovine kidney LMr PTP. The assay was performed as described in "Methods", in the absence (V_0) and in the presence (V) of 1 mM of guanosine (■), adenosine (▲), uric acid (○), or guanine (▼). Bars represent the s.e. of quadruplicate determinations.

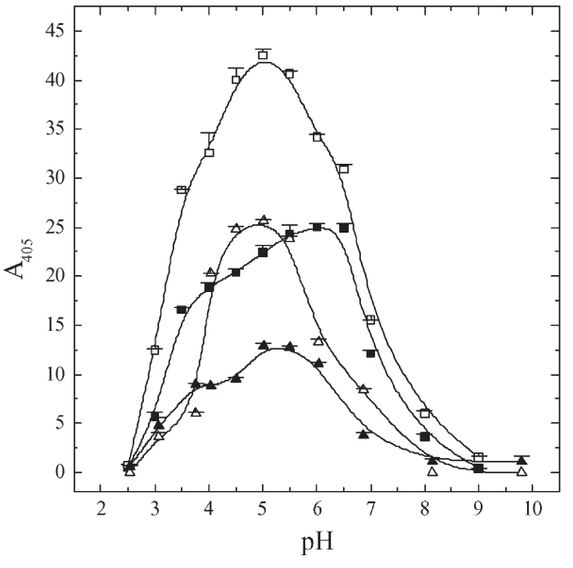


FIGURE 3 pH dependence of LMr PTP activity. The enzyme activity was determined in the absence (open symbols) and in the presence of 1 mM or 0.1 mM uric acid (closed symbols) using, respectively, *p*-NPP (■) or Tyr-P (▲) as substrate. Bars represent the s.e. of quadruplicate determinations.

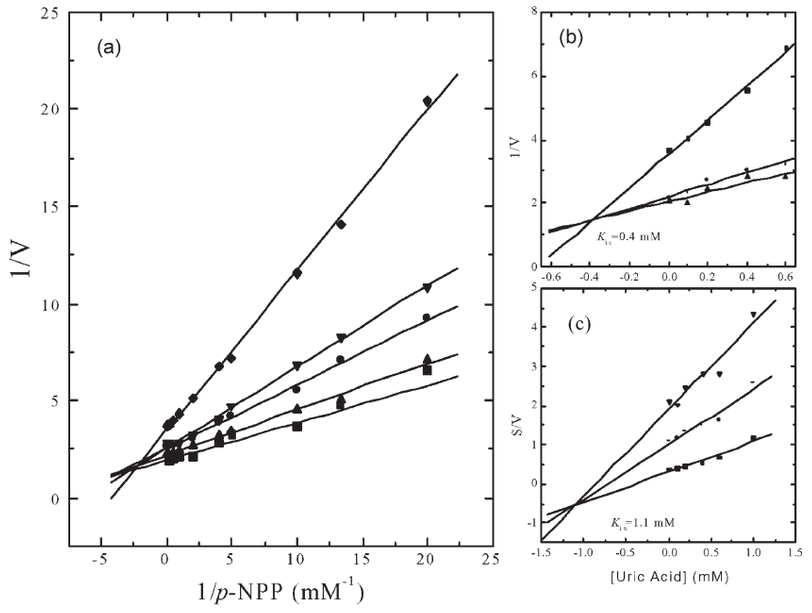
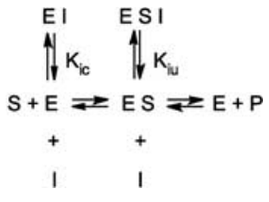


FIGURE 4 Inhibition of *p*-NPP hydrolysis by uric acid. (a) *p*-NPP hydrolysis was determined in the absence (■) and in the presence of 0.2 (▲), 0.4 (○), 0.6 (▼), or 1.0 mM (◆) of uric acid. (b) and (c) Replots of 1/V and S/V, respectively, as a function of uric acid concentration, in the presence of 0.1 (■), 0.5 (○), or 1.0 mM (▼) of *p*-NPP.

tight network of hydrogen bonds allowing the active site to adopt the most favorable geometry. Thus, at pH 5.0 (reaction medium), there could be electrostatic interactions between uric acid (partially ionized) and histidine/arginine residues. However above pH 7.0, the His residue lost its positive charge, decreasing the effect of uric acid.

A Lineweaver–Burk plot indicated a mixed type inhibition for the hydrolysis of *p*-NPP (Fig. 4a), with both specific and catalytic effects being present, i.e. both V_{app}/K_{mapp} and V_{app} varied with the uric acid concentration. According to Cornish-Bowden,[32] the simplest mechanism for this behavior is one in which the inhibitor (I) can bind to the free enzyme to produce an enzyme-inhibitor (EI) complex (dissociation constant = K_{ic}) and to the enzyme-substrate complex to form an enzyme-substrate-inhibitor (ESI) complex (dissociation constant = K_{iu}), as in the following scheme:



The values of K_{ic} (0.4 mM) and K_{iu} (1.1 mM) can be obtained from plots of 1/V *versus* inhibitor concentration [I], and S/V *versus* [I], respectively (Fig. 4b, c).[32,33] In contrast, the inhibition of Tyr-P hydrolysis by uric acid was of the competitive type (Fig. 5) with a K_i value of 0.05 mM, calculated from the plot of slope *versus* [I] (inset). The existence of two models of inhibition could occur since the two PTP isoforms have distinct susceptibilities to purine compounds. In IF2, Trp-49 and Asn-50 are involved in the purine-enzyme interaction, while in IF1, Tyr-49 and Glu-50 are involved.[15] Since these amino acid residues modulate substrate specificity, and purine compounds can bind to the ES complex, the conformational structure of the enzyme will be dependent on both the purine and substrate bound to the enzyme. This observation strongly supports our findings that the type of inhibition varied with the substrate and purine used.

Our results also allow us to correlate the inhibition mechanism by uric acid with the two activation mechanisms proposed for purine modulation of phosphatase activities. In this context, Tanizaki *et al.*[12] suggested that purine compounds interacted with the enzyme-substrate (ES) complex via uncompetitive activation and more recently, Cirri *et al.*[15] have shown that guanosine and cGMP interacted with the free enzyme, as well as with the enzyme-substrate complex.

The inhibition of LM_r PTP by uric acid was reversible. Dilution of the uric acid-enzyme mixture to a non-inhibitory concentration of uric acid led to the recovery of enzyme activity after a 12 min incubation on ice; about 80% recovery was observed at zero time (results not shown).

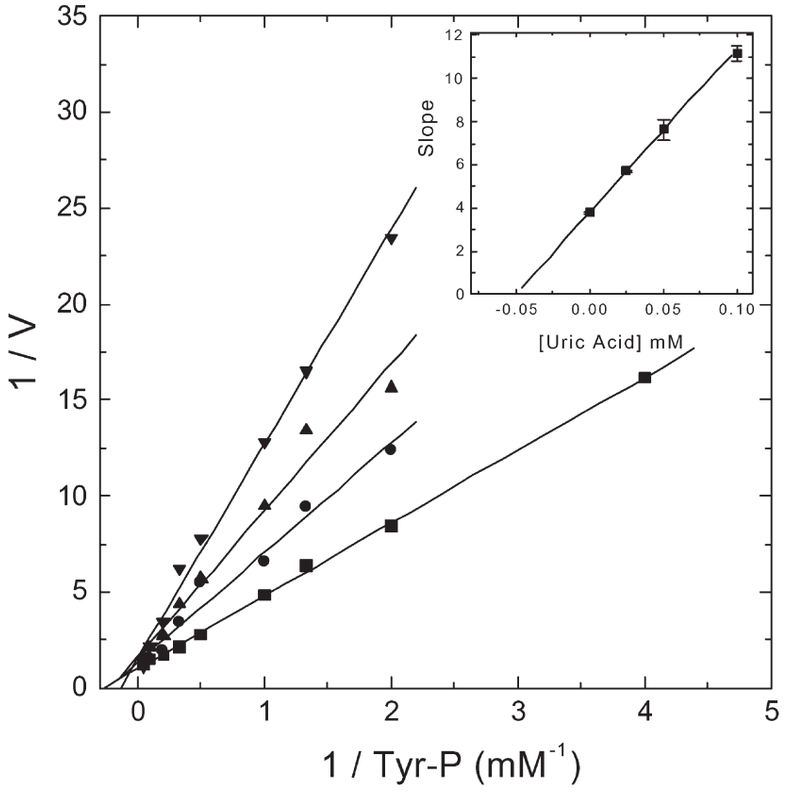


FIGURE 5 Double reciprocal plot of the effect of uric acid on the rate of Tyr-P hydrolysis. Tyr-P hydrolysis was determined in the absence (■) and in the presence of 0.025 (○), 0.05 (▲), 0.1 mM (▼) of uric acid as indicated. Inset: Dixon plot.

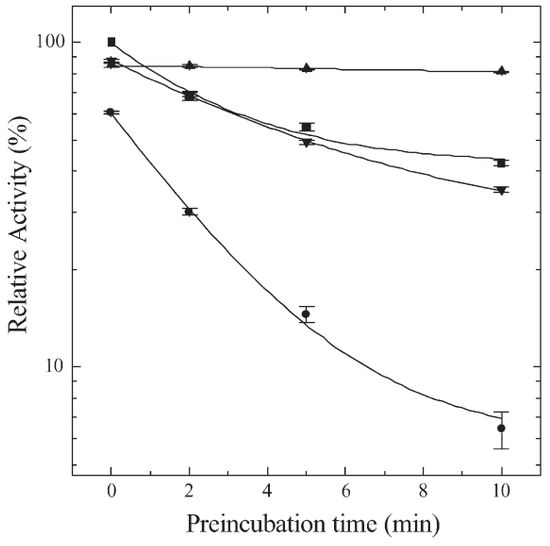


FIGURE 6 Effect of inorganic phosphate on the thermal inactivation of LMr PTP. The enzyme was preincubated at 45°C in the absence (■) or presence of 5 mM of uric acid (○), 10 mM of Pi (▲), or 5 mM of uric acid plus 10 mM of Pi (▼). At different times, the residual activity was measured taking 50 μL of preincubated enzyme and using *p*-NPP as substrate. Bars represent the s.e. of quadruplicate determinations.

The inclusion of 1 mM dithiothreitol or reduced glutathione in the reaction medium did not prevent the inhibitory effect of uric acid (results not shown). It is worthwhile mentioning that the uric acid itself can act as an antioxidant.[34,35]

Heating LM_r PTP to 45°C reduced enzyme activity by 60% after 10 minutes, and by 95% in the presence of 1 mM uric acid (Fig. 6). Inorganic phosphate, a classical competitive inhibitor of LM_r PTP, protected the enzyme against thermal inactivation and against uric acid inhibition, suggesting that this interaction of the purine analog with the enzyme occurred at the active site.

We concluded that uric acid inhibition was reversible, and depended on the substrates, with mixed and competitive types inhibitions when *p*-NPP and Tyr-P were used as substrates, respectively. Our results suggested also that the interaction of the enzyme with uric acid occurred at the active site, but did not involve SH residues. However, the mechanism of interaction of purine compounds with the active site of acid phosphatases has been well established only recently by Wang *et al.*,[36] through a crystal structure of yeast LM_r PTP complexed with

adenine. In this crystal structure, adenine molecule was found to be bound in the active site cavity, between the side chains of two large hydrophobic residues. These authors also observed that an ordered water molecule found in the proximity to the bound phosphate ion, present in the active site, was the nucleophile that participated in the dephosphorylation of the phosphoenzyme intermediate. This last observation could explain the differential effect of the purine compound depending on the substrate, considering that, in each case, distinct water organization could be occurring in the active site.

Finally, considering that the hydrolyses of the physiological substrates Tyr-P and FMN were almost completely inhibited by uric acid, we can suggest that this inhibition could represent a regulatory pathway for this family of phosphotyrosine protein phosphatases.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Fundo de Apoio ao Ensino e à Pesquisa/UNICAMP and CAPES. We are grateful to Dr. Stephen Hyslop (Departamento de Farmacologia, FCM, UNICAMP) for helpful discussions and for critically reading the manuscript.

References

[1] Camici, G., Manao, G., Cappugi, G., Modesti, A., Stefani, M. and Ramponi, G. (1989) *J. Biol. Chem.* **264**, 560–2567.
[2] Zhang, Z.-Y. and Van Etten, R.L. (1990) *Arch. Biochem. Biophys.* **282**, 39–49.
[3] Ramponi, G. and Stefani, M. (1997) *J. Biochem. Cell Biol.* **29**, 279–292.
[4] Tabernero, L., Evans, B.N., Tishmack, P.A., Van Etten, R.L. and Stauffacher, C.V. (1999) *Biochemistry* **38**, 11651–11658.
[5] Thonks, N.K. and Neel, B.G. (1996) *Cell* **87**, 365–368.
[6] Jia, Z. (1997) *Biochem. Cell. Biol.* **75**, 17–26.
[7] Burke, T.R. and Zhang, Z.-Y. (1998) *Biopolymers* **47**, 225–241.
[8] Zhang, Z-Y. (2001) *Curr. Opin. Chem. Biol.* **5**, 416–423.
[9] Davis, J.P., Zhou, M.-M. and Van Etten, R.L. (1994) *J. Biol. Chem.* **269**, 8734–8740.
[10] 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.
[11] Zhang, M., Van Etten, R.L. and Stauffacher, C.V. (1994) *Biochemistry* **33**, 11097–11105.
[12] Tanizaki, M.M., Bittencourt, H.M.S. and Chaimovich, H. (1977) *Biochim. Biophys. Acta* **485**, 116–123.
[13] Baxter, J.H. and Suelter, C.H. (1985) *Arch. Biochem. Biophys.* **239**, 29–37.
[14] Dissing, J., Rangaard, B. and Christensen, U. (1993) *Biochim. Biophys. Acta* **1162**, 275–282.
[15] Cirri, P., Caselli, A., Manao, G., Camici, G., Polidori, R., Cappugi, G. and Ramponi, G. (1995) *Biochim. Biophys. Acta* **1243**, 129–135.
[16] Cirri, P., Chiarugi, P., Camici, G., Manao, G., Pazzagli, L., Caselli, A., Barghini, I., Capuggi, G., Raugei, G. and Ramponi, G. (1993) *Biochim. Biophys. Acta* **1161**, 216–222.
[17] Stefani, M., Caselli, A., Bucciantini, M., Pazzagli, L., Dolfi, F., Camici, G., Manao, G. and Ramponi, G. (1993) *FEBS Lett.* **326**, 131–134.
[18] Marzocchini, R., Bucciantini, M., Stefani, M., Tadei, N., Thunnissen, M.G.M.M., Nordlund, P. and Ramponi, G. (1998) *FEBS Lett.* **426**, 52–56.
[19] Ramponi, G., Manao, G., Camici, G., Cappugi, G., Ruggiero, M. and Bottaro, B.P. (1989) *FEBS Lett.* **250**, 469–473.
[20] Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, F., Berti, A. and Ramponi, G. (1995) *FEBS Lett.* **372**, 49–53.
[21] Fuchs, K.R., Shekels, L.L. and Bernlohr, D.A. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1598–1605.
[22] Granjeiro, J.M., Ferreira, C.V., Jucá, M.B., Taga, E.M. and Aoyama, H. (1997) *Biochem. Mol. Biol. Int.* **41**, 1201–1208.
[23] Caselli, A., Taddei, M.L., Manao, G., Camici, G. and Ramponi, G. (2001) *J. Biol. Chem.* **276**, 18849–18854.
[24] Ioshida, H. and Tamiya, N. (1971) *J. Biochem.* **69**, 525–534.
[25] Taga, E.M. and Van Etten, R.L. (1982) *Arch. Biochem. Biophys.* **214**, 505–515.
[26] Saeed, A., Tremori, E., Manao, G., Camici, G., Cappugi, G. and Ramponi, G. (1990) *Physiol. Chem. Phys. Med. NMR* **22**, 81–94.
[27] Wurzinger, K.H., Novotny, J.E. and Mohrenweiser, H.W. (1985) *Mol. Cell. Biochem.* **66**, 126–136.
[28] Granjeiro, J.M., Taga, E.M. and Aoyama, H. (1997) *Ann. Acad. Bras. Ci.* **69**, 451–460.
[29] Ferreira, C.V., Taga, E.M. and Aoyama, H. (2000) *J. Enz. Inhib.* **15**, 403–410.
[30] Zhang, Z.-Y. and Van Etten, R.L. (1991) *Biochemistry* **30**, 8954–8959.
[31] Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666.
[32] Cornish-Bowden, A. (1995) *Fundamentals of Enzyme Kinetics*. (Portland Press Ltd. London).
[33] Dixon, M. and Webb, E.C. (1979) *Enzymes*, 3rd. Ed. (Academic Press, New York).
[34] Ames, B.N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981) *Proc. Natl Acad. Sci. U.S.A.* **78**, 6858–6862.
[35] Becker, B.F. (1993) *Free Rad. Biol. Med.* **14**, 615–631.
[36] Wang, S., Stanflacher, C.V. and Van Etten, R.L. (2000) *Biochemistry* **39**, 1234–1242.