Biochemical and Biophysical Research Communications **280,** 319–327 (2001)
doi:10.1006/bbrc.2000.4094, available online at http://www.idealibrary.com on IDEAL®

Intramolecular Interactions in Protein Tyrosine Phosphatase RPTPμ: Kinetic Evidence

Alexandru R. Aricescu,*[,1] Tudor A. Fulga,*[,2] Valeriu Cismasiu,*
Roger S. Goody,† and Stefan E. Szedlacsek*[,3]

*Department of Enzymology, Institute of Biochemistry, Splaiul Independentei 296, 77700, Bucharest, Romania; and
†Department of Physical Biochemistry, Max-Planck Institute for Molecular Physiology,
Otto-Hahn Strasse 11, D-44227 Dortmund, Germany

Received November 7, 2000

The receptor-like protein tyrosine phosphatase RPTPμ contains three intracellular domains: the juxtamembrane (JM) and two phosphatase domains (D1 and D2). D1 is catalytically active *in vitro*. The functional roles of JM and D2 are still unclear. To find out whether and how they modulate the phosphatase activity of D1, we compared the enzymatic characteristics of two constructs, containing a truncated JM and either D1 or both phosphatase domains. *p*-Nitrophenyl phosphate and two peptide substrates were efficiently dephosphorylated by both constructs. The specificity constant of D1 alone was up to 50% higher. D2 induces (a) decreased K_m values for peptide substrates, (b) decreased catalytic efficiency for these substrates, (c) shifting of the optimal pH to slightly lower values, and (d) looser binding of competitive inhibitors. These data suggest that the phosphatase activity of D1 is negatively modulated and its ligand binding capacity is sensibly modified by domain D2, having possible functional significance.

Key Words: **protein–tyrosine phosphatase; RPTPμ; intramolecular interactions; enzyme kinetics.**

Abbreviations used: PTP, protein–tyrosine phosphatase; RPTP, receptor-like protein–tyrosine phosphatase; aa, amino acids; JM, juxtamembrane domain (aa 765–923); D1 and D2, phosphatase domains; GST, glutathione *S*-transferase; pNPP, *p*-nitrophenyl phosphate.

[1] Present address: Institute of Child Health, Neural Development Unit, University College London, 30 Guilford Street, London WC1N 1EH, UK.

[2] Present address: European Molecular Biology Laboratory, Structural Biology Programme, Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

[3] To whom correspondence and reprint requests should be addressed. Fax: +40-1-223-9068. E-mail: Stefan.Szedlacsek@biochim.ro.

Protein tyrosine phosphatases (PTPs) participate in the regulation of cell growth, differentiation and intercellular communication. This family of enzymes is divided into two groups: receptor-like (RPTPs) and nonreceptor PTPs (for review, see Refs. 1 and 2). The intracellular regions of RPTPs are similar for the great majority of representatives, whereas the extracellular domains exhibit a large variability. Most RPTPs have two phosphatase domains in their cytoplasmic regions: the membrane proximal domain D1 and the membrane distal, D2. Despite the sequence homology of D1 and D2, frequently only D1 is catalytically active *in vitro*. Occasionally, D2 may display weak enzyme activity as in the case of RPTPα (3, 4), CD45 (5), or RPTP-LAR (6). An important question in this respect is whether domain D2, albeit catalytically inactive, modulates the substrate specificity of D1 or plays a regulatory role in its catalytic activity. Although this question is still controversial, there are several reports supporting the involvement of domain D2 in modulatory interactions. Thus, domain D2 of RPTPδ was proved to bind to and inhibit the enzyme activity of domain D1 of RPTPσ (7). Intramolecular interactions are also possible, as demonstrated in the case of domains D1 and D2 of CD45 (8).

RPTPμ is an interesting representative of RPTPs since its ectodomain promotes cell-to-cell adhesion in a homophilic, Ca^{2+} independent manner (9). Similar homotypic adhesion has also been demonstrated for two RPTPs homologous to RPTPμ: RPTPκ (10) and RPTPλ (11). Another common characteristic of RPTPμ (12), κ (13) and λ (11) seems to be their specific association with the catenin/cadherin complex. The putative site of this interaction is the juxtamembrane (JM) domain. This segment of 158 amino acids (aa 765–923), about twice longer in RPTPμ than in other PTPs, has notable sequence similarity with members of the cadherin family (14).

0006-291X/01 $35.00

Previous studies indicate that the JM segment also influences the PTPase activity of the intracellular region, whereby removal of the first 53 aa of the JM region of RPTPμ produced a 2-fold decrease of activity (15). In the same report it was shown that D2 has no enzymatic activity. It might be inferred that domain D2 has no contribution to the overall PTPase activity of the intracellular region. Surprisingly, the construct lacking D2 but having the intact JM segment, displayed a 10-fold lower enzyme activity compared to the activity of the whole intracellular region (15). These experiments suggest that D2 modulates the activity of D1; at the same time, D2 might interfere with the JM region in influencing the enzymatic activity of D1. Indeed, a recent report (16) demonstrates, using the yeast two-hybrid system, that a part of the JM domain of RPTPμ can interact with both D1 and D2 domains in an intramolecular fashion. In addition, a fragment of JM (aa 814–922) was found to be essential for D1 to be catalytically active.

Despite the important features of RPTPμ, systematic studies concerning the enzymatic characteristics of this receptor enzyme have not yet been reported. Here we investigate the stability and kinetic behavior of two constructs, containing the JM fragment essential for catalytic activity plus either D1 or both D1 and D2. We present kinetic evidence that the phosphatase activity of D1 is modulated by D2, alone or in conjunction with the JM domain.

MATERIALS AND METHODS

Materials. Restriction endonucleases, glutathione-Sepharose 4B and thrombin were purchased from Amersham Pharmacia Biotech. *p*-Nitrophenyl phosphate (pNPP) and *p*-nitrophenol, vanadate and common use chemicals were from Sigma. Phosphotyrosine containing peptides P1: DGDFEEIPEEpYLQ (hirudine 53–65) and P2: EGPWLEEEEEApYGWMDF (human gastrin 1–17) were kindly provided by Dr. H. Voss. They yielded single peaks on analytical HPLC.

Constructs and protein expression. A *Bgl*II–*Eco*RI fragment (bp 2450–3565) from pB5 [kindly provided by Dr. M. F. G. B. Gebbink (15)] was subcloned into pGEX-KT plasmid (17), between *Bam*HI and *Eco*RI sites, resulting in pΔJM-D1 (RPTPμ aa 818–1190). The 782-bp *Eco*RI (bp 3566–4347) insert of pB5, encoding the D2 domain, was subcloned into pΔJM-D1, resulting in pΔJM-D1D2 (RPTPμ aa 818–1452). The ΔJM-D1– and ΔJM-D1D2–glutathione *S*-transferase fusion protein constructs were expressed in *E. coli* DH5α. The cultures were grown in LB medium with 100 μg/ml ampicillin, until the OD_{600} reached 0.5–0.6. Expression of fusion proteins was induced by adding 100 μM IPTG. Cells were allowed to grow overnight at 26°C.

Protein purification. The harvested cells were resuspended in PBST buffer (137 mM NaCl, 3 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1% Triton X-100, 0.4 mM PMSF, pH 7.4) and disrupted by sonication (5 pulses of 15 s). The fusion proteins were purified on glutathione–Sepharose 4B, then digested with thrombin according to the manufacturer's protocol; SDS–PAGE revealed approximately 90% purity. Further purification was performed by ion-exchange chromatography on Mono Q (Amersham Pharmacia Biotech), using for equilibration 20 mM Tris–HCl, pH 8.0, 10 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.02% NaN_3. To elute the proteins adsorbed on the column a 0–0.5 M NaCl gradient was applied. The peaks having the highest specific activity were collected and pooled. Finally, the buffer was changed and the pure enzyme solution was concentrated on Centricon-10 (Amicon). Where not specified otherwise, the pure enzyme was stored at 4°C in buffer PCB (see below).

Enzyme stability tests. The specific activities toward pNPP of the purified ΔJM-D1 and ΔJM-D1D2, kept at 4 or 22°C in four buffers (PBSA: 140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.02% NaN_3, 10% glycerol added after thrombin cleavage, pH 7.5; PBSG: same buffer but glycerol added before thrombin cleavage; PCB: 20 mM Tris–HCl, 10 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.02% NaN_3, pH 8.0; PCBI: 25 mM imidazole, 2 mM DTT, 1 mM EDTA, 10% glycerol, 0.02% NaN_3, pH 7.2) were measured at various storage times. Selwyn's test of the enzyme stability (18) was performed at 37°C in the assay buffer described below, using 10 mM pNPP as substrate.

Phosphatase assays. The PTPase activity of ΔJM-D1 and ΔJM-D1D2 was assayed at 37°C in a 1-ml reaction mixture containing appropriate substrate concentrations and 25 mM imidazole, pH 7.4, 2 mM DTT, 5 mM EDTA (assay buffer). When pNPP was used as substrate, the absorbance increase at 405 nm was continuously recorded and the initial rate calculated using the UV-KinLab software (Perkin–Elmer). Correction was made for the nonenzymatic hydrolysis of the substrate. The steady-state parameters were determined from a direct fit of $v = f[S]$ data to the Michaelis–Menten equation using the program EZ-FIT (19). Similar approaches were performed using the tyrosine phosphorylated peptides as substrates, except that absorbance increase was recorded at 280 nm. The progress of reaction was followed using the experimentally determined difference between the extinction coefficients of phosphorylated and corresponding dephosphorylated peptide.

The pH dependence of the PTPase activity was studied using the following buffers: pH 3.4–5.4, 50 mM acetate; pH 5.6–6.4, 50 mM citrate; pH 6.8–7.4, 25 mM imidazole; pH 7.8–8.6, 50 mM Tris; all the buffers contained 2 mM DTT and 5 mM EDTA. For each pH value, the extinction coefficients of *p*-nitrophenol (product of pNPP substrate hydrolysis, absorbing at 405 nm) was initially determined. In case of peptide substrates, the pH dependence study was carried out without correction of the extinction coefficients for different pH values (see Refs. 20 and 21).

Inhibition experiments. The inhibition patterns and constants for the reaction products, vanadate and Zn^{2+} ions, were determined using pNPP as substrate. Initially, vanadate and Zn^{2+} were incubated with the enzyme in absence of substrate, and the reaction was started by pNPP addition. The initial rate data obtained for different substrate and inhibitor concentrations were considered in order to obtain the inhibition constants, by using the program EZ-FIT (19). If the inhibition constant K_{iu}—responsible for the uncompetitive binding of inhibitor to the reaction intermediate—was much higher than K_{ic}—responsible for the competitive binding of inhibitor to the enzyme—then the regression procedure was repeated for the case of a competitive inhibitor.

RESULTS

Cloning, Expression, and Purification of RPTPμ Constructs

To study the influence of D2 on the kinetic properties of domain D1 we cloned and expressed in *E. coli*, as glutathione *S*-transferase (GST) fusion proteins, domain D1 (ΔJM-D1) and the intracellular region containing D1 and D2 (ΔJM-D1D2), both lack-

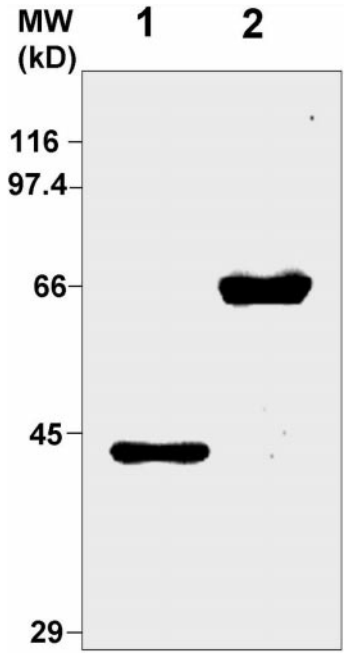


FIG. 1. SDS–PAGE analysis of the purified ΔJM-D1 and ΔJM-D1D2 proteins. The recombinant GST fusion proteins were expressed in *E. coli*, purified on glutathione–Sepharose 4B and GST was cleaved off by thrombin digestion. The RPTPμ constructs were further purified on Mono Q and eluted with a 0–0.5 M NaCl gradient. Fractions of the major peak eluted from the Mono Q column, having the highest specific activity, were concentrated and analyzed by SDS–PAGE, after reduction by 2-mercaptoethanol. Lane 1, ΔJM-D1; lane 2, ΔJM-D1D2.

ing the initial 53 amino acids of the JM region. The fusion proteins were purified first on glutathione–Sepharose 4B, then GST was removed by thrombin cleavage and the RPTPμ fragments were further purified on a MonoQ column. SDS–PAGE of purified ΔJM-D1 and ΔJM-D1D2 proteins showed that both protein preparations were electrophoretically homogeneous (Fig. 1).

Effects of Buffers and Storage Conditions on the Specific Activity

To explore the effect of D2 on the stability of the activity of ΔJM-D1 under various storage conditions, we used four buffers: PBSA, PBSG, PCB, and PCBI (Table 1). PBSA differs from PBSG with respect to glycerol, which was added before (PBSA) or after (PBSG) the thrombin cleavage from the glutathione–Sepharose beads of ΔJM-D1 or ΔJM-D1D2, respectively. The specific activity of both proteins was measured immediately after preparation, after 28 days of storage at 4°C, after 7 days at 22°C and after 28 days of storage at 22°C. In some cases, minor amounts of precipitated protein were formed; the precipitate was always removed by centrifugation before measuring the specific activity. The small increase of the specific activity under certain situations might be caused by the precipitation of otherwise enzymatically inactive forms, which occurred during the storage time. The presence of D2 did not influence the enzyme stability under the conditions tested.

Selwyn's Test of Enzyme Inactivation

It is more relevant for the physiological stability of an enzyme to check whether the enzyme activity remains constant in the presence of substrate, in a buffer simulating physiological conditions. For this purpose, Selwyn's test (18) is an important tool. It is based on the fact that, as long as the rate equation corresponding to the enzymatic reaction is linear in enzyme concentration, the product concentration is determined solely by the product of time and enzyme concentration. In other words, when representing the progress curves—where the abscissa corresponds to the time multiplied by the enzyme concentration—at different enzyme concentrations, all the points should fall on a single curve, providing that the enzyme concentration during the reaction does not vary with time.

TABLE 1

Stability of Purified Proteins under Different Storage Conditions

Construct	Buffer	a	b	c	d
ΔJM-D1	PBSA	1.37	1.42 (103)	1.6 (116)	1.27 (93)
	PBSG	1.58	1.43 (90)	1.8 (131)	1.31 (83)
	PCB	1.28	0.93 (73)	1.35 (105)	1.2 (94)
	PCBI	1.27	1.45 (114)	1.59 (125)	1.32 (104)
ΔJM-D1D2	PBSA	0.52	0.43 (83)	0.53 (101)	0.52 (100)
	PBSG	0.5	0.43 (86)	0.53 (101)	0.51 (99)
	PCB	0.5	0.44 (88)	0.54 (104)	0.56 (108)
	PCBI	0.56	0.41 (73)	0.59 (113)	0.47 (84)

Note. Specific activities (μmol/min/mg enzyme) of purified proteins using pNPP as substrate were determined. Before the activity assay, the purified proteins were stored at (a) −70°C (initial values); (b) 4°C, 28 days; (c) 22°C (RT), 7 days; and (d) 22°C, 28 days, in the following buffers: PBSA, PBSG, PCB, and PCBI (see Materials and Methods). In parentheses, the relative activities (percentages, compared to the initial values) are given.

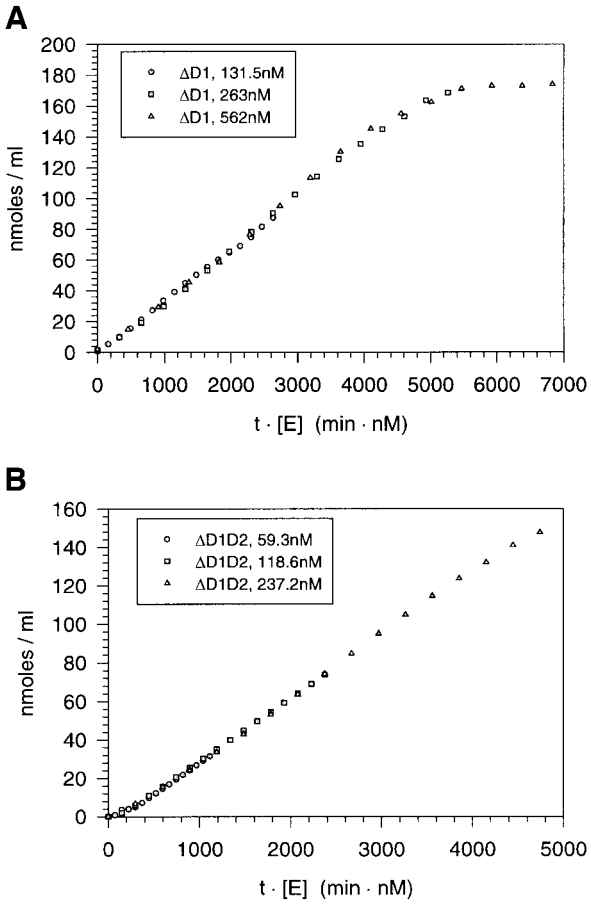


FIG. 2. Selwyn's test of enzyme inactivation for (A) ΔJM-D1 and (B) ΔJM-D1D2. Selwyn's test was performed at 37°C in assay buffer, using 10 mM pNPP and the enzymes at the concentrations mentioned in the insets.

Applying Selwyn's test in case of both ΔJM-D1 and ΔJM-D1D2 (Fig. 2), it can be seen that both enzymes are stable in presence of pNPP as substrate, at 37°C in 0.1 M imidazole buffer at pH 7.4, for at least 15 min. Superimposition of the experimental progress curves, when enzyme concentration varies in a 4-fold range, suggests the lack of enzyme dimerization or other oligomerizations associated with changes in the specific enzyme activity.

Kinetic Parameters for Different Substrates

pNPP and two tyrosine phosphorylated peptides (P1 and P2) were tested as substrates of ΔJM-D1 and ΔJM-D1D2. The pH of the assay buffer for all kinetic measurements was 7.4. Although nonoptimal, this value was preferred in order to have the kinetic parameters determined at the physiological pH. In this way, it has been also possible to compare our results to those reported in Ref. 15. To establish whether peptides P1 and P2 are substrates or not for RPTPμ, the absorption spectra of peptides were recorded before and after incubation with ΔJM-D1 and ΔJM-D1D2. Figure 3 shows that both peptides are substrates of ΔJM-D1D2. Similar determinations showed that peptides P1 and P2 are also substrates of ΔJM-D1 (data not shown). The maximal decrease of absorbance is at 280 nm; therefore, this wavelength was selected to record the progress of enzyme catalyzed dephosphorylation of peptides P1 and P2. As expected, the K_m for pNPP was one order of magnitude higher than that for the peptide substrates (Table 2). For ΔJM-D1, the K_m was twofold lower than for ΔJM-D1D2 when pNPP was used as substrate, while for the peptide substrates, ΔJM-D1 had an approximately twofold higher K_m than ΔJM-D1D2. As the k_{cat} values were about twice higher for ΔJM-D1 than for ΔJM-D1D2, the specificity constants were of the same order of magnitude for both enzyme forms. Nevertheless, the specificity constant of ΔJM-D1 was slightly different in the case of P2, being approximately 50% higher than that of ΔJM-D1D2. The non-peptide substrate, pNPP, also gave a 50% higher k_{cat}/K_m value for ΔJM-D1 compared to the corresponding value of ΔJM-D1D2. The specificity constant for the peptide substrate was much higher (about 3 orders of magnitude) than for the nonpeptide substrate pNPP.

pH Dependence of Enzyme Activity for Different Substrates

As shown in Fig. 4, the optimal pH for pNPP hydrolysis is 4.8, while for the peptide substrates it was 5.4 and respectively 5.8 [much lower than those reported in (15) and (22)]. In the case of pNPP and P1, the optimal pH values are respectively the same for ΔJM-D1 and ΔJM-D1D2. However, when P2 was used as substrate, the optimal pH was shifted from 5.8 for ΔJM-D1 to 5.4 for ΔJM-D1D2. Both ΔJM-D1 and ΔJM-D1D2 display tyrosine phosphatase activity over a broad pH interval: 3.4–8.6. Excepting some extreme pH values, the activity of ΔJM-D1 was higher than that of ΔJM-D1D2 over the whole pH range mentioned, irrespective of the substrate used. The maximal activity of ΔJM-D1 was higher than that of ΔJM-D1D2 with approximately the same percentage of 50–60% for all three substrates considered (50% for P1 and around 63% for pNPP and P2).

Inhibition by Phosphate, p-Nitrophenol, Vanadate, and Zn^{2+}

There is considerable evidence supporting the idea that PTPs and dual-specific phosphatases have a common catalytic mechanism, employing a thiol-phosphate enzyme-intermediate and two transition states (23). Product inhibition studies may be used to confirm a suggested mechanism while the inhibition with specific inhibitors may provide information about the transition state geometry.

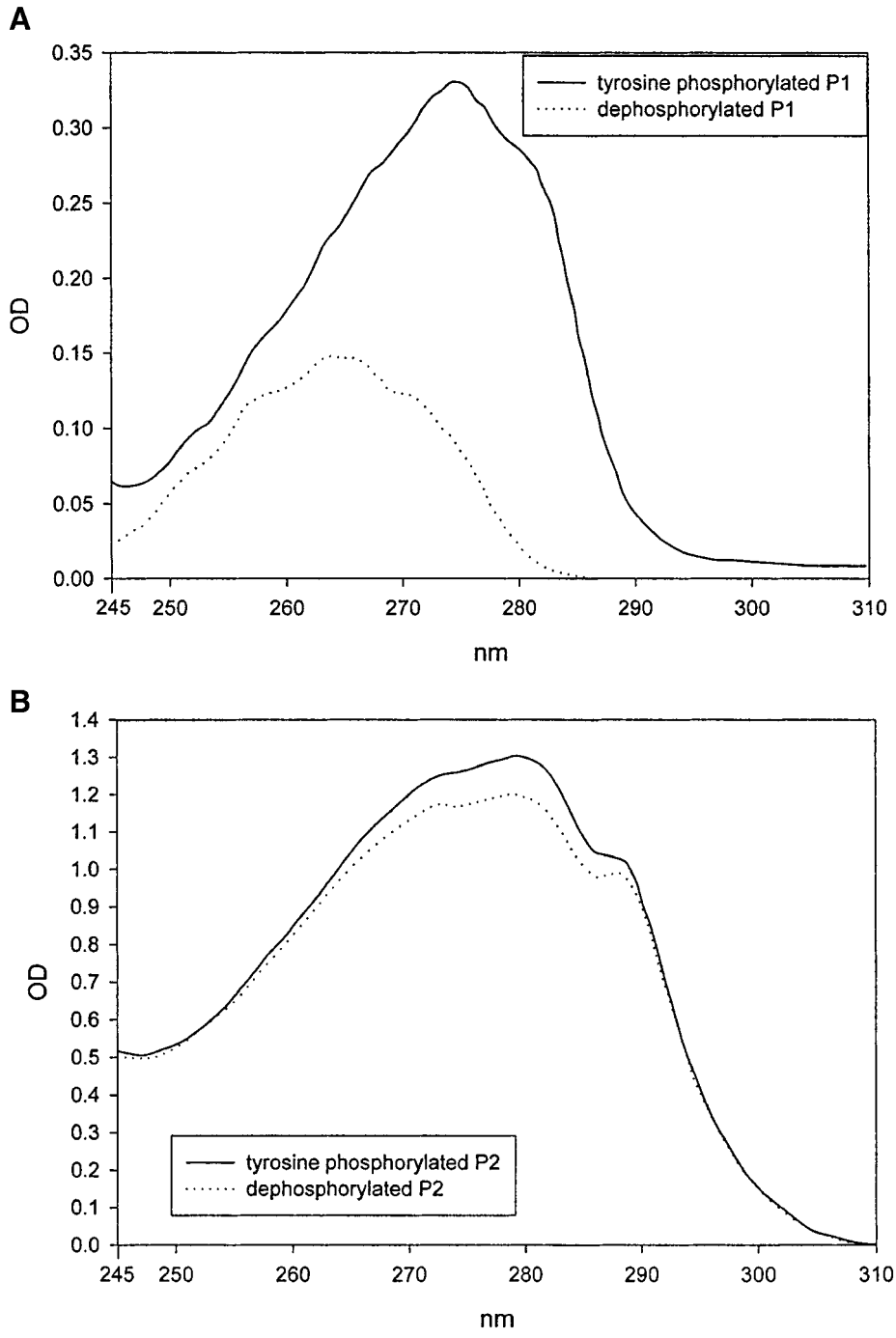


FIG. 3. Absorption spectra of tyrosine phosphorylated peptides P1 (A) and P2 (B). UV spectra of tyrosine phosphorylated peptides, before (—) and after (· · ·) 10 min treatment with purified ΔJM-D1D2. The concentration of peptides was 250 μM and enzyme was added to a final concentration of 0.28 μM in assay buffer.

Product inhibition studies on catalytically active PTPase domains support the substituted-enzyme mechanism (4, 24). To examine the influence of D2 on the product inhibition pattern of RPTPμ, both reaction products of the pNPP substrate (i.e., phosphate and *p*-nitrophenol) were tested as inhibitors of ΔJM-D1 and ΔJM-D1D2. Table 3 shows that phosphate is a competitive inhibitor while *p*-nitrophenol is a mixed type inhibitor of ΔJM-D1 and ΔJM-D1D2. Although the inhibition patterns relative to both constructs were unchanged, there are some differences in the inhibition constants. Thus, phosphate binds more than 3-fold stronger to the ΔJM-D1D2 construct than to ΔJM-D1. In contrast, *p*-nitrophenol binds 3.3-fold stronger to the

TABLE 2

Steady-State Kinetic Constants for ΔJM-D1 and ΔJM-D1D2

	ΔJM-D1			ΔJM-D1D2		
Construct	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
pNPP	0.54 ± 0.015	1796.64 ± 126	300.67 ± 12	0.69 ± 0.03	3502.35 ± 370	197.01 ± 11
Peptide P1	33.31 ± 1.59	184.81 ± 21.1	$0.18 \pm 0.023 \times 10^6$	14.67 ± 1	95.38 ± 9.75	$0.15 \pm 0.011 \times 10^6$
Peptide P2	37.69 ± 2.65	187.02 ± 20.5	$0.21 \pm 0.018 \times 10^6$	15.09 ± 0.97	102.12 ± 13.03	$0.14 \pm 0.023 \times 10^6$

Note. The kinetic parameters k_{cat}, K_m, and k_{cat}/K_m were determined when pNPP, peptide P1, or peptide P2 was used as substrate, by direct fit of $v = f[S]$ data to the Michaelis–Menten equation using the nonlinear regression program EZ-FIT (19).

reaction intermediate of ΔJM-D1D2 than that of ΔJM-D1 while the competitive inhibitory effect is comparable in both situations.

The affinity toward phosphate is relatively low compared to other PTPs but similar to RPTPα (4). The inhibition pattern of *p*-nitrophenol found for ΔJM-D1 and ΔJM-D1D2 (mixed type) is in agreement with theoretical expectations (25) but differs from that found by Wu *et al.* (4) and Zhang (24) (noncompetitive type). It is worth mentioning that noncompetitive inhibition is an unlikely particular case of mixed type inhibition (25).

Vanadate and Zn^{2+} were also tested as inhibitors of ΔJM-D1 and ΔJM-D1D2. Vanadate, the most potent inhibitor of PTPs, was found to behave as a competitive inhibitor with an inhibition constant in the micromolar range. However, differences were observed in its efficiency toward the inhibition of the RPTPμ constructs considered. Vanadate anion binds almost 3 fold more tightly to ΔJM-D1 than to ΔJM-D1D2, while, surprisingly, Zn^{2+} was found to be an inhibitor of ΔJM-D1 as potent as vanadate. Moreover, the difference between the Zn^{2+} inhibition of ΔJM-D1 and ΔJM-D1D2 is still higher in this case, the inhibition constant of ΔJM-D1D2 being 17-fold higher than the inhibition constant of ΔJM-D1.

Some of our preliminary results concerning the inhibition of both RPTPμ constructs suggest that vanadate and Zn^{2+} act as slow-binding inhibitors. Further experiments must be performed in order to determine accurately all inhibition parameters corresponding to this particular type of enzyme inhibition (26, 27).

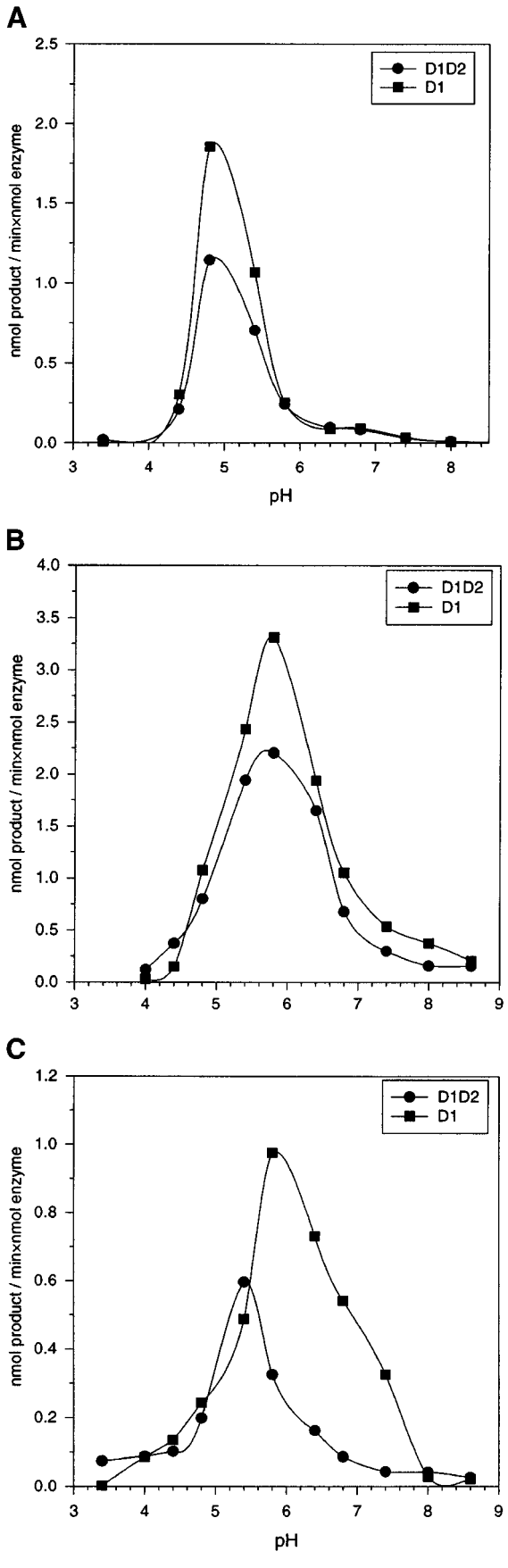
DISCUSSION

Gebbink *et al.* (15) reported that mutation of Cys-1095 to Ser in domain D1 of RPTPμ abolishes the enzymatic activity of the whole intracellular region. This suggests that D2 is an inert and catalytically neutral segment of RPTPμ. The stability tests at different temperatures in the presence or absence of substrate, which show a high stability both of ΔJM-D1 and ΔJM-D1D2, are in agreement with this hypothesis. Nevertheless, the other results obtained here support a modulatory effect of D2 on the enzymatically active domain D1 rather than the non-involvement of D2. Thus, ΔJM-D1 is more active than ΔJM-D1D2, as reflected by the turnover number and specificity constants for both peptide and nonpeptide substrates. The decrease of ΔJM-D1D2 activity compared to ΔJM-D1 is approximately 50%. Both constructs display a specificity constant 3 orders of magnitude higher for peptide substrates than those for pNPP. This preference of RPTPμ for peptide substrates is much more pronounced than in the case of other RPTPs. For instance, the specificity constant of RPTPα for pNPP is only 3.5-fold lower than for the peptide substrate RR-src (28). It is worth mentioning that both tyrosine phosphorylated peptides tested in this study proved to be excellent synthetic substrates for RPTPμ, so far not reported in the literature as substrates of RPTPs. Particularly, the assay in the presence of P1 is very simple, due to the relatively large increase of absorbance after dephosphorylation.

The pH-dependence study showed that the activity at the optimal pH value of ΔJM-D1 was again 50–60% higher than the corresponding activity of ΔJM-D1D2 for all tested substrates. Interestingly, in the case of P2 but not in the case of P1, the presence of D2 shifted the optimal pH from 5.8 to 5.4. This may be attributed to the contribution of D2 to the overall substrate specificity of ΔJM-D1D2.

The optimal pH values reported here are different from the values (7.4–7.5) found by Gebbink *et al.* (15) and Brady-Kalnay and Tonks (22). This may be explained by the influence of the initial 53 aa which are missing in our constructs but present in that used in (15) and (22).

The inhibition experiments demonstrate that strong competitive inhibitors such as vanadate and Zn^{2+} have higher affinity for domain D1 when the non-catalytic domain D2 is absent. The slightly distorted trigonal bipyramidal geometry of the vanadate complex mimics the transition state for the hydrolysis of the thiophosphate enzyme intermediate (23), explaining the high affinity of vanadate to the active site of phosphatases. As D2 is devoid of enzymatic activity, it may be sup-



posed that there is no direct tight binding of vanadate to D2. Thus, the difference in the affinity of vanadate for ΔJM-D1 compared to ΔJM-D1D2 may be attributed to the involvement of D2 in modulation of the conformation of D1 at the active site. The same modulatory effect of D2 on D1 may be responsible for the different K_m values of ΔJM-D1 and ΔJM-D1D2 for pNPP, peptide P1 and peptide P2, respectively.

To explore the possibility that domain D2 influences the relative ratio between the rates of formation and of breakdown of the reaction intermediate, fast kinetic measurements were also performed (data not shown). At a pNPP concentration of 5 mM, both for ΔJM-D1 and ΔJM-D1D2 no "burst" was detected at enzyme concentrations as high as 40 μM. Thus, in both cases, the rate-limiting step appears to be the formation of the covalent cysteinylphosphate intermediate.

The results reported in (15) suggest a modulatory role of D2 and of the JM region on the catalytic activity of D1. Supposing that D2 and the initial 53 aa (of the JM region) exert their influence independently of each other, in the case of our constructs ΔJM-D1D2 should have had a much higher activity than ΔJM-D1. Our results clearly contradict this expectation, ΔJM-D1D2 being catalytically less active than ΔJM-D1. A possible explanation for this fact is that D2 and the initial segment of the JM region influence the catalytic properties of D1 in a synergistic manner. Thus, it might be imagined that D2 alone diminishes the activity of D1 but when interacting with the initial JM region, the effect is converted to the activation of the catalytic function of D1. Indeed, this type of interaction was demonstrated for another RPTPase, CD45, whereby a noncovalent association takes place between the N-terminal 27-kDa fragment and the C-terminal 53-kDa fragment of the cytoplasmic region (8).

In a recent study, Feiken *et al.* (16) demonstrate using the yeast two-hybrid system that a fragment of the JM region of RPTPμ can interact with both D1 and D2 domains and that this interaction is intramolecular rather than intermolecular. On the other hand, Zondag *et al.* (29) have recently shown that RPTPμ binds and dephosphorylates the catenin $p120^{ctn}$ and that both JM and D2 domains contain binding sites for $p120^{ctn}$. The fact that JM and D2 can bind each other, and that they may act synergistically in binding and presenting substrates to D1, is in agreement with our kinetic data which suggest that the phosphatase activity of D1 is modulated by D2 and JM in a concerted manner.

FIG. 4. pH-dependence of ΔJM-D1 and ΔJM-D1D2 activity. The PTPase activity was assayed at 37°C and different pH values using (A) pNPP, (B) peptide P1, or (C) peptide P2 as substrate and the following buffers: pH 3.4-5.4, 50 mM acetate; pH 5.6-6.4, 50 mM citrate; pH 6.8–7.4, 25 mM imidazole; pH 7.8–8.6, 50 mM Tris. All buffers contained 2 mM DTT and 5 mM EDTA.

TABLE 3

Inhibitory Effect of Various Compounds on ΔJM-D1 and ΔJM-D1D2 Activity

		K_{ic} (μM)	K_{iu} (μM)	Inhibition pattern
ΔJM-D1	Phosphate*	$113.8 \pm 6.3 \times 10^3$	—	Competitive
	p-nitrophenol*	285.61 ± 22	624.39 ± 66	Mixed
	Vanadate	6.65 ± 0.68	—	Competitive
	Zn^{2+}	3.38 ± 0.68	—	Competitive
ΔJM-D1D2	Phosphate*	$34.85 \pm 2.5 \times 10^3$	—	Competitive
	p-nitrophenol*	387.67 ± 13	190.47 ± 17	Mixed
	Vanadate	19.06 ± 2.8	—	Competitive
	Zn^{2+}	66.347 ± 2.8	—	Competitive

Note. Purified proteins, pNPP as substrate, as well as inhibitors and reaction products (*) were used in the enzyme activity assays. Using the program EZ-FIT (19), the inhibition constants K_{ic} and K_{iu} were determined from steady-state measurements for different substrate and inhibitor concentrations.

The crystal structure of the D1 domain of RPTPα shows a homodimeric organization in which part of the JM region forms a helix-turn-helix segment inserted into the active site of the dyad-related D1 monomer (30). The authors propose that this dimeric structure would result in downregulation of the catalytic activity. Our data obtained by using Selwyn's test of enzyme inactivation show that, under the range of enzyme concentrations tested, no dimerization, resulting in enzyme inhibition, takes place. The results of Hoffmann *et al.* (31) also support an absence of dimer formation or inhibition by dimerization in the case of RPTPμ domain D1. On the other hand, the results of Bilwes *et al.* (30) show that principally, the interaction between domain D2 of RPTPα (having high homology with domain D1) and the JM region is possible. The recently reported crystal structure of the tandem phosphatase domains of RPTP-LAR (32) is also in agreement with our results. Thus, a monomeric form was observed, in contrast to the dimeric blocked orientation model suggested by Bilwes (30).

The data presented here suggest that domain D2 of RPTPμ modulates the enzymatic activity of domain D1 via intramolecular interactions, most probably in conjunction with the JM domain, thus contributing to better understanding of the regulation of this important family of receptor enzymes.

ACKNOWLEDGMENTS

Part of this work was done in the Max-Planck Institute for Biophysics in Frankfurt/M and supported by a grant from Alexander von Humboldt Foundation to S. E. Szedlacsek. Support through NATO Linkage Grant LG972981 is also acknowledged. We are very grateful to Professor Hartmut Michel and Dr. Helmut Reilaender for help and encouragement. The authors are also indebted to Dr. Octavian Barzu and Iain McKinnell for critical reading of the manuscript.

REFERENCES

1. Li, L., and Dixon, J. E. (2000) Form, function, and regulation of protein tyrosine phosphatases and their involvement in human diseases. *Semin. Immunol.* **12,** 75–84.
2. Neel, B. G., and Tonks, N. K. (1997) Protein tyrosine phosphatases in signal transduction. *Curr. Opin. Cell Biol.* **9,** 193–204.
3. Wang, Y., and Pallen, C. J. (1991) The receptor-like protein tyrosine phosphatase HPTP alpha has two active catalytic domains with distinct substrate specificities. *EMBO J.* **10,** 3231–3237.
4. Wu, L., Buist, A., den Hertog, J., and Zhang, Z.-Y. (1997) Comparative kinetic analysis and substrate specificity of the tandem catalytic domains of the receptor-like protein–tyrosine phosphatase alpha. *J. Biol. Chem.* **272,** 6994–7002.
5. Tan, X., Stover, D. R., and Walsh, K. A. (1993) Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* **268,** 6835–6838.
6. Pot, D. A., Woodford, T. A., Remboutsika, E., Haun, R. S., and Dixon, J. E. (1991) Cloning, bacterial expression, purification, and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase. *J. Biol. Chem.* **266,** 19688–19696.
7. Wallace, M. J., Fladd, C., Batt, J., and Rotin, D. (1998) The second catalytic domain of protein tyrosine phosphatase delta (PTP delta) binds to and inhibits the first catalytic domain of PTP sigma. *Mol. Cell. Biol.* **18,** 2608–2616.
8. Felberg, J., and Johnson, P. (1998) Characterization of recombinant CD45 cytoplasmic domain proteins. Evidence for intramolecular and intermolecular interactions. *J. Biol. Chem.* **273,** 17839–17845.
9. Gebbink, M. F. G. B., van Etten, I., Hateboer, G., Suijkerbuijk, R., Beijersbergen, R. L., van Kessel, A. G., and Moolenaar, W. H. (1993) Cell–cell adhesion mediated by a receptor-like protein tyrosine phosphatase. *J. Biol. Chem.* **268,** 16101–16104.
10. Jiang, Y., Wang, H., D'Eustachio, P., Musacchio, J., Schlessinger, J., and Sap, J. (1993) Cloning and characterization of R-PTP-kappa, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region. *Mol. Cell. Biol.* **13,** 2942–2951.
11. Cheng, J., Wu, K., Armanini, M., O'Rouke, N., Dowbenko, D., and Lasky, L. A. (1997) A novel protein–tyrosine phosphatase related to the homotypically adhering kappa and mu receptors. *J. Biol. Chem.* **272,** 7264–7277.
12. Brady-Kalnay, S. M., Mourton, T., Nixon, J. P., Pietz, G. E., Kinch, M., Chen H., Brackenbury, R., Rimm, D. L., Del Vecchio, R. L., and Tonks, N. K. (1998) Dynamic interaction of PTPmu with multiple cadherins *in vivo. J. Cell Biol.* **141,** 287–296.
13. Fuchs, M., Muller, T., Lerch, M., and Ullrich, A. (1996) Association of human protein–tyrosine phosphatase kappa with members of the armadillo family. *J. Biol. Chem.* **271,** 16712–16719.

14. Tonks, N. K., Yang, Q., Flint, A., Gebbink, M., Franza, B., Hill, D., Sun, H., and Brady-Kalnay, S. (1992) Protein tyrosine phosphatases: The problems of a growing family. *Cold Spring Harbor Symp. Quant. Biol.* **57,** 87–94.
15. Gebbink, M. F. G. B., Verheijen, M. H. G., Zondag, C. M., van Etten, I., and Moolenaar, W. H. (1993) Purification and characterization of the cytoplasmic domain of human receptor-like protein tyrosine phosphatase RPTP mu. *Biochemistry* **32,** 13516–13522.
16. Feiken, E., van Etten, I., Gebbink, M. F. G. B., Moolenaar, W. H., and Zondag, G. C. M. (2000) Intramolecular interactions between the juxtamembrane domain and phosphatase domains of receptor protein–tyrosine phosphatase RPTPmu. Regulation of catalytic activity. *J. Biol. Chem.* **275,** 15350–15356.
17. Hakes, D. J., and Dixon, J. E. (1992) New vectors for high level expression of recombinant proteins in bacteria. *Anal. Biochem.* **202,** 293–298.
18. Selwyn, M. J. (1965) A simple test for inactivation of an enzyme during assay. *Biochem. Biophys. Acta* **105,** 193–195.
19. Perrella, F. W. (1988) EZ-FIT: A practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. *Anal. Biochem.* **174,** 437–447.
20. Apostol, I., Kuciel, R., Wasylewska, E., and Ostrowski, W. S. (1985) Phosphotyrosine as a substrate of acid and alkaline phosphatases. *Acta Biochim. Polonica* **32,** 187–197.
21. Zhang, Z.-Y., Maclean, D., Thieme-Sefler, A. M., Roeske, R., and Dixon, J. E. (1993) A continuous spectrophotometric and fluorimetric assay for protein tyrosine phosphatase using phosphotyrosine-containing peptides. *Anal. Biochem.* **211,** 7–15.
22. Brady-Kalnay, S. M., and Tonks, N. K. (1993) Purification and characterization of the human protein tyrosine phosphatase, PTP mu, from a baculovirus expression system. *Mol. Cell. Biochem.* **127/128,** 131–141.
23. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E (1996) Visualization of intermediate and transition-state structures in protein–tyrosine phosphatase catalysis. *Proc. Natl. Acad. Sci. USA* **93,** 2493–2498.
24. Zhang, Z.-Y. (1995) Kinetic and mechanistic characterization of a mammalian protein–tyrosine phosphatase, PTP1. *J. Biol. Chem.* **270,** 11199–11204.
25. Cornish-Bowden, A. (1995) Fundamentals of Enzyme Kinetics, 2nd ed., Portland Press, London.
26. Szedlacsek, S. E., and Duggleby, R. G. (1995) Kinetics of slow and tight-binding inhibitors. *Methods Enzymol.* **249,** 144–180.
27. Szedlacsek, S. E., Ostafe, V., Duggleby, R. G., Serban, M., and Vlad, M. O. (1990) Progress-curve equations for reversible enzyme-catalysed reactions inhibited by tight-binding inhibitors. *Biochem. J.* **265,** 647–653.
28. Lim, K. L. Kolatkar, P. R., Ng, K. P., Ng, C. H., and Pallen, C. J.(1998) Interconversion of the kinetic identities of the tandem catalytic domains of receptor-like protein–tyrosine phosphatase PTPalpha by two point mutations is synergistic and substrate-dependent. *J. Biol. Chem.* **273,** 28986–28993.
29. Zondag, G. C. M., Reynolds, A. B., and Moolenaar, W. H. (2000) Receptor protein–tyrosine phosphatase RPTPmu binds to and dephosphorylates the catenin p120(ctn). *J. Biol. Chem.* **275,** 11264–11269.
30. Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996) Structural basis for inhibition of receptor protein–tyrosine phosphatase-alpha by dimerization. *Nature* **382,** 555–559.
31. Hoffmann, K. M. V., Tonks, N. K., and Barford, D. (1997) The crystal structure of domain 1 of receptor protein–tyrosine phosphatase mu. *J. Biol. Chem.* **272,** 27505–27508.
32. Nam, H. J., Poy, F., Krueger, N. X.., Saito, H., and Frederick, C. A. (1999) Crystal structure of the tandem phosphatase domains of RPTP LAR. *Cell* **97,** 449–457.