Effects of modification of the hydrophobic C-1–C-16 segment of tautomycin on its affinity to type-1 and type-2A protein phosphatases

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Among the naturally occurring toxins that are known to have specific inhibitory effects on type-1 and type-2A protein phosphatases (PP1 and PP2A), tautomycin (TM) is unique in that it exhibits significantly higher affinity to PP1 than to PP2A. The ratio of the dissociation constant for the PP1–TM interaction to that for the PP2A–TM interaction (the PP1/PP2A ratio) is 0.01–0.03. The aim of the present study was to evaluate the possible contributions of the C-1–C-16 segment of TM to the affinity characteristics of the toxin. The relatively hydrophobic segment contains a spiroketal motif whose enantiomeric form is present in okadaic acid (OA), which exhibits exceedingly higher affinity to PP2A than to PP1. We therefore synthesized two TM analogues: TM1 in which the side chains of the spiroketal motif of TM were removed but its absolute configuration was retained,

and TM2 in which the spiroketal motif of TM1 was replaced with its enantiomeric form. The effects of TM, TM1 and TM2 on the activities of the native catalytic subunits of PP1 (PP1C) and PP2A and a recombinant γ isoform of PP1 (PP1 γ) were examined. The PP1/PP2A ratio determined thereby was 0.2–0.5 for TM1 and 5–10 for TM2. Both the presence of the side chains and the stereochemistry of the spiroketal moieties may be major determining factors for the affinity characteristics of TM. We also show that a monoclonal antibody raised against OA binds to TM2 albeit with much lower affinity than to OA, whereas it exhibits no measurable affinities to TM and TM1.

Key words: enantiomer, enzyme inhibitor, okadaic acid, structure–affinity relationship.

INTRODUCTION

Type-1 and type-2A protein phosphatases (PP1 and PP2A) are two of the four major enzymes that dephosphorylate serine and threonine residues of proteins [1,2]. These enzymes, between which the architecture of the catalytic domain is thought to be closely conserved [3], play critical roles in determining the level of phosphorylation and hence the biological activities of many proteins. There are several naturally occurring toxins which are now widely used as research tools because of their specific and potent inhibitory effects on these enzymes. They include okadaic acid (OA) [4–6], calyculin A [7], microcystin-LR [8], cantharidin [9,10] and fostriecin [11], as well as tautomycin (TM) [12,13], with which this paper is concerned. TM, first isolated as an antibiotic from the bacterium *Streptomyces spiroverticillatus* [14], is unique among the toxins in that it binds to and inhibits PP1 with higher affinity than it does PP2A [12,15]. The ratio of the dissociation constant for the PP1–TM interaction to that for the PP2A–TM interaction (the PP1/PP2A ratio) is in the range 0.01–0.03 (see Table 3, below). None of the other toxins are known to give a PP1/PP2A ratio significantly smaller than unity. Although experiments using various derivatives and fragments of TM have suggested several moieties as being essentially important for its inhibitory actions on PP1 and PP2A [16,17], no specific structural factors responsible for its characteristic affinity to the enzymes have been identified until now.

The present study was initiated to evaluate the contributions of the C-1–C-16 segment of TM (see Figure 1) to its binding to PP1 and PP2A. According to the current binding model for the phosphatase inhibitors, this relatively hydrophobic segment, which contains a spiroketal motif with two side chains, is supposed to be accommodated in a site that is slightly apart from

the catalytic centre of the enzyme (see Discussion). We therefore expected that chemical modifications of this segment might yield some TM derivatives retaining a measurable inhibitory activity. One could evaluate the relative contributions of structural components belonging to this segment in the binding of TM to PP1 and PP2A by comparing the affinities of the original toxin and such derivatives. It is known that even slight modification of the rest of the molecule tends to be very destructive and give only completely inactive derivatives [17,18]. We were also particularly interested in the fact that the enantiomeric form of the spiroketal motif is present in the OA molecule (see Figure 1), which, in contrast to TM, exhibits exceedingly higher affinity to PP2A than to PP1 [4,6,15]. Since there are numerous examples of stereo-specific interaction of a macromolecule with a relatively small ligand, it seemed to us natural to speculate that the stereochemistry of the spiroketal motif might be a key factor in determining the affinity characteristics of the toxins.

For these reasons we have chemically synthesized two TM analogues: TM1 in which the side chains of the spiroketal motif are removed but its stereochemistry retained, and TM2 in which the spiroketal motif of TM1 is replaced with its enantiomeric form (see Figure 1). We show here that these derivatives indeed retain considerable inhibitory activities against PP1 and PP2A. The PP1/PP2A ratio, determined by dose-inhibition analyses using the native catalytic subunits of PP1 (PP1C) and PP2A and a recombinant γ isoform of PP1 (PP1 γ), is in the range 0.2–0.5 with TM1 and 5–10 with TM2. The marked increases in the ratio caused by the derivatization of TM to TM1 and TM2 indicate that the stereochemistry of the spiroketal motif as well as the presence of its side chains are important factors for the characteristic affinities of TM to PP1 and PP2A. Some implications for the structure of the toxin-binding site will be discussed.

Abbreviations used: PP1 and PP2A, type-1 and type-2A protein phosphatases; PP1C, catalytic subunits of PP1; PP1 γ , recombinant γ isoform of PP1; TM, tautomycin; OA, okadaic acid; pNPP, *p*-nitrophenyl phosphate; PMLC, phosphorylated myosin light chain.

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Figure 1 Chemical structures of TM, its analogues and OA

The chemical structures of TM and its analogues used in the present experiments are shown. The structure of OA is also shown for comparison. The backbone carbon atoms of TM and OA are numbered. To avoid confusion, we refer to corresponding carbon atoms of TM, TM1 and TM2 by the same numbers. Note that the spiroketal motifs of TM and TM1 and those of TM2 and OA (enclosed with dashed lines) are mutually enantiomeric. Ionic functional groups are represented by their forms prevailing in aqueous solution at biological pH. We also synthesized a derivative of TM1 in which the hydroxy group at C-22 is methylated (22-*O*-methyl TM1; not shown).

Here we also show that a monoclonal antibody (IgG1) raised against OA [19,20] binds to TM2 albeit with much lower affinity than to OA, whereas it exhibits no measurable affinities to TM and TM1. It has been shown that the IgG1 binds strongly to the C-15–C-38 fragment of OA, which retains the spiroketal motif [20]. Since OA possesses an identical spiroketal motif to that of TM2 (Figure 1), the success of the synthesis of TM1 and TM2 gave us an opportunity to evaluate the capability of the IgG1 to recognize the stereochemistry of the spiroketal motif. The present results indicate that the IgG1 does recognize the stereochemistry.

EXPERIMENTAL

Materials

TM was purified from a culture of *S. spiroverticillatus* by the method described previously [14,21]. TM1 and 22-*O*-methyl TM1 were synthesized by a slight modification of the method that we developed for total synthesis of TM [22,23]. TM2 was synthesized by a method described elsewhere [24]. OA was given kindly by Dr Yasumasa Tsukitani (Fujisawa Pharmaceutical Co., Tokyo, Japan). Murine IgG1 monoclonal antibody against OA, prepared by the method of Usagawa et al. [19], was provided kindly by Dr Takeshi Yasumoto (Japan Food Research Laboratories, Tokyo, Japan).

p-Nitrophenyl phosphate (*p*NPP; disodium salt) was from Sigma (catalogue code 104). Phosphorylase *b* (catalogue code P-6635) and phosphorylase kinase (catalogue code P-2014) purified from rabbit skeletal muscle were also purchased from Sigma. [γ -

32 P]ATP (catalogue code NEG-002Z) was obtained from Amersham Japan (Tokyo, Japan). All other chemical reagents used were of analytical grade.

The protein phosphatase inhibitors were dissolved in pure DMSO and added to aqueous buffers. The maximum concentration of DMSO in reaction mixtures was 0.01 % (v/v). Enzyme activities were not affected by addition of this amount of the solvent.

Preparation and assay of protein phosphatases

The catalytic subunits of PP1 (PP1C) and PP2A were prepared from rabbit skeletal muscle essentially by the method of Tung et al. [25]. A full-length cDNA encoding PP1 γ [26–29] was expressed in *Escherichia coli* (provided kindly by Dr Patricia Cohen, University of Dundee, Dundee, U.K.), as described by Alessi et al. [30]. The expressed PP1 γ was purified by a procedure essentially similar to that used for purification of the native PP1C, involving chromatographic steps with the use of Q-Sepharose XL, heparin-Sepharose, phenyl-Sepharose, Sephacryl S-200 and Mono-Q (all purchased from Amersham Pharmacia Biotech, Uppsala, Sweden). Buffers used for the expression and purification of PP1 γ contained 1 mM MnCl₂. SDS/PAGE of the preparations of PP1C, PP1 γ and PP2A revealed single polypeptide bands with apparent molecular masses of 35–36 kDa. The molar concentrations of the phosphatases were determined by a titration procedure ([31], but see also [31a]) using microcystin-LR, a tight-binding inhibitor of both PP1 and PP2A [8,15], as the standard. The sample of microcystin-LR used for this purpose was purified from a culture of the green alga *Microcystis aeruginosa* and quantified meticulously by weighing in the laboratory of Dr Ken-ichi Harada (Meijo University, Nagoya, Japan).

Myosin light chain (20 kDa) was purified from chicken gizzard essentially by the method of Hathaway and Haeblerle [32], and it was converted into the 32 P-phosphorylated form (PMLC) using chicken gizzard myosin light-chain kinase isolated by the method of Ngai et al. [33]. 32 P-Phosphorylated phosphorylase *a* was prepared from phosphorylase *b* using phosphorylase kinase by the method of Antoniw et al. [34].

All assays of phosphatase activities were carried out at 25 °C essentially by the procedures described in previous papers ([4,31], but see also [31a]). Briefly, *p*NPP phosphatase activities were measured by monitoring the increase in the absorbance at 405 nm resulting from accumulation of the reaction product *p*-nitrophenol, using either a Microscan MS 96-well microplate reader (Labsystems Oy, Helsinki, Finland) or a U-1100 spectrophotometer (Hitachi, Tokyo, Japan) connected to a pen recorder. The buffer used contained 40 mM Tris (base), 34 mM MgCl₂, 4 mM EDTA (free acid) and 4 mM D,L-dithiothreitol. No bivalent cation other than Mg²⁺ was added. The pH of this solution (used without titration) was 8.4, which is optimal for the *p*NPP phosphatase activities of PP1 and PP2A ([31], but see also [31a]). For isotopic measurements using either PMLC or phosphorylase *a* as substrate we used buffer of the following composition: 40 mM Tris, 100 mM KCl and 2 mM D,L-dithiothreitol (pH 7.4, adjusted with HCl). This solution was supplemented with 1 mM MnCl₂ for the assays of PP1C and PP1 γ .

Analysis of data

Estimation of K_i , K_0 and K_i

In previous experiments [6,15,31,31a,35,36] on the inhibition of protein phosphatases by their tight-binding inhibitors we have shown that the steady-state dose-inhibition relationships at

Scheme 1 Binding of inhibitor to enzyme and its removal by antibody

various enzyme concentrations are well described by a general model function:

$$\zeta = \zeta(I_t) = \frac{E_t - I_t - K_t + \sqrt{(E_t - I_t - K_t)^2 + 4E_t K_t}}{2E_t} \quad (1)$$

where $\zeta(I_t)$ denotes the fractional activity at a given total inhibitor concentration, I_t , and E_t represents the total concentration of the enzyme. Eqn (1) is readily derived using steady-state assumptions, considering the reduction of the free-inhibitor concentration to be caused by binding to the enzyme [37,38].

If E_t is sufficiently smaller than K_t (say, $E_t < 0.1K_t$), then eqn (1) is well approximated by the classical model function:

$$\zeta(I_t) = \left(1 + \frac{I_t}{K_t}\right)^{-1} \quad (2)$$

In the present experiments we estimate the values of K_t by fitting either eqn (1) or eqn (2) to (I_t, ζ) data, depending on the cases.

Generally, K_t is a function of S (or rather of S/K_m):

$$K_t = K_t(S) = K_0 \cdot \left(1 + \frac{S}{K_m}\right) / \left(1 + \frac{K_0}{K_t} \cdot \frac{S}{K_m}\right) \quad (3)$$

When one intends to study the structure–affinity relationship of enzyme inhibitors as we do in this paper, it is desirable to obtain estimates of K_0 since it is independent of the type and concentration of the substrate used. Theoretically, the value of K_0 and that of K_t can be estimated by fitting eqn (3) to a set of (S, K_t) data. It should be noticed, however, that (S, K_t) data have to be collected over a sufficiently wide range of S (say, $0 < S/K_m < 5$) for reliable estimation of K_0 and K_t by this procedure.

It is useful to consider the following three limiting forms of eqn (3). (i) In competitive inhibition, where $K_t \rightarrow \infty$, it reduces to:

$$K_t(S) = K_0 \cdot \left(1 + \frac{S}{K_m}\right) \quad (4)$$

(ii) In (pure) non-competitive inhibition,

$$K_t = K_0 = K_t \text{ (constant)} \quad (5)$$

(iii) In uncompetitive inhibition, $K_0 \rightarrow \infty$, and then eqn (3) converges to:

$$K_t(S) = K_1 \cdot \left(\frac{K_m}{S} + 1\right)$$

Another very useful limiting form of $K_t(S)$ is:

$$\lim_{S \rightarrow 0} K_t(S) = K_0 \quad (6)$$

Thus, K_t approximates closely to K_0 if the substrate concentration is sufficiently low (say $S/K_m < 0.1$).

Estimation of K_a

Suppose that the steady-state reaction velocity at fixed S , E_t and I_t is measured in the presence of various total concentrations (A_t) of an antibody, A, which reversibly binds to the inhibitor, I (Scheme 1). If it is now assumed that the A–I complex has no inhibitory effect on the enzyme, the fractional activity, ζ , is given as a root of the following cubic equation:

$$a\zeta^3 + b\zeta^2 + c\zeta + d = 0 \quad (7)$$

where $a = E_t(K_a - K_t)$, $b = (A_t + E_t)K_t - (E_t - I_t - K_t)(K_a - K_t)$, $c = -K_t(A_t + E_t - I_t + K_a - K_t)$ and $d = -K_t^2$. To solve this equation using the trigonometric formula for roots (see e.g. pp. 102–130 of [39]), we define the following parameters:

$$p = \frac{3ac - b^2}{9a^2}, \quad q = \frac{2b^3 - 9abc + 27ad}{27a^3},$$

$$r = \frac{q}{2|p|^{2/3}}$$

and

$$\theta = \frac{\arccos r}{3} \quad (\text{if } K_a > K_t)$$

or

$$\frac{\arccos r}{3} - \frac{2\pi}{3} \quad (\text{if } K_a < K_t)$$

Then, noting that $0 < \zeta < 1$, we have:

$$\zeta = \zeta(A_t) = -\frac{b}{3a} + 2|p|^{1/2} \cos \theta \quad (8)$$

In the present experiments we measure the dependence of ζ on A_t at fixed E_t and I_t . We then fit eqn (8) to the (A_t, ζ) data obtained and thereby estimate the value of K_a .

Fitting and statistics

The model functions were fitted to data by non-linear least-squares regression with the use of the Origin software, version 6.0 (Microcal Software, Northampton, MA, U.S.A.), which utilizes the Marquardt–Levenberg algorithm (see e.g. p. 302 of [40]). In the fitting we used as weight the reciprocal of the square of the standard error (S.E.) at each value of the independent variable (see pp. 9–15 of [41]). The values of parameters obtained with S.E. by the regression were compared by a modified t test (see pp. 15–18 of [41]). Differences were taken as statistically significant when a two-tailed probability (P) of less than 0.05 was obtained.

RESULTS

Estimation of K_m and V

To estimate the values of K_m and V which were necessary for the dose-inhibition analyses (see below), the initial steady-state activities (v) of the enzyme preparations were measured using various concentrations (S) of pNPP (0.2–20 mM), PMLC (0.5–25 μ M) and phosphorylase a (0.2–20 μ M) as substrates in the absence of inhibitors. Non-linear least-squares regression fitted well the Michaelis–Menten equation to the (S, v) data obtained

Table 1 K_m and V

The enzyme activities were measured using *p*NPP (0.2–20 mM), PMLC (0.5–25 μ M) or phosphorylase *a* (0.2–20 μ M) as substrate. The values of K_m and V were calculated with S.E. by fitting the Michaelis–Menten equation $v = VS/(K_m + S)$ to the (S, v) data by non-linear least-squares regression (results not shown). n denotes the total number of measurements; 1 unit of activity is the amount catalysing the release of 1 μ mol of P_i /min. See the text for further details.

Enzyme	Substrate	K_m (μ M)	V (units/mg of protein)	n
PP1C	<i>p</i> NPP	16000 $\pm$ 2000	0.3 $\pm$ 0.1	66
	PMLC	9 $\pm$ 1	18 $\pm$ 6	60
	Phosphorylase <i>a</i>	7 $\pm$ 1	72 $\pm$ 3	30
PP1 γ	<i>p</i> NPP	2700 $\pm$ 400	10 $\pm$ 1	32
	PMLC	11 $\pm$ 2	17 $\pm$ 2	60
	Phosphorylase <i>a</i>	7 $\pm$ 1	83 $\pm$ 7	30
PP2A	<i>p</i> NPP	4300 $\pm$ 500	96 $\pm$ 7	66
	PMLC	47 $\pm$ 8	17 $\pm$ 4	60
	Phosphorylase <i>a</i>	33 $\pm$ 5	7 $\pm$ 1	30

(results not shown). Table 1 gives the values of K_m and V estimated by the regression.

Using either PMLC or phosphorylase *a* as substrate, we obtained almost the same values of K_m and V for PP1C and PP1 γ (Table 1). This is consistent with the report of Alessi et al. [30] concerning the substrate specificity of these enzymes. When *p*NPP was used as substrate, however, these enzymes gave considerably different values of K_m and V ; the ratios of K_m and V for PP1 γ in relation to those values for PP1C were 0.3 and 30 respectively. Taking advantage of the high activity of recombinant PP1 γ towards the artificial substrate *p*NPP, colorimetric assay methods have been developed for sensitive detection of protein phosphatase inhibitors like microcystins [42,43]. The values of K_m and V for PP2A (Table 2) were within the range as reported in our previous papers [31,31a,35].

Figure 2 Effects of TM and its analogues on PP1C and PP2A

The activities of PP1C (filled symbols) and PP2A (open symbols) were measured in the presence of various concentrations of TM (●, ○), TM1 (■, □), TM2 (▲, △) and 22-*O*-methyl TM1 (◆, ◇), using PMLC (0.2–1 μ M) as the substrate. The activities are normalized to those in the absence of the inhibitors. Solid curves are least-squares fits of eqns (1) or (2). See the text for further details.

Estimation of K_0 and K_i

Figure 2 shows the effects of TM, TM1, TM2 and 22-*O*-methyl TM1 on the PMLC phosphatase activities of PP1C (1–5 nM) and PP2A (0.5–5 nM). TM showed a much higher affinity to PP1C than it did to PP2A, confirming previous observations. Although the affinities to PP1C and PP2A were reduced markedly in all three derivatives examined, TM1 and TM2 still inhibited the enzymes in a dose-dependent manner over the concentration range 10 nM–10 μ M (Figure 2). In contrast, no measurable affinity was retained in 22-*O*-methyl TM1. The assays were

Table 2 Dissociation constants associated with the interaction of protein phosphatases with TM and its analogues

Inhibitory effects of TM and its derivatives were examined on PP1C, PP1 γ and PP2A using *p*NPP, PMLC or phosphorylase *a* as substrate. Eqns (1) or (2) were fitted to each set of (I_i, ζ) data obtained to estimate the value of K_i . We used relatively low concentrations (0.2–1 μ M) of PMLC and phosphorylase *a* (such that $S < 0.1K_m$) to estimate K_0 values as the same as K_i (see eqn 7). For PP1 γ and PP2A, assays were also carried out using different concentrations (0.2–20 mM) of *p*NPP. The (S, K_i) data obtained thereby were used to calculate the values of K_0 and K_i by regression (see Figure 3). The Table includes the ratios of the K_0 values for PP1C and PP1 γ to those for PP2A (1C/2A or 1 γ /2A). The *p*NPP phosphatase activity of PP1C was measured with a fixed concentration (1.6 mM; i.e. $S = 0.1K_m$) of *p*NPP only. The K_i ($\approx K_0$) values for the interaction of OA with PP1C and PP2A obtained by previous experiments are included for comparison. n denotes the total number of measurements. See the text for further explanations.

Compound	Substrate	PP1C		PP1 γ			PP2A			K_0 ratio	
		K_0 (nM)	n	K_0 (nM)	K_i (nM)	n	K_0 (nM)	K_i (nM)	n	1C/2A	1 γ /2A
TM	<i>p</i> NPP	0.32 $\pm$ 0.01	158	0.41 $\pm$ 0.03	3.0 $\pm$ 0.4	209	15 $\pm$ 2	180 $\pm$ 10	312	0.02	0.03
	PMLC	0.28 $\pm$ 0.05	144	0.35 $\pm$ 0.04	—	144	21 $\pm$ 4	—	144	0.01	0.02
	Phosphorylase <i>a</i>	0.36 $\pm$ 0.07	152	0.29 $\pm$ 0.08	—	72	19 $\pm$ 5	—	72	0.02	0.02
TM1	<i>p</i> NPP	—	—	90 $\pm$ 20	340 $\pm$ 90	56	260 $\pm$ 30	2100 $\pm$ 400	217	—	0.4
	PMLC	70 $\pm$ 8	144	97 $\pm$ 7	—	144	270 $\pm$ 20	—	144	0.3	0.4
	Phosphorylase <i>a</i>	65 $\pm$ 5	72	102 $\pm$ 6	—	144	290 $\pm$ 30	—	72	0.2	0.4
TM2	<i>p</i> NPP	—	—	1300 $\pm$ 500	7800 $\pm$ 900	62	160 $\pm$ 20	1150 $\pm$ 90	387	—	8
	PMLC	1090 $\pm$ 90	72	1200 $\pm$ 300	—	72	210 $\pm$ 20	—	144	5	6
	Phosphorylase <i>a</i>	980 $\pm$ 300	72	1300 $\pm$ 200	—	72	190 $\pm$ 30	—	48	5	7
22- <i>O</i> -Methyl TM2	<i>p</i> NPP	> 10 000	38	> 10 000	—	38	> 10 000	—	38	—	—
	PMLC	> 10 000	48	> 10 000	—	48	> 10 000	—	48	—	—
OA	<i>p</i> NPP	150 $\pm$ 30	48	100 $\pm$ 20	110 $\pm$ 30	48	0.032 $\pm$ 0.002	—	213	4700	3200

Figure 3 Dependence of K_i on S

The K_i for the inhibition of the *p*NPP phosphatase activities of PP1 γ (a) and PP2A (b) by TM was determined at various substrate concentrations, S . Vertical bars indicate S.E.M. Eqn (3) was fitted to the data by non-linear least-squares regression with the use of the values of K_m given in Table 2. Elevation of S resulted in a rightward shift of the dose-inhibition relationship (results not shown) and an increase in the K_i . The values of K_0 and K_i estimated by the fitting are listed with S.E. and total numbers of measurements in Table 2. Graphs of eqns (4) and (5) are also shown for comparison (dotted lines). See the text for further explanations.

carried out using low concentrations ($S/K_m \leq 0.1$) of PMLC as the substrate, so that the K_0 values could be directly estimated from the dose-inhibition relationships using the approximation $K_0 \approx K_i$ (see eqn 6). The model functions (eqn 1 for TM and eqn 2 for TM1 and TM2) appeared to fit the (I_i , ζ) data satisfactorily. The K_0 values obtained by the regression are listed in Table 2. Essentially similar dose-inhibition curves (results not shown) and K_i values ($\approx K_0$; Table 2) were obtained when low concentrations of phosphorylase *a* or *p*NPP was used as the substrate. (For the dependence of K_i on the substrate concentration, see below.) PP1 γ (0.1–2 nM) exhibited essentially the same susceptibilities to the inhibitors as did PP1C (Table 2).

The extent of the changes in affinity associated with the modification of the C-16–C-22 segment of TM was substantially larger in PP1 (PP1C and PP1 γ) than in PP2A (Table 2). In PP1, the removal of the side chains (TM1) caused a 200–300-fold increase in the K_0 value, which was further increased 15-fold by the enantiomeric modification of the spiroketal motif (TM2). In PP2A, on the other hand, the K_0 , which was increased 15-fold by the derivatization to TM1, was slightly but significantly decreased by the enantiomerization (TM2). Consequently, the PP1/PP2A

Figure 4 Recovery of the PP2A activity suppressed with TM2 by application of the monoclonal antibody

(a) Dephosphorylation of *p*NPP by PP2A was continuously monitored with a spectrophotometer. The reaction was started by adding *p*NPP (3 mM) to the buffer containing PP2A (2 nM), and after the control activity (dashed line; $I_i = 0$) was measured, TM2 (1 μ M) was added. Addition of the monoclonal antibody (IgG1) in the presence of TM2 resulted in an immediate recovery of the activity. (b) In similar experiments, the steady-state activity at fixed concentrations of enzyme ($E_i = 2$ nM), substrate ($S = 3$ mM) and inhibitor ($I_i = 1$ μ M) was measured after addition of various concentrations of IgG1 ($A_i = 0$ –2 μ M). The values (ζ) normalized to the activity in the absence of the inhibitor and IgG1 are plotted against A_i . The curve is the least-squares fit of eqn (8). The estimate of K_a obtained by the fitting is given in Table 4. See the text for further explanations.

ratio of K_0 , which was in the range 0.01–0.03 with TM, increased to 0.2–0.4 with TM1 and to 5–8 with TM2. Thus the characteristic difference between the affinity of TM to PP1 and that to PP2A became narrower with TM1 and was even inverted with TM2.

As shown in Figure 3, the K_i values for the inhibition of PP1 γ (1 nM) and PP2A (1 nM) increased when the concentration of *p*NPP was increased up to 20 mM. These observations may suggest a mutually extruding interaction of the enzyme and substrate on the enzyme molecule (see Discussion). By fitting eqn (3) to the (S , K_i) data we estimated K_0 and K_i (Table 2). The K_0 values obtained by this procedure agreed well with the estimates obtained by the assays using low concentrations of PMLC or phosphorylase *a* as substrates (Table 2). This seems reasonable since K_0 reflects the affinity of the free enzyme (rather than of its substrate-bound form) to the inhibitor. The finite values of K_i indicate that the binding of the substrate *p*NPP and that of the inhibitors are not completely exclusive (see Discussion).

Reduction of the affinity of TM associated with elevation of the substrate concentration was also observed when PMLC was

Table 3 The affinity of the monoclonal antibody

The values of the dissociation constant K_d for the interaction of the monoclonal antibody (0–2000 nM) with TM and its analogues were estimated from its ability to restore the activity of PP2A against 3 mM *p*NPP in their presence (see Figure 4). The total concentration of PP2A in the reaction mixture was fixed at 2 nM. The K_d value for OA previously estimated by enzyme immunoassay [20] is listed for comparison. n denotes the number of experiments.

Compound	I_i (nM)	K_d (nM)	n
TM	100	$> 10^4$	4
TM1	1000	$> 10^4$	4
TM2	1000	310 ± 10	24
OA	—	0.1	—

Table 4 Recovery of the PMLC phosphatase activity by addition of a higher concentration of IgG1

The activity of the PMLC phosphatase activity of PP2A was measured using 2 μ M PMLC as the substrate in the absence and presence of TM2 (250 nM) and IgG1 (50 μ M). TM2, which inhibited the activity by about 50% in the absence of IgG1, produced no significant inhibition in the presence of 50 μ M IgG1.

TM2 (nM)	IgG1 (μ M)	Activity (units/mg of protein)	n
0	0	0.70 ± 0.05	10
250	0	0.33 ± 0.08	10
250	50	0.68 ± 0.05	10

used as the substrate. When the inhibitory effect of TM on the activities of the phosphatases towards 10 μ M PMLC was examined, the following K_i values were obtained: 0.49 ± 0.06 nM ($n = 72$) for PP1C, 0.52 ± 0.05 nM ($n = 72$) for PP1 γ and 23 ± 5 nM ($n = 56$) for PP2A. The values of K_i for PP1C and PP1 γ were significantly ($P < 0.01$) larger than the K_0 values for the inhibition of these enzymes at the lower substrate concentrations (Table 2). In contrast, elevation of the substrate concentration caused no discernible change in the affinity of PP2A (Table 1). Since K_i is generally a function of S/K_m as defined by eqn (3), the apparently different effects of changing the substrate concentration on the affinity to TM are probably attributable to the difference of the K_m values of the enzymes for the substrate (Table 1).

Estimation of K_d

As shown in Figure 4(a), addition of the IgG1 (1 μ M) caused a partial but significant recovery of the *p*NPP phosphatase activity in the presence of TM2 (1 μ M). Such a phenomenon would most simply be interpreted as resulting from sequestration of the inhibitor by its binding to the antibody (Scheme 1). No recovery of the phosphatase activity was observed when the IgG1 (2 μ M) was added after the activity had been partially inhibited by either TM (100 nM) or TM1 (1 μ M). Therefore the recovering effect is not likely to be produced by a direct interaction of the IgG1 with the enzyme. Figure 4(b) shows the steady-state fractional activity ζ at fixed E_t (1 nM), S (3 mM) and I_i (1 μ M) values as a function of A_i (0–2 μ M). Eqn (8) appears to fit the (A_i , ζ) data satisfactorily. Table 3 gives the dissociation constant estimated by the regression using the values of K_m , K_0 and K_i given in Tables 1 and 2.

In Scheme 1, on which eqn (8) is based, it is assumed that the A–I complex retains no inhibitory effect on the enzyme. One could argue that the binding of the IgG1 might only partially

inactivate the inhibitory effect of TM2 on PP2A, because the fractional activity ζ was only 0.54 even at the highest antibody concentration examined in the experiments, as shown in Figure 4(b). However, we observed that the presence of a higher concentration (50 μ M) of the IgG1 nearly abolished ($\zeta = 0.97$) the inhibition of the PMLC phosphatase activity of PP2A by 250 nM TM2, which inhibited the activity by about 50% in the absence of the antibody (Table 4). [Limited availability of the IgG1 did not allow us to conduct experiments using this concentration of IgG1 in our *p*NPP phosphatase assay system, which required a relatively large volume (> 0.2 ml) of reaction mixture.] If the A–I complex had retained a measurable activity, a recovery of the activity to this extent should not have occurred, no matter how high the concentrations of IgG1 added in the presence of the inhibitor might have been.

In a previous experiment using a similar protocol we showed that the IgG1 recovered the *p*NPP phosphatase activity of PP2A inhibited by OA in a highly time-dependent manner [35]. However, such time-dependence was not observed in the present experiment (Figure 4a), indicating that the dissociation of TM2 from PP2A is a much faster process.

DISCUSSION

The present experiments have shown that TM1 and TM2, both of which lack the two side chains, retain considerable affinities to PP1 (PP1C or PP1 γ) and PP2A. From the K_0 values obtained (Table 2) the standard free-energy change (ΔG) for the binding of TM is estimated, by the equation $\Delta G = RT \ln K_0$, to be -54 kJ/mol for PP1 (PP1C or PP1 γ) and -44 kJ/mol for PP2A. Similar calculations estimate that the percentage of the absolute value of ΔG ($|\Delta G|$) for either of the derivatives to that for TM falls in the range 60–75% with PP1 and 85–90% with PP2A. Considering the relatively large size of the side chains which constitute 15% of the total molecular mass of TM, the reduction of the $|\Delta G|$ caused by their removal seems to be very small. It is known that even slight modifications of the rest of the molecule tend to cause a much more drastic reduction of the affinity [17,18]. The seemingly small extent of the effect of the removal of the side chains may be related to the fact that they are located at the position most distal to the maleic half-acid group, which supposedly interacts with the catalytic centre (see below).

The TM affinity of PP1 is much more liable to be impaired by the modifications of the C-1–C-16 segment of TM than that of PP2A (Table 2). In PP1, the removal of the side chains (TM1) causes a 200–300-fold increase in the K_0 , which is increased further (15-fold) by the enantiomeric modification of the spiroketal motif (TM2). In PP2A, however, the K_0 is increased only 15-fold by the derivatization to TM1 and it is slightly but significantly decreased by the enantiomerization. The wide and characteristic difference between the affinity of TM to PP1 and that to PP2A is therefore substantially narrowed with TM1 and even inverted with TM2 (Table 2). Both the presence of the side chains and the stereochemistry of the spiroketal motif thus appear to be important factors determining the unique affinity characteristics of TM. The inversion of the affinity sequence resulting from the enantiomeric modification is particularly interesting because OA, which has an identical spiroketal motif to that of TM2, is known to exhibit, in contrast to TM or TM1, exceedingly higher affinity to PP2A than to PP1. Chemical modifications of OA involving its spiroketal motif (removal, enantiomerism, methylation etc.) would be very useful for further studies of the relationship between the stereochemistry and affinity characteristics. We have previously shown that

38-methyl OA (also called dinophysistoxin-1) gives a significantly larger PP1/PP2A ratio of K_0 than does OA [6].

According to the structure of a co-crystal of PP1 and microcystin-LR revealed by X-ray analysis [44], the toxin binds to the enzyme in such a way that the circular component of the toxin forms indirect co-ordinate bonds at its carboxylate group and carbonyl oxygen with the catalytic centre, whereas the long non-polar side chain is accommodated in the hydrophobic surface groove which directly extends from the catalytic centre. Although it is not very easy to predict the three-dimensional structure of TM because of its poor crystallizability and flexible backbone structure, semi-empirical calculations incorporating the data of NMR spectroscopy have generated several energetically realistic solution structures of TM, some of which are superimposable on the solution structure of microcystin [18,45]. It has been shown by competition experiments *in vitro* that the binding of microcystin and other phosphatase inhibitors including TM to PP1 or PP2A is mutually exclusive [8,12,15,46]. Moreover, the architecture of the catalytic domain is expected to be closely preserved between PP1 and PP2A on the basis of the sequence data [3]. It therefore seems reasonable to assume that TM binds to PP1 and PP2A essentially in the same manner as microcystin binds to PP1. If so, the maleic half-acid group (and possibly also one or more of the three hydroxy groups) of TM would interact with the catalytic centre, whereas the C-1–C-16 segment would be docked in the hydrophobic groove. The marked difference of the effects of modifying the C-1–C-16 segment on the affinity to PP1 and PP2A observed in the present experiments might imply some structural difference between the enzymes in the hydrophobic groove. It has been shown that the spectrum of inhibitor sensitivity of PP1 is largely changed by mutational substitutions of several amino acid residues near the C-terminus such as Tyr-272, which reside within the active site [47–49]. Mutagenetic experiments targeting the sequence related to the surface groove would also be highly interesting.

We have shown that the K_i increases as the substrate concentration is elevated (see Results). The increase in K_i is clearly observed not only with TM but also with TM1 and TM2. This also supports the notion that the side chains are not directly involved in the interaction with the catalytic centre. As for the *p*NPP phosphatase activities of PP1 γ and PP2A, the K_i converges to a finite value, K_1 , as the substrate concentration tends to a level much higher than the K_m , suggesting that the binding of the substrate *p*NPP and that of the inhibitors are not completely exclusive; or, in other words, that an ESI complex exists. The decrease in $|\Delta G|$ caused by the binding of *p*NPP would be estimated, using the formula $RT\ln(K_i/K_0)$, to be within a narrow range, 3–6 kJ/mol. This may indicate that TM, TM1 and TM2 interact with the catalytic centre in a mutually similar way, leaving a similar binding space for *p*NPP. The present experiments, however, do not decide whether phosphopeptides also bind to the enzyme simultaneously with the inhibitors to give an ESI complex. It is possible that during interaction with the inhibitor the catalytic centre is less accessible to a large phosphopeptide substrate than to a small one like *p*NPP. Indeed, it is known that the *p*NPP activity of type-2B protein phosphatase (calcineurin) is resistant to complexes of some immunophilins and their immunosuppressant ligands, which strongly inhibit the activities of the enzyme towards its phosphopeptide substrates [50].

There are several lines of evidence indicating the essential importance of the hydroxy group at C-22 for the inhibitory action of TM [17,18]. As is often the case with hydrogen bonds, the bond formation between the hydroxy group and its acceptor on the enzyme molecule appears to be highly dependent on the

direction of the hydroxy group as the donor. Kawamura et al. [17] have reported that its epimeric translocation resulted in more than 10^3 -fold increases in the ID_{50} associated with the inhibition of PP1 and PP2A. We have now shown that the affinities of TM1 to PP1 and PP2A are abolished by methylation of the hydroxy group (22-*O*-methyl TM1). The hydroxy group of TM1 is thus likely to keep nearly the same relative position with respect to its acceptor as does that of TM.

Previously Hokama et al. [20] have reported that the IgG1 antibody exhibits a very high affinity to the C-15–C-38 fragment of OA, which retains the spiroketal motif of OA. We have now presented the results that indicate that the IgG1 binds to TM2, though with much lower affinity ($K_a = 310$ nM) than to OA ($K_a = 0.1$ nM), whereas it exhibits no measurable affinities to TM and TM1 (Table 4). The antibody thus appears to recognize the stereochemistry of the spiroketal motif. Since TM2 exhibits no inhibitory effect on PP2A in the presence of a sufficiently high concentration of the IgG1, the binding of IgG1 to the spiroketal motif alone seems to be enough to abolish the inhibitory activity completely. This suggests that the presence of the spiroketal motif is a prerequisite for the interaction of the rest of the molecule with the enzyme, although the spiroketal motif itself is probably not directly involved in the interaction with the catalytic centre (see above).

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