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Inhibition of *myo*-Inositol Monophosphatase Isoforms by Aromatic Phosphonates

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Abstract—α-Hydroxyphosphonates are moderately potent (K_i = 6–600 μM) inhibitors of the enzyme *myo*-inositol monophosphatase (McLeod et al., *Med. Chem. Res.* **1992**, *2*, 96). Hydroxy-[4-(5,6,7,8-tetrahydronaphtyl-1-oxy)phenyl]methyl phosphonate (**3**) was resynthesized and its inhibitory potency towards the recombinant bovine brain enzyme confirmed (K_i = 20 μM). Similar aromatic difluoro-, keto-, and ketodifluorophosphonates (**5**, **7**, **9**) were inactive. Compound **3** was 15-fold less active on the human as compared to the bovine enzyme. Molecular modeling suggested that the hydrophobic part of the inhibitor interacts with amino acid side chains that are located at the interface between the enzyme subunits in an area (amino acids 175–185) with low similarity between the two isozymes. Phe-183 in the human enzyme was replaced with leucine, the corresponding residue in the bovine isoform. The three isozymes (human wild-type, bovine wild-type and human F183L) had similar kinetic properties, except that the bovine enzyme was less effectively inhibited by high concentrations of the activator Mg^{2+}. The F183L mutant enzyme had a twofold increased affinity for compound **3** as compared to the human wild-type form. We conclude that residue 183 contributes to the binding of aromatic hydroxyphosphonates to IMPase, but it is not the only determining factor for inhibitor specificity with respect to different isozymes.

Introduction

myo-Inositol monophosphatase (E.C. 3.1.3.25, IMPase), which hydrolyzes inositol monophosphate isomers to produce free inositol, is an important enzyme within the inositol lipid signaling system. Due to its uncompetitive inhibition by lithium, IMPase was proposed as the target of lithium therapy in bipolar disorder.[1–5] According to the hypothesis, inhibition would lead to a depletion of intracellular inositol pools and reduced resynthesis of membrane inositol lipids. Hyperactive cells, as they may occur in mania, would subsequently be forced to slow down, provided their stimulation is linked to the inositol lipid second messenger system and they have limited access to dietary inositol.[6]

A variety of studies carried out over the last few years have provided important and exhaustive information regarding the structure, mechanism and kinetic properties of *myo*-inositol monophosphatase. Both the bovine and human isoforms have been cloned and produced in large quantities in bacterial expression systems.[7–9] The three-dimensional structure of the enzyme shows a large hydrophilic active site with several acidic residues that are involved in substrate and metal ion binding.[10–12] The enzyme is activated by Mg^{2+} in a nonlinear fashion,[13] which is explained by the requirement for more than one metal ion for substrate binding and catalysis.[9,14–16] Catalytic mechanisms were proposed with roles for two Mg^{2+} ions in binding the phosphate moiety of the substrate, activating a water nucleophile and

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facilitating ester hydrolysis.[12,16,17] More recently, structural and kinetic evidence was provided, which clearly points to a third metal ion contributing to enzyme catalysis.[18,19] Lithium inhibits the phosphatase reaction uncompetitively by binding to an enzyme phosphate complex and preventing the dissociation of inorganic phosphate.[1,20] Even though widely used in the treatment of bipolar disorder, lithium has an unfavorable side effect profile and a very narrow therapeutic window. Consequently, the design and evaluation of novel and selective IMPase inhibitors, both as pharmacological tools and leads for drug development, has received much attention. Most of the active compounds described so far were phosphate, phosphonate or bisphosphonate derivatives.[21–28] Non-hydrolysable bisphosphonic acids, for example, are potent inhibitors with affinities in the nanomolar range,[27] but are highly charged and therefore have low bioavailability in the brain.[29] A promising novel concept of inhibiting *myo*-inositol monophosphatase and other metal containing enzymes based on the chelating properties of the tropolone ring was recently discovered, but the in vivo properties of such compounds still need to be evaluated.[30,31]

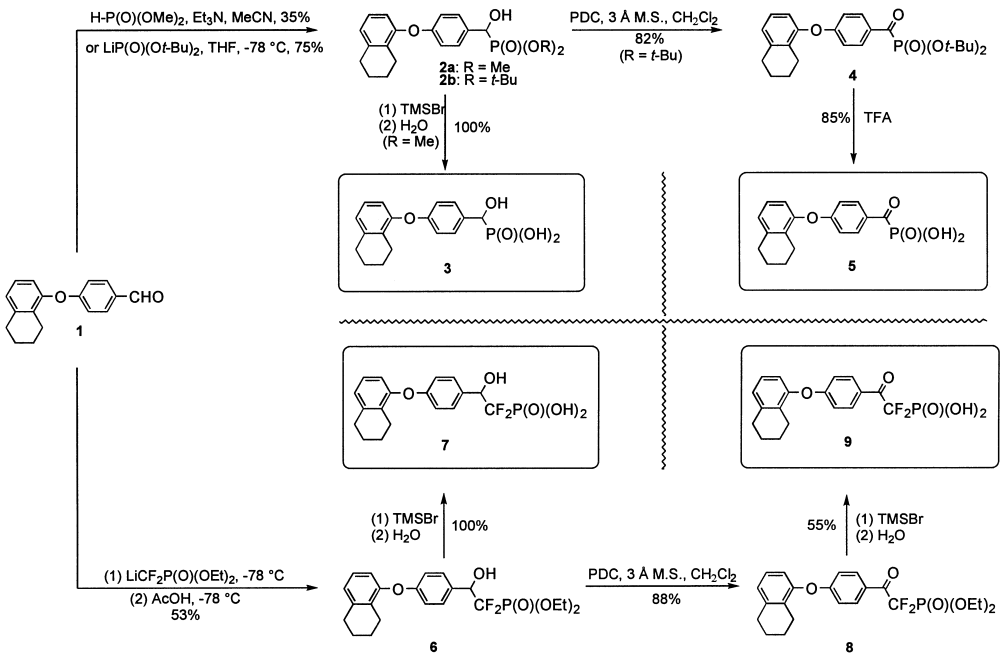
McLeod et al.[24] have synthesized a series of hydroxyphosphonate derivatives, which inhibit the enzyme from bovine brain in the low micromolar range, but have reported no data on the human brain enzyme. These may be interesting compounds since they are not as highly charged as the bisphosphonate series and therefore have a better chance to cross the blood–brain barrier. We have extended their series to include difluoro-, keto-, and ketodifluorophosphonates and report here the effect of these compounds on both bovine and human brain IMPase.

Results and Discussion

Chemistry

Aldehyde **1** was readily obtained from 1-hydroxy-5,6,7,8-tetrahydronaphtalene and *p*-fluorobenzaldehyde under basic conditions (Scheme 1).[24] Reaction with dimethyl or di-*tert*-butyl phosphite in the presence of a base gave the expected adducts **2a** and **2b**, respectively. Dimethyl phosphonate **2a** was transformed into the corresponding acid **3**.[24] Oxidation of di-*tert*-butyl phosphonate **2b** was performed using pyridinium dichromate (PDC) and furnished the oxophosphonate **4** in good yield. This ester was hydrolyzed to the acid by treatment with trifluoroacetic acid.

An analogous strategy was followed for β-hydroxy and β-oxo-α,α-difluorophosphonates **7** and **9**. Thus reaction of aldehyde **1** with diethyl α-lithio-α,α-difluoromethylphosphonate at low temperature led to the formation of the expected adduct 6 in fair yield. Sequentially treating **6** with trimethylsilyl bromide (TMSBr) and water liberated acid **7** in quantitative yield. β-Hydroxy-α,α-difluoromethylenephosphonate **6** was also oxidized by PDC to afford the corresponding oxophosphonate **8** in excellent yield. Deprotection as above ($TMSBr/H_2O$)



Scheme 1. Synthesis of phosphonates **3** and **5** and difluorophosphonates **7** and **9**.

led to the isolation of β-oxo-α,α-difluorophosphonic acid **9**.[32]

Inhibition of bovine and human IMPase

Enzyme activity was measured in a standard assay, where the production of inorganic phosphate is detected through its complexation with ammonium molybdate.[33] With DL-inositol 1-phosphate (Ins(1)P) as the enzyme substrate, neither **3**, **5**, **7**, nor **9**, at a concentration of 0.5 mM, inhibited human recombinant *myo*-inositol monophosphatase (IMPase). Bovine IMPase, on the other hand, was inhibited by **3** with an IC_{50} value of 60 μM. Assuming competitive inhibition and based on a K_m value of 0.1 mM,[9] this corresponds to a K_i of 20 μM, in agreement with the original result ($K_i = 32$ μM) by MacLeod et al.[24] The bovine isoform was not inhibited by 0.5 mM of **5**, **7**, or **9**. Inhibition by **3** was also measured in a continuous photometric assay.[13] Again, the IC_{50} was 60 μM for the bovine enzyme, but less than 20% inhibition was found at 0.5 mM compound with the human isozyme. In order to rule out a possible problem related to the bacterial expression of the human enzyme, we also purified the enzyme from human brain. Tissue and recombinant enzyme had very similar kinetic properties and were both inhibited equally well by lithium ($K_i = 0.2$ mM), but neither one by compound **3**.

Structural interpretation

Previous comparisons of bovine and human IMPase did not indicate any major differences regarding their affinities for substrates, substrate analogues and other inhibitors including a bisphosphonate derivative.[8,29] It therefore appears that the structural difference between the two isozymes that accounts for the difference in inhibition by **3**, is not located within the substrate/phosphate binding pocket. However, it cannot be too distant from the active site, since **3** is a competitive inhibitor versus inositol 1-phosphate.[24]

A molecular model for the bovine IMPase structure was generated based on the 3-dimensional structure of the human enzyme[10] by amino acid replacement followed by energy minimization and inhibitor **3** was tentatively docked into the active site (Fig. 1). For positioning the

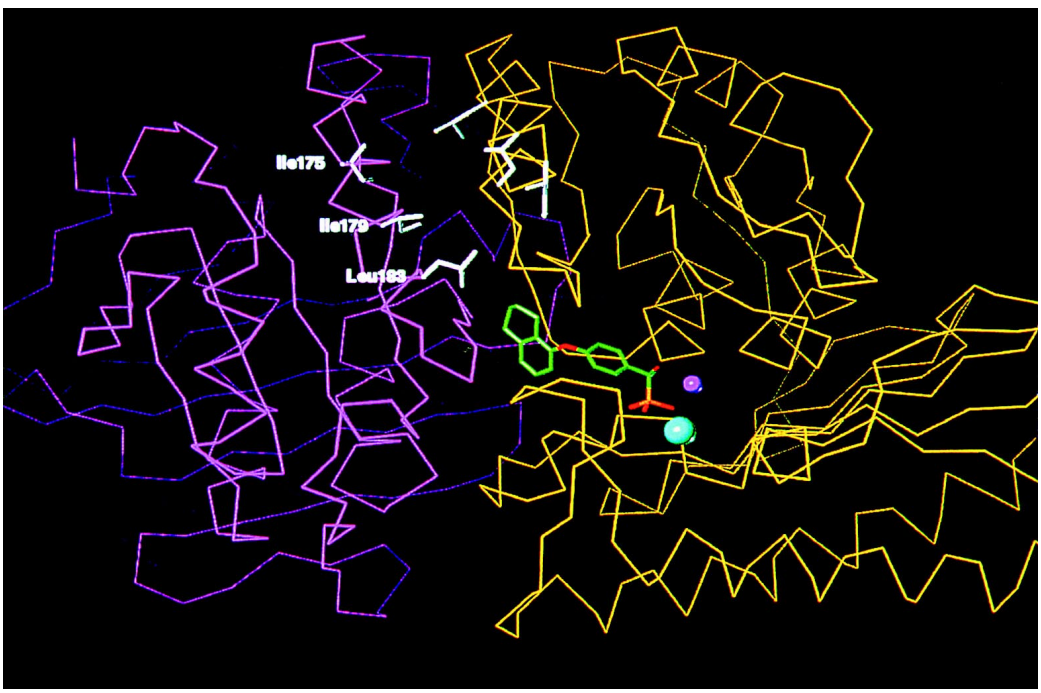


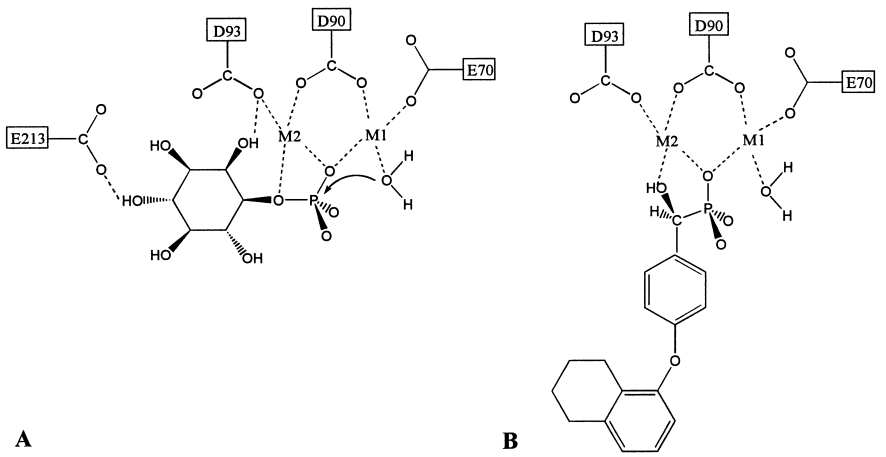
Figure 1. Proposed mode of binding of **3** to the active site of bovine *myo*-inositol monophosphatase. The model of bovine IMPase based on the X-ray structure of the human enzyme[10] is shown with two metal ions bound. A gadolinium ion (blue sphere) occupies metal binding site M1, whereas binding site M2 (magenta sphere) is presumably occupied by lithium, invisible though in the X-ray structure.[11] M1 and M2 bind two Mg^{2+} ions in catalytically active complexes.[16] Compound **3** was placed in the active site in such a way that the phosphonate moiety takes the place of the sulfate ion, which corresponds to the phosphate binding site in enzyme–substrate complexes.[11] In this model, one phosphonate oxygen interacts with both metal ion binding sites and the α-hydroxy group is in contact with M2 (see also Scheme 2B). The hydrophobic part of the inhibitor occupies a pocket that extends from the metal binding sites towards the interface between the two subunits.

hydroxyphosphonate moiety we took into account the information from the published X-ray structures of enzyme complexes with sulfate,[10] inorganic phosphate[12] and inositol 1-phosphate.[11] These as well as kinetic and spectroscopic evidence[9,14,20,34,35] indicate strong interactions between at least two metal ions and the phosphate moiety as main contributors to substrate affinity. Metal ion M1 binds to one of the phosphate oxygens and to two acidic protein residues, Glu-70 and Asp-90. It may also ligate and activate a water nucleophile for in-line attack on the phosphoester[16] (Scheme 2A). Metal ion M2 interacts with two phosphate oxygens including the ester oxygen, as well as three acidic residues, Asp-90, Asp-93, and Asp-220. It should be noted that alternative hydrolytic mechanisms have been proposed with the water nucleophile being activated by M2 or even a third metal ion M3 (not shown in Scheme 2A) for a non-inline attack on the phosphoester.[17,18] We assumed a similar arrangement in our model for hydroxyphosphonate binding with one of the phosphonate oxygens binding to both metal ions and an additional interaction between the α-hydroxy group and metal ion M2 (Scheme 2B). In enzyme–substrate complexes, the 2- and 4-OH groups of the inositol ring bind to enzyme residues in the substrate binding pocket, in particular Asp-93 and Glu-213 (Scheme 2A). In contrast, the aromatic part of **3** points away from this site and into a region, which extends towards the interface between the two enzyme subunits (Fig. 1, Scheme 2B). Other known inhibitors of IMPase most likely exploit interactions similar to those in Scheme 2: chelation of metal ions, hydrogen bonds in the inositol binding site and additional, most likely hydrophobic, interactions in a large empty pocket between the active site and the subunit interface. Inositol phosphate derivatives, where the 6-OH group of the inositol ring had been replaced with long aliphatic/aromatic side chains are excellent inhibitors of *myo*-inositol monophosphatase with K_i values as low as 70 nM.[22] It is reasonable to assume that these side chains occupy the same pocket as the tetrahydronaphtyloxyphenyl group of inhibitor **3**. A similar mode of binding may be proposed for a series of hydroxymethylenebisphosphonic acid derivatives.[26] In contrast, 4-hydroxyphenoxymethylenebisphosphonates, in particular the thoroughly studied compound L690330,[27,29] still have the phosphoester oxygen, instead of the α-hydroxy group. According to Scheme 2, these compounds may rather interact with acidic residues in the inositol binding site such as Glu-213, presumably through hydrogen bonding involving the hydroxyl group in para position of the phenyl ring. Following a different concept, inositol phosphate analogues have been designed, where the 6-OH group of the inositol ring had been replaced with an ethylene glycol side chain, providing an additional chelating interaction with metal ion M2.[23] A totally unrelated class of inhibitors, namely hydroxytropolone derivatives, have recently been described.[30,31] These appear to be very efficient chelators of the Mg^{2+} ions in the active site of IMPase, with the three hydroxyl groups of the tropolone ring superimposing on the water and two phosphate oxygens in Scheme 2A.

Human and bovine IMPase are 85% identical with respect to their primary sequence.[8] However, the percentage of identical amino acids is considerably reduced between residues 174 and 187:

bovine	**170 ETVRI I LSNI ERLLCLPI HGI 190**
human	**170 ETVRMVLSNMEKLFCI PVHGI 190**

The X-ray structure of the human enzyme[10] reveals that this stretch corresponds to the α-helix which is the major contact between the two enzyme subunits. This helix in



Scheme 2. Binding of D-inositol 1-phosphate (A) and proposed binding of **3** (B) to the active site of IMPase. Some interactions are not shown for reasons of clarity.

one subunit is adjacent to the active site of the other subunit. Our docking result suggests that the tetrahydronaphtyl part of **3** may be located closely to the helix mentioned above, and in particular could make a direct hydrophobic contact with residue 183, which is a leucine in the bovine, but a phenylalanine in the human enzyme (Fig. 1). The difference in size of these two residues could be an explanation for the species dependent inhibition by **3**. Alternatively, the differences at the dimer interface might slightly modify the shape of the pocket available for compound **3**.

Mutagenesis

Phe-183 in the human enzyme was replaced with leucine. A summary of all kinetic constants for human and bovine inositol monophosphatase, as well as the F183L mutant is given in Table 1. The mutant enzyme has the same kinetic characteristics as wild-type human IMPase. For example, it is activated by low, but inhibited by high concentrations of Mg^{2+}. The activation phase is non-linear with a Hill coefficient of 2 and the optimal concentration of Mg^{2+} is about 0.5 mM. In agreement with a previous report,[8] the bovine enzyme has a significantly higher K_i value for the inhibition by Mg^{2+}, which is also reflected by a shift of the optimal Mg^{2+} concentration from 0.5 mM for the human to about 1 mM for the bovine isoform. The difference in K_m for Ins(1)P is also significant, but this is probably not due to a higher affinity of the human isozyme for the substrate. Rather it is a consequence of its lower K_i for magnesium, because at 1 mM, the metal ion is already inhibitory with human IMPase. Since inhibition is uncompetitive with respect to Ins(1)P[13], this causes the substrate K_m to drop. In fact, at 0.5 mM Mg^{2+}, the K_m of the human enzyme for Ins(1)P increases by two–threefold.

Table 1. Kinetic and inhibition constants of IMPase isozymes at pH 7.5 and 37 °C

Constant[a]	Human	Isozyme F183L	Bovine
$k_{cat}(s^{-1})$[b]	22	31	26
$A^{0.5}$(mM)[b]	0.16	0.12	0.2
A_{opt}(mM)[c]	0.5	0.5	1.0
n[b]	1.8	2.0	1.8
K_m(mM)[d]	0.03	0.07	0.1
$K_{i,Mg}$(mM)[b]	2.1	1.5	5.2

[a]Standard errors were below 25% for all constants.
[b]k_{cat} (turnover number), $A_{0.5}$ (the concentration of magnesium giving half-maximal velocity, n (Hill coefficient), and $K_{i,Mg}$ (Mg^{2+} inhibition constant) were obtained by varying the concentration of Mg^{2+} over a wide range at 0.5 mM Ins(1)P. Data were fitted by the Hill equation, modified to account for inhibition at higher concentrations of magnesium.[13] $v = k_{cat}[E][Mg^{2+}]^n/(A_{0.5}{}^n + [Mg^{2+}]^n(1 + [Mg^{2+}]/K_i))$. [E], the total enzyme concentration, was determined from the UV spectrum.[40]
[c]Optimal Mg^{2+} concentration, estimated from the Mg^{2+} activation/inhibition curve.
[d]Michaelis constant for Ins(1)P. The substrate concentration was varied over a ninefold range up to 0.5 mM at 1 mM Mg^{2+}, initial velocities were fitted by the Michaelis–Menten equation.

A different assay system with 4-nitrophenyl phosphate as the substrate was used to compare the inhibition of the different isozymes by **3**. 4-Nitrophenyl phosphate was previously characterized as a substrate of IMPase.[9,33] The assay is more precise and less susceptible to interference with higher inhibitor concentrations than the discontinuous measurement using molybdate complexation of inorganic phosphate or the multicomponent coupled systems. The K_m values for 4-nitrophenyl phosphate and the IC_{50} values for **3** are summarized in Table 2. The apparent affinities for the inhibitor are about threefold higher than in the assay systems with inositol 1-phosphate. This is due to the much higher concentrations of Mg^{2+} that have to be used with the less efficient substrate.[13] (For example, the K_i for human IMPase with 4-nitrophenyl phosphate as the substrate increases from 75 μM at 5 mM $MgCl_2$ to about 200 μM at 1 mM Mg^{2+}.) As a consequence, inhibition of the human enzyme now becomes more easily detectable, but the IC_{50} value is still about 15-fold higher as compared to the bovine isoform.

In order to correctly compare the affinity of **3** for the different enzymes, a complete inhibition study was carried out to determine K_i values (Table 2). Inhibition by **3** versus 4-nitrophenyl phosphate was competitive with bovine and human IMPase, as well as F183L (data not shown). The K_i with the bovine enzyme was 15-fold

Table 2. Inhibition of IMPase isozymes by compound **3** at pH 7.5 and 37 °C with 4-nitrophenyl phosphate as the substrate

Isozyme	K_m,[a] mM	IC_{50},[b] μM	K_i,[c] μM
Bovine	2.0 ± 0.2	20 ± 2	5.0 ± 0.4
Human	1.4 ± 0.1	310 ± 40	75 ± 9
F183L	2.0 ± 0.2	75 ± 10	36 ± 2
F183A	1.8 ± 0.2	150 ± 10	n.d.[d]

[a]The substrate concentration was varied over a ninefold range up to 10 mM at 5 mM $MgCl_2$.
[b]IC_{50} values were determined by varying the inhibitor concentration between 0 and 200 μM at 5 mM $MgCl_2$, and 2 mM 4-nitrophenyl phosphate.
[c]K_i values were determined by varying the substrate over a ninefold range up to 10 mM at 5 mM $MgCl_2$ and different, fixed concentrations of inhibitor (0, 25, 50 and 100 μM with bovine IMPase; 0, 100, 200, and 400 μM with human IMPase; 0, 50, 100, and 200 μM with F183L). Data were fitted with the program COMP.[41]
[d]Not determined.

lower as compared to human IMPase. The F183L mutation increases the affinity of the human enzyme for **3** by about twofold. This result shows that our original hypothesis was correct and residue 183 is somehow involved in the binding of **3**. However, it is only in part responsible for the difference between human and bovine IMPase regarding the inhibition by α-hydroxyphosphonates. Other amino acids must be involved either through a direct interaction with the inhibitor or indirectly by increasing its interaction with F183. It may also be possible that the size of Phe-183 interferes with the binding of **3** in the human enzyme. However, replacing phenylalanine 183 with alanine was less effective than the leucine mutation (Table 2). An attempt to produce more 'bovine-like' double mutants by introducing additional mutations at positions 175 and 179 was equally unsuccessful (data not shown). This is not too surprising, since the distance between these two residues and the phosphate binding site in the published X-ray structure is about 19 and 24 Å, as compared to about 12 Å for Phe-183 (Fig. 1).

Conclusion

The α-hydroxyphosphonate **3** is a moderately potent inhibitor of bovine *myo*-inositol monophosphatase, but much less efficient on the human isozyme. This is a somewhat surprising result, since such differences are not found with other substrates or substrate analogues. We propose that the hydrophobic side chain of **3** points towards the interface between the two enzyme subunits and that residue 183 at this interface is in part responsible for the species selectivity. A F183L mutant has an increased affinity for the inhibitor as compared to wild-type human IMPase. The difference is about twofold and consistent with an increase in hydrophobic binding due to one additional methylene unit.[36,37] It therefore appears that a leucine in this position contributes to the affinity of **3** for bovine IMPase through a favourable hydrophobic interaction with the tetrahydronaphtyl moiety of the inhibitor.

Experimental

Materials

DL-Inositol 1-phosphate was purchased from Bachem and 4-nitrophenyl phosphate from Sigma. All reagents for the polymerase chain reaction were obtained from Perkin Elmer. The PCR products were purified using the Gene-Clean kit (Bio 101). Restriction enzymes, other DNA modifying enzymes and corresponding buffers plus the vector pUC19 were from New England Biolabs. The vector, pT7.6, containing the promotor for T7 RNA polymerase plus ampicillin resistance and used for expression of the mutant protein was a gift from Stan Tabor (Harvard University). All oligonucleotides were synthesized with an Applied Biosystems Model 381A DNA synthesizer using phosphoramidate chemistry. The Applied Biosystems automated DNA sequencer Model 373A and the corresponding Taq di-deoxy terminator sequencing kit were used for sequencing and the analysis was aided by the University of Wisconsin Genetic Computer Group program. The components for the growth and propagation of all the *E. coli* strains were obtained from Difco (Detroit, USA) or Sigma. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Clonetech (Ozyme). The competent strain of *E. coli* JM109 was supplied by Stratagene. The strain for expression of the proteins was BL21(DE3)pLys S and was donated by Raymond Leppick. Unless otherwise stated, chemical reagents were obtained from commercial sources and used without further purification. Tetrahydrofuran (THF) was distilled under nitrogen from sodium/benzophenone immediately *prior* to use. Di-*iso*-propylamine and triethylamine were distilled from calcium hydride and stored under nitrogen over 3 Å molecular sieves. Reactions involving LDA and TMSBr were conducted under an inert atmosphere. Drying of the organic extract was carried out using $MgSO_4$. Chromatography was performed using Merck 60 230-400 mesh silica gel according to the procedure published by Still (abbreviation for the elution solvents are the following: H = heptane, C = methylene chloride, A = ethyl acetate, E = diethyl ether).[38] Unless otherwise stated, ^{1}H NMR, ^{19}F NMR, proton-decoupled ^{31}P NMR, and proton-decoupled ^{13}C NMR spectra were recorded in deuterated chloroform at 200, 188, 81, and 50 MHz, respectively; chemical shifts are expressed in ppm downfield from internal or external tetramethylsilane, hexafluorobenzene, phosphoric acid and deuterated chloroform, respectively.

Chemistry

4-(5,6,7,8-Tetrahydronaphtyloxy)benzaldehyde (1). To a slurry of sodium hydride (400 mg, 10 mmol, 60% grade in mineral oil, prewashed under argon with pentane (3×3 mL) in dimethylformamide (DMF) (25 mL) was added at room temperature 1-hydroxy-5,6,7,8-tetrahydronaphtalene (1.24 g, 10 mmol) in portions over a 5 min period of time. The resultant mixture was stirred for 30 min and 4-fluorobenzaldehyde (1.24 g, 10 mmol) was added dropwise. The solution was heated at 70 °C for 2 h and cooled down. DMF was evaporated under reduced pressure and the residue was treated with water and extracted with methylene chloride (CH_2Cl_2) (3×20 mL). Drying of the combined organic extracts and evaporation of the volatiles left a crude product, which was chromatographed and eluted with H/C (75/

25) to give the desired product as a colorless oil (1.83 g, 67%). ^{1}H NMR: δ 1.70–1.88 (m, 4H), 2.55–2.62 (m, 2H), 2.80–2.88 (m, 2H), 6.78–7.20 (m, 5H), 7.78–7.88 (m, 2H), 9.90 (s, 1H).

Dimethyl 1-[4-(5,6,7,8-tetrahydronaphtyloxy)phenyl]-1-hydroxymethylphosphonate (2a). A mixture of aldehyde **1** (756 mg, 3 mmol), dimethylphosphite (363 mg, 3.3 mmol) and triethylamine (303 mg, 3.3 mmol) in acetonitrile (6 mL) was refluxed for 18 h. The volatiles were evaporated and the residue was treated with water. Extraction of the resultant aqueous phase with CH_2Cl_2 (3×20 mL), drying of the organic phases and evaporation afforded a crude yellow oil, which was subjected to chomatography. Elution with ethyl acetate delivered 350 mg of colorless, oily product **2a** (32%). ^{1}H NMR: δ 1.72–1.79 (m, 4H), 2.6–2.7 (m, 2H), 2.78–2.88 (m, 2H), 3.68 (d, 3H, J = 8.4 Hz), 3.73 (d, 3H, J = 8.4 Hz), 4.23 (dd, 1H, J = 5.6, 9.5 Hz), 5.01 (dd, 1H, J = 5.6, 10.4 Hz), 6.71 (d, 1H, J = 8.0 Hz), 6.87–6.93 (m, 3H), 7.06 (t, 1H, J = 7.8 Hz), 7.38–7.48 (m, 2H); ^{31}P NMR: δ 24.26 (s, 1P). M.S. (C.I./NH_3): 380 ($M + NH_4^+$), 363 ($M + H^+$).

Di-*tert*-butyl 1-[4-(5,6,7,8-tetrahydronaphtyloxy)phenyl]-1-hydroxymethylphosphonate (2b). Di-*tert*-butyl phosphite (776 mg, 4 mmol) was added dropwise to a cooled (−78 °C) THF/hexanes solution of LDA (4 mmol), prepared at 0 °C (30 min of stirring) from di*iso*propylamine (405 mg, 4 mmol) in THF (6 mL) and *n*-butyllithium (2.5 mL of a 1.6 N solution in hexanes, 4 mmol). The resultant milky mixture was stirred for 45 min, after which a THF (2 mL) solution of aldehyde **1** (1009 mg, 4 mmol) was added drop by drop. After stirring for an additional 20 min at the same temperature, the mixture was warmed up to room temperature and stirring was continued for 1 h. The solution was poured into brine (40 mL) extracted with diethyl ether (3×20 mL) and the combined extracts were dried. Evaporation and chromatography (elution: H/E (1/4)) gave 1.38 g of product **2b**, which was recrystallized from C/H (1.34 g in 3 crops, 75%). Melting point 150–151 °C. ^{1}H NMR: δ 1.41 (s, 9H), 1.45 (s, 9H), 1.72–1.79 (m, 4H), 2.6–2.7 (m, 2H), 2.78–2.88 (m, 2H), 4.41 (dd, 1H, J = 5.0, 11.8 Hz), 4.81 (dd, 1H, J = 5.0, 9.5 Hz), 6.68 (d, 1H, J = 7.3 Hz), 6.87–6.93 (m, 3H), 7.04 (t, 1H, J = 7.8 Hz), 7.38–7.48 (m, 2H); ^{31}P NMR: δ 14.45 (s, 1P). M.S. (C.I./NH_3): 464 ($M + NH_4^+$), 447 ($M + H^+$).

Diethyl 1-[4-(5,6,7,8-tetrahydronaphtyloxy)phenyl]-2-hydroxy-1,1-difluoroethylphosphonate (6). To a THF/hexanes solution of LDA (3.8 mmol), prepared as above and cooled at −78 °C, was added dropwise a THF (2 mL) solution of diethyl difluoromethylphosphonate (716 mg, 3.8 mmol) and stirring was continued for 30 min. To the resultant orange solution was added a THF solution of aldehyde **1** (960 mg, 3.8 mmol). After 1 h of additional stirring, glacial acetic acid (229 mg, 3.8 mmol) was added and the solution was warmed to room temperature. It was then poured into brine (40 mL) and extracted with CH_2Cl_2 (3×20 mL). The combined organic extracts were dried and evaporated to give a crude reddish oil, which was chromatographed. Elution H/E (65/35, then 7/3) furnished 1.12 g of product **6**, recrystallized from C/H (1.01 g, 53%). Melting point 134–135 °C. ^{1}H NMR: δ 1.28–1.39 (m, 6H), 1.70–1.82 (m, 4H), 2.6–2.7 (m, 2H), 2.78–2.88 (m, 2H), 4.14–4.34 (m, 5H), 4.98–5.20 (dm, 1H, J = 20.3 Hz), 6.72 (d, 1H, J = 8.2 Hz), 6.88–6.96 (m, 3H), 7.08 (t, 1H, J = 7.8 Hz), 7.44 (d, 2H, J = 8.6 Hz); ^{19}F NMR: δ 36.43 (ddd, 1F, J = 20.3, 105.6, 303 Hz), 47.30 (ddd, 1F, J = 6.6, 100.6, 303 Hz). ^{31}P NMR: δ 7.59 (dd, 1P, J = 100.1, 105.1 Hz). MS (C.I./NH_3): 458 ($M + NH_4^+$), 441 ($M + H^+$).

Diethyl 1-[4-(5,6,7,8-tetrahydronaphtyloxy)phenyl]-2-oxo-1,1-difluoroethylphosphonate (8). A mixture of 2-hydroxy-1,1-difluorophosphonate **2b** (440 mg, 1 mmol), pyridinium dichromate (PDC, 752 mg, 2 mmol) and 3 Å molecular sieves (440 mg, beads, 8–12 mesh) in CH_2Cl_2 (10 mL) was stirred for 48 h at room temperature. Diethyl ether (20 mL) was then added while stirring and the mixture was filtered through a plug of celite. Evaporation of the volatiles and chromatography of the residual orange oil (elution: H/E (2/3)) led to the isolation of 388 mg of colorless, oily product (88%). ^{1}H NMR: δ 1.28–1.37 (t, 6H, J = 6.6 Hz), 1.70–1.85 (m, 4H), 2.52–2.62 (m, 2H), 2.78–2.88 (m, 2H), 4.24–4.44 (m, 4H), 6.82 (d, 1H, J = 7.8 Hz), 6.88–6.96 (m, 2H), 6.99 (d, 1H, J = 6.8 Hz), 7.14 (t, 1H, J = 7.7 Hz), 8.11 (d, 2H, J = 9.0 Hz); ^{19}F NMR: δ 51.95 ppm (d, 1F, J = 96.4 Hz); ^{31}P NMR: δ 4.34 (t, 1P, J = 96.4 Hz). M.S. (C.I./NH_3): 439 ($M + NH_4^+$), 457 ($M + H^+$).

Ditert-butyl 1-[4-(5,6,7,8-tetrahydronaphtyloxy)phenyl]-1-oxo-methylphosphonate (4). The procedure hereabove was used starting from di-*tert*-butyl 1-hydroxyphosphonate **2b** (981 mg, 2.2 mmol). Chromatography and elution (H/E (3/7) gave 802 mg of light-yellow, oily product (82%). ^{1}H NMR: δ 1.54 (s, 18H), 1.72–1.79 (m, 4H), 2.53–2.63 (m, 2H), 2.78–2.88 (m, 2H), 6.82 (d, 1H, J = 7.8 Hz), 6.87–6.94 (m, 2H), 6.98 (d, 1H, J = 7.8 Hz), 7.13 (t, 1H, J = 7.8 Hz), 8.22–8.32 (m, 2H); ^{31}P NMR: δ–7.0 (s, 1P).

1-[4-(5,6,7,8-Tetrahydronaphtyloxy)phenyl]-1-oxomethylphosphonic acid (5). Di-*tert*-butyl 1-oxophosphonate **4** (223 mg, 0.5 mmol) was dissolved in trifluoroacetic acid (5 mL) and the solution was stirred for 2 h at room temperature. Removal of the solvent under reduced pressure left 148 mg of pure product (1H and ^{31}P NMR spectroscopy) (83%). Melting point 164–165 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.68–1.80 (m, 4H), 2.50–

2.60 (m, 2H), 2.75–2.85 (m, 2H), 6.78 (d, 1H, $J=7.8$ Hz), 6.82–6.92 (m, 2H), 6.97 (d, 1H, $J=7.6$ Hz), 7.11 (t, 1H, $J=7.7$ Hz), 8.10–8.32 (m, 2H); ^{31}P NMR ($CDCl_3$/DMSO-d_6): δ 1.0 (br. s, 1P). MS (C.I./NH_3): 350 ($M+NH_4^+$). Anal. calcd for $C_{17}H_{17}O_5P$: C: 61.45; H: 5.16. Found: C: 60.96; H: 5.13.

Dealkylation using TMSBr/H_2O sequence. Trimethylsilyl bromide (3.3 equiv for **2a** and **6**; 2.2 equiv for **8**) was added at room temperature to a solution of starting dialkyl phosphonate (1 equiv) in acetonitrile (4 mL per mmol of starting material). Stirring was continued for 24 h and the solution was then concentrated by evaporation of the volatiles under reduced pressure. The residue was taken up with toluene (3 mL) and evaporated; this was repeated twice to ensure the complete removal of any HBr. The residual oil was dissolved in acetonitrile (3 mL) and water was (3 mL) added. After 16 h of stirring, the solution was evaporated to furnish the pure product.

1-[4-(5,6,7,8-Tetrahydronaphtyloxy)phenyl]-1-hydroxymethylphosphonic acid (3). The reaction was carried out on a 0.5 mmol scale; 167 mg (100%) of creamy powder. Melting point 153–154 °C. ^{1}H NMR (CD_3CN/DMSO-d_6): δ 1.70–1.80 (m, 4H), 2.54–2.64 (m, 2H), 2.72–2.82 (m, 2H), 4.75 (d, 1H, $J=12.5$ Hz), 6.68 (d, 1H, $J=7.2$ Hz), 6.82 (d, 2H, $J=8.5$ Hz), 6.90 (d, 1H, $J=7.7$ Hz), 7.07 (t, 1H, $J=7.7$ Hz), 7.35–7.45 (m, 2H); ^{13}C NMR (CD_3CN/DMSO-d_6): δ 23.23, 23.42, 23.99, 29.99, 70.94 (d, $J=161$ Hz), 117.21, 118.34, 125.87, 127.15, 129.83, 129.93, 134.29, 140.43, 154.85, 158.04; ^{31}P NMR (CD_3CN/DMSO-d_6): δ 22.30 (br, s, 1P). Anal. calcd for $C_{17}H_{19}O_5P$: C: 61.08; H: 5.73. Found: C: 60.25; H: 5.52.

1-[4-(5,6,7,8-Tetrahydronaphtyloxy)phenyl]-2-hydroxy-1,1-difluoroethylphosphonic acid (7). The reaction was carried out on a 0.5 mmol scale; 199 mg (100%) of white solid. Melting point 165–166 °C. ^{1}H NMR (CD_3CN/DMSO-d_6): δ 1.70–1.80 (m, 4H), 2.55–2.65 (m, 2H), 2.74–2.84 (m, 2H), 5.05 (dd, 1H, $J=5.0$, 22.7 Hz), 6.71 (d, 1H, $J=7.9$ Hz), 6.84 (d, 2H, $J=8.7$ Hz), 6.93 (d, 1H, $J=7.5$ Hz), 7.10 (t, 1H, $J=7.8$ Hz), 7.40 (d, 2H, $J=8.5$ Hz); ^{13}C NMR (CD_3CN/DMSO-d_6): δ 23.23, 23.44, 23.99, 29.99, 73.23 (m), 117.06, 118.31, 126.10, 127.21, 130.15, 130.84, 131.74, 140.55, 154.66, 158.94; ^{19}F NMR (CD_3CN/DMSO-d_6): δ 34.35 (ddd, 1F, $J=22.7$, 101.0, 297.4 Hz), 47.30 (ddd, 1F, $J=4.2$, 96.4, 297.4 Hz); ^{31}P NMR (CD_3CN/DMSO-d_6): δ 7.64 (t, 1P, $J=99.0$ Hz). Anal. calcd for $C_{18}H_{19}F_2O_5P.0.75H_2O$: C: 54.34; H: 5.19. Found: C: 54.71; H: 5.14.

1-[4-(5,6,7,8-Tetrahydronaphtyloxy)phenyl]-2-oxo-1,1-difluoroethylphosphonic acid (9). The reaction was carried out on a 0.6 mmol scale; 128 mg (55%) of white solid after recrystallization from chloroform/heptane. Melting point 186–187 °C. ^{1}H NMR ($CDCl_3$/DMSO-d_6): δ 1.70–1.82 (m, 4H), 2.52–2.62 (m, 2H), 2.78–2.88 (m, 2H), 6.80 (d, 1H, $J=7.8$ Hz), 6.86–6.93 (m, 2H), 6.93 (d, 1H, $J=7.2$ Hz), 7.13 (t, 1H, $J=7.7$ Hz), 8.16 (d, 2H, $J=8.9$ Hz); ^{19}F NMR ($CDCl_3$/DMSO-d_6): δ 50.52 ppm (d, 1F, $J=92.4$ Hz); ^{31}P NMR ($CDCl_3$/DMSO-d_6): δ 2.62 (t, 1P, $J=93.1$ Hz). MS (C.I./NH_3): 439 ($M+NH_4^+$), 457 ($M+H^+$). Anal. calcd for $C_{18}H_{17}F_2O_5P$: C: 56.55; H: 4.48. Found: C: 56.45; H: 4.38.

Molecular modeling

Modeling was done using Sybyl 5.32. (Tripos Ass., St. Louis, MO 62177). A model for the bovine IMPase structure was generated by amino acid replacement in the X-ray structure of the human enzyme, followed by energy optimization with the Kollman all atom parameter set for 200 steps using a conjugate gradient minimizer. Docking of **3** in the active site was done manually and aimed at optimizing the interactions of the phosphonate group with the bimetallic center in the enzyme and at avoiding unfavorable steric contacts. It is obvious that the resulting complex is tentative but useful for giving a possible explanation for the species differences which could be tested experimentally.

Enzyme assays

For the determination of IC_{50} values and with inositol 1-phosphate as the substrate, release of inorganic phosphate was measured in a discontinuous assay, based on the method by Attwood et al.[33] The enzyme (5–10 pmol) was incubated at 37 °C in 50 mM Tris–Cl, pH 7.5, in the presence of 0.2 mM ($2\times K_m$) inositol 1-phosphate, 0.5 mM (with human IMPase) or 1 mM (bovine IMPase) $MgCl_2$, and different concentrations of inhibitor. The total assay volume was 0.5 mL. Aliquots of 100 μL were removed at different time points and added to 100 μL of a phosphate reagent in a microtiter plate. The absorbance at 340 nm was determined in a plate reader (Dynatech MR 5000) and the absorbance change per minute calculated by linear regression. Alternatively, in particular for the more detailed kinetic studies (Table 1), continuous, coupled assay systems were used.[9,13] With 4-nitrophenyl phosphate, the formation of nitrophenolate was monitored at 400 nm ($\varepsilon=16.7\times10^3\ M^{-1}\ cm^{-1}$).

Mutagenesis

The wild-type clone of human *myo*-inositol monophosphatase was obtained by RT-PCR as previously described for the bovine enzyme.[9] Oligonucleotides were based on the published sequence for the human isoform.[8]

The mutant clones were prepared by PCR using the technique of overlap extension[39] and the GeneAmp reagents (Perkin Elmer) according to the manufacturer's instructions. For F183L the following oligonucleotide primers were used: P1 (5′-CTAATATGGAAAAGCT-**T**CTTTGCATTCCTGTT-3′) and P2 (5′-GAACAG-GAATGCAAA**G**AAGCTTTTCCATATTAG-3′) are complementary and contain the mutant sequence as noted in bold print. P3 (5′-**GAGCTCCTGCAG**A-GGAGGAATATAAT*ATG*GC-TGATCCTT-3′) and P4 (5′-**GTCGACTCTAGA**TTAATCTTCGTCGTC-3′) flank the entire coding sequence of the human *myo*-inositol monophosphatase cDNA plus restriction sites for subcloning purposes (outlined in bold print). P3 also contains a ribosome binding site with spacer for prokaryotic expression (underlined) and the start codon, *ATG*. The same strategy was applied for the other mutants: P3 and P4 were kept the same and P1 and P2 designed according to the desired target sequence. The full length mutated DNA was digested with Pst I and Xba I and subcloned first into the vector pUC19 for sequencing and subsequently into the expression vector, pT7.6. The proteins were expressed in *E. coli* strain BL21(DE3)pLysS and purified to apparent homogeneity as previously described for the bovine enzyme.[9] The concentrations of the purified enzymes were determined from the UV spectra using an extinction coefficient $\varepsilon = 26\ 200\ M^{-1}\,cm^{-1}$.[40] Specific activities were 15 U/mg for the wild-type human enzyme, 24 U/mg for F183L, 19 U/mg for F183A, 19 U/mg for F183L/M179I, 18 U/mg for F183L/V175I, and 24 U/mg for the bovine enzyme. They were determined in a standard assay with 4 mM 2-glycerophosphate and 2 mM $MgCl_2$ at pH 7.5 and 37 °C.[33]

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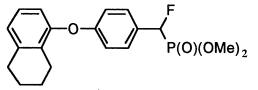
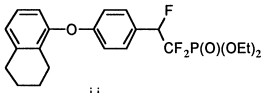
with diethylaminosulfur trifluoride (DAST) at room temperature; however the products could not be converted to the corresponding acids. No reaction was observed between compound **8** and DAST.



i



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