Spatial Conformation and Topography of the Tyrosine Aromatic Ring in Substrate Recognition by Protein Tyrosine Kinases†

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Received October 25, 2005

The side chain orientation of the tyrosine residue included in a peptide, which is an excellent substrate of Syk tyrosine kinase, was fixed in different conformations by either incorporating the tyrosine in cyclic structures (6-OH-Tic, 5-OH-Aic, and Hat derivatives) or adding a sterically bulky substituent in the tyrosine side chain moiety (β-MeTyr). Synthetic peptides containing tyrosine analogues displaying different side chain orientations were analyzed by NMR techniques and tested as potential substrates of the nonreceptor tyrosine kinases Syk, Csk, Lyn, and Fyn. The "rotamer scan" of the phosphorylatable residue generated optimal substrates in terms of both phosphorylation efficiency and selectivity for Syk tyrosine kinase, while the peptidomimetics were not recognized by the other tyrosine kinases. In particular, L-β-MeTyr and D-Hat containing peptides resulted to be both suitable substrates for the specific monitoring of Syk and consensus sequence scaffolds for the design of potential inhibitors highly selective for this tyrosine kinase.

Introduction

Protein phosphorylation is one of the main post-translational modifications that regulate many vital cellular processes. In particular, protein tyrosine kinases (PTKs) catalyze the transfer of the γ-phosphoryl group of ATP to the tyrosine residues of proteins, and the investigation of the molecular bases for PTK recognition is an active area of research in cell signal transduction, which regulates cell cycle, growth, and differentiation.[1] Defining the structural requirements of natural and unnatural PTK substrates, which enable the recognition by the kinase catalytic domains, has proven to be helpful in piecing together signaling pathways. In addition peptides containing specific consensus sequences are potentially useful inhibitory compounds that could get insights into the biological role of signaling proteins. While the properties of the PTK nucleotide binding site are well-studied and used to generate highly specific inhibitors, relatively little is known about features and topography of the phosphorylatable tyrosine side chain that might enhance or counteract the enzyme binding and/or catalysis.

A strategy to increase the selectivity of PTK peptide substrates and, at the same time, to study the preferred topography of the target tyrosine side chain could take advantage of the finding that the introduction of local or global constraints in the phosphorylatable residue is variably tolerated by the different classes of tyrosine kinases (ref 2 and references therein).

We have previously demonstrated that the octapeptide EDDEYEEV is an excellent substrate of the nonreceptor tyrosine kinase Syk,[3] which displays a marked preference for tyrosine residues located in a highly acidic sequence.[4] On the other hand, most PTKs do not display specificities strictly dependent on the sequence containing the phosphoacceptor site, but recognize their targets through adhesion modules located outside the catalytic domain.[5] In the present paper we describe a series of derivatives of the peptide EDDEYEEV, which contain various conformationally restricted analogues of the phosphorylatable tyrosine (Tyr5) (Figure 1).

Local conformational restriction of the side chain mobility was achieved by replacement of Tyr5 with β-methyltyrosine (β-MeTyr), whereas substitution of 5-hydroxy-2-aminoindan-2-carboxylic acid (5-OH-Aic), 6-hydroxy-2-aminotetraline-2-carboxylic acid (Hat), or 6-hydroxy-tetrahydro isoquinoline-3-carboxylic acid (6-OH-Tic) resulted in the conformational restriction of both the side chain and the peptide backbone. To distinguish whether the interaction of 5-OH-Aic or Hat derivatives with the enzyme was influenced by the introduced side chain conformational constraint per se or by the local restriction of the peptide backbone around the 5-position, the analogue containing L-C$^{\alpha}$-methyltyrosine (L-α-MeTyr) was synthesized. On the other hand, previous studies showed that the substitution of an *N*-methyl-tyrosine residue for the tyrosine residue has a deleterious effect on the substrate recruitment by Src-like kinases as well as by Syk kinase.[6] In addition, since 5-OH-Aic can be considered as a conformationally restricted analogue of the non-natural *m*-tyrosine amino acid (L-mTyr), as well as the 6-OH-Tic residue, the L-mTyr5 derivative of the parent sequence was prepared and characterized to explore the effect of the presence of the hydroxyl group at the *meta* position of the ring in protein kinase recognition.

Here we show that the conformational restriction of the tyrosine side chain mobility is well tolerated by Syk, while the unnatural aromatic analogues are not recognized by the catalytic domain of the nonreceptor tyrosine kinases Csk and Src-related. Thus these peptidomimetics are both useful substrates for the specific monitoring of Syk and peptide scaffolds for the design of inhibitors highly selective for this tyrosine kinase.

† Deceased November 21, 2005. This paper is dedicated to the memory of Dr. Gianfranco Borin, an outstanding and enthusiastic mentor, a dear friend and colleague.

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10.1021/jm051080q CCC: $33.50
Published on Web 02/16/2006

H-Glu-Asp-Asp-Glu-**Xaa**-Glu-Glu-Val-OH

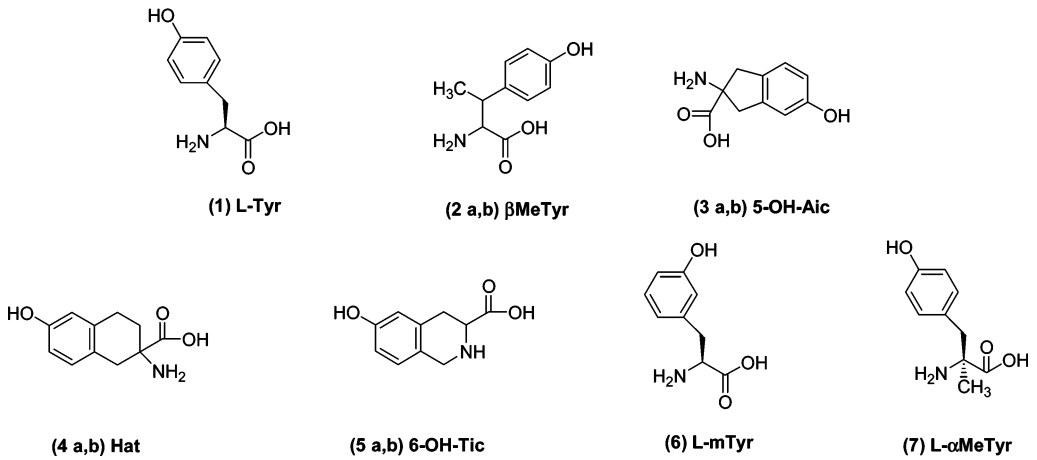

Figure 1. Structural formulas of tyrosine analogues.

Table 1. Analytical Data of EDDE-Xaa-EEV Analogues

		MW				amino acid ratio in acidic hydrolysate			
	peptide	calcd for		found	t_r (min)[a]	Asp (2)	Glu (4)	Val (1)	Xaa (1)
1	EDDE-(L)**Tyr**-EEV*	$C_{42}H_{58}N_8O_{22}$	1026.37	1027.38	27.4[b,c]	2.03	4.06	1.00	0.97
2a	EDDE-(L)***β*-MeTyr**-EEV	$C_{43}H_{60}N_8O_{22}$	1040.38	1041.42	23.8[b,c]	2.05	4.10	1.00	0.96
2b	EDDE-(D)***β*-MeTyr**-EEV	$C_{43}H_{60}N_8O_{22}$	1040.38	1041.43	24.7[b,c]	2.06	4.09	1.00	0.98
3a	EDDE-**5-OH-Aic**-EEV	$C_{43}H_{58}N_8O_{22}$	1038.37	1039.47	21.2[b,d]	2.06	4.11	1.00	nd[f]
3b	EDDE-**5-OH-Aic**-EEV	$C_{43}H_{58}N_8O_{22}$	1038.37	1039.47	26.4[b,d]	2.06	4.08	1.00	nd
4a	EDDE-**Hat**-EEV	$C_{44}H_{60}N_8O_{22}$	1052.39	1053.40	19.1[b,e]	2.07	4.10	1.01	nd
4b	EDDE-**Hat**-EEV	$C_{44}H_{60}N_8O_{22}$	1052.39	1053.41	20.9[b,e]	2.06	4.09	1.00	nd
5a	EDDE-**6-OH-Tic**-EEV	$C_{43}H_{58}N_8O_{22}$	1038.37	1039.40	17.4[b,d]	2.07	4.12	1.01	0.99
5b	EDDE-**6-OH-Tic**-EEV	$C_{43}H_{58}N_8O_{22}$	1038.37	1039.41	19.5[b,d]	2.05	4.10	1.00	0.98
6	EDDE-(L)**mTyr**-EEV	$C_{42}H_{58}N_8O_{22}$	1026.37	1027.42	23.5[b,c]	2.02	4.08	1.00	1.01
7	EDDE-(L)**αMeTyr**-EEV	$C_{43}H_{60}N_8O_{22}$	1040.38	1041.43	27.2[b,c]	2.01	4.05	1.01	0.99

[a] Xaa = Tyr, *β*-MeTyr, 5-OH-Aic, Hat, 6-OH-Tic, mTyr, αMeTyr. [b] Elution conditions: eluents (A) 0.05% TFA in water, (B) 0.05% TFA in 9:1 v/v MeCN/water; column Jupiter C18, 10 *μ*m, 250·4.6 mm; flow rate 1 mL/min; detection at 216 nm; isocratic elution at low B percentage for 3 min and then linear gradient (see footnotes *c*, *d*, and *e*). [c] Linear gradient 10 to 30% B in 40 min. [d] Linear gradient 12 to 18% B in 40 min. [e] Linear gradient 15 to 18% in 40 min. [f] Not detected.

Results

Peptide Synthesis. Racemic erythro-*β*-MeTyr, 6-OH-Tic, 5-OH-Aic, and Hat tyrosine constraints were synthesized by Tourwé's group as previously described,[7−10] whereas L-mTyr was synthesized by reaction of *m*-methoxybenzyl bromide with the Schiff base of glycine ethyl ester according to the procedure described by Lecointe et al.[11] Tyrosine analogues were incorporated into the peptide structure after protection of the amino group as an Fmoc derivative.[12]

Peptides were prepared by the solid phase method on a Wang resin according standard Fmoc/HBTU protocol.[12] To overcome low yields and subsequent side reactions, the coupling of Glu4 to the secondary amino group of tetrahydroisoquinoline derivative as well as to the amino groups of C^{α}-tetrasubstituted residues was performed using HATU as coupling reagent.[13] Following the removal of the Fmoc group, side chain protecting groups were left on during cleavage from the resin by TFA standard treatment (see Experimental Section). Crude products were purified by preparative reversed phase chromatography. In the case of the peptides synthesized using the racemic mixture of a tyrosine constraint, the enantiomers were separated and assigned as the **a** or **b** form depending on their elution times from the reversed phase column. By comparison with the elution of EDDE-Xaa-EEV derivatives containing L- or D-Htc analogues at the 5-position,[2] **a**-derivatives, which were first eluted from the column, might correspond to the L-enantiomers, while **b**-derivatives might display the D-conformation. The correct composition of the synthetic peptides was confirmed by both ESI-MS and amino acid analyses of the acidic hydrolysates (Table 1).

Peptide Analysis by NMR. NMR investigation of Tyr5-substituted peptides was performed using standard one-dimensional and two-dimensional homonuclear and heteronuclear techniques in DMSO-d_6 solution at 298 K. The complete sequential assignment was based on the $C^{\alpha}H$ (residue *i*)-NH (residue $i + 1$) homonuclear NOE effect.[14] The chemical shift assignment of proton resonances, as well as the NH−$C^{\alpha}H$ vicinal coupling constants, and the corresponding dihedral φ and ψ angles, as derived from the Karplus-type equation of Bystrov,[15] have been determined and reported in the Supporting Information (Tables S1 and S2, respectively). The NH chemical shifts shifted linearly in the temperature interval 288−318 K, indicating that conformational changes do not occur in this temperature range.[16] The temperature dependence of NH chemical shifts of the synthetic peptides is reported in Table S3 (Supporting Information). Temperature gradients indicative of NH involved in intramolecular hydrogen bonding (more positive than -2 ppb·K^{-1}) are exhibited by Glu4 (peptides **2** and **6**), 5-OH-Aic (peptide **3**), and Glu6 (peptide **4**) residues. Only a few nonsequential ROESY cross peaks are observed for the investigated peptides, which in addition to NH−$C^{\alpha}H$ coupling constants greater than 7.5−8 Hz are indicative of the existence of extended conformations in DMSO solution.

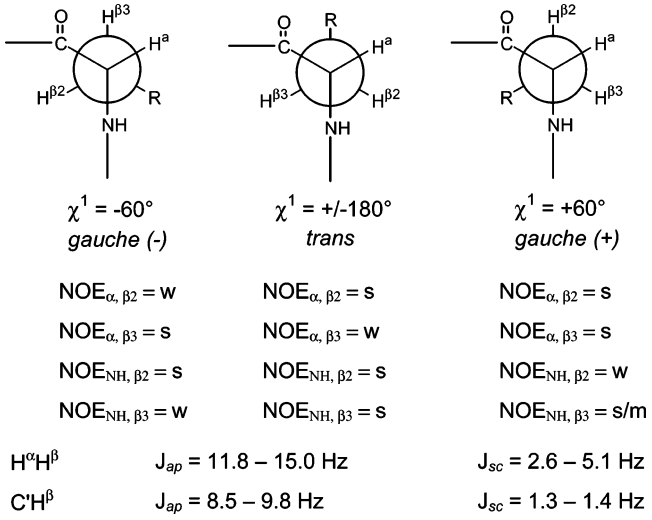

Figure 2. Staggered side chain conformers and their NMR characteristics.

Table 2. Kinetic Constants for Syk Tyrosine Kinase of Tyr5-Substituted Analogues

	peptide	k_{cat} (min^{-1})	K_m (μM)	efficiency k_{cat}/K_m
1	EDDE-**(L)Tyr**-EEV	70.1	3.6	19.47
2a	EDDE-**(L)*β*-MeTyr**-EEV	65.8	6.5	10.12
2b	EDDE-**(D)*β*-MeTyr**-EEV	50.8	63.2	0.80
3a	EDDE-**5-OH-Aic**-EEV	65.2	17.3	3.77
3b	EDDE-**5-OH-Aic**-EEV	15.1	145.6	0.10
4a	EDDE-**Hat**-EEV	5.5	98.0	0.06
4b	EDDE-**Hat**-EEV	40.5	3.6	11.25
5a	EDDE-**6-OH-Tic**-EEV	8.8	124.5	0.07
5b	EDDE-**6-OH-Tic**-EEV	5.9	136.1	0.04
6	EDDE-**(L)mTyr**-EEV	34.7	5.5	6.31
7	EDDE-**(L)αMeTyr**-EEV	19.1	87.7	0.22

The side chain conformation of the tyrosine constrained analogues, as well as the stereospecific assignment of $^{\beta 2}$H and $^{\beta 3}$H protons,[17] could be simply deduced from $^{\alpha}$H−$^{\beta}$H vicinal coupling constants ($^3J_{\alpha,\beta}$) and by the ROE pattern observed between the backbone and the side chain protons ($^{\alpha}$H−$^{\beta}$H, NH−$^{\beta}$H) (Figure 2).[18]

In the tyrosine constraints lacking $^{\alpha}$H or NH protons these data are not sufficient to determine the orientation of the side chain with respect to the backbone, since we could not unambiguously assign the prochiral β-protons. In conjugation with partial homonuclear data ($^3J_{\alpha,\beta}$ or NOE contacts), a qualitative or quantitative (not easily accessible in natural abundance samples at mM peptide concentration) knowledge of the $^3J_{C'H\beta}$ heteronuclear coupling constants could unambiguously distinguish the three staggered conformations about the $C^{\alpha}-C^{\beta}$ bond.[19]

The introduction of a β-methyl substituent into the Tyr residue strongly affects the side chain orientation rather than the peptide backbone conformation. The methylation of the β-carbon virtually eliminates the trans χ^1-rotamers in the erythro isomers (2*S*,3*S* and 2*R*,3*R*) and strongly reduces the population of the gauche (+) in the (2*S*,3*R*) threo isomer and of the gauche (−) rotamer in the (2*R*,3*S*) threo isomer.[7] The NMR data of bioactive β-MeTyr peptide **2a** (bioactivity is discussed in the next section and summarized in Table 2) (Table S4), in addition to the RP-HPLC elution profile, permit the assignment of a gauche (−) side chain conformation with an absolute erythro (2*S*,3*S*) configuration (Table S4).

On the other hand, the introduction of the bicyclic structures 5-OH-Aic, Hat, and 6-OH-Tic in the peptide produces conformational constraints both in the side chain and in the peptide backbone at the 5-position. In particular, in 5-OH-Aic and Hat residues the phenolic moiety is covalently linked to the α-carbon atom through a one or two carbon bridge, respectively. In these peptides, the aromatic ring is forced to adopt an orientation almost perpendicular to the plane of its adjacent backbone atoms. Crystallographic and mechanicistic studies on the corresponding phenylalanine constraints Aic (2-aminoindan-2-carboxylic acid) and Atc (2-aminotetraline-2-carboxylic acid) show that the torsional angles of the indane derivative are limited to values of $\chi^1 = -80°$, $\chi^2 = -20°$, and $\chi^1 = -160°$, $\chi^2 = +20°$, whereas the Atc residue is able to adopt essentially only two side chain conformations characterized by the torsional angles $\chi^1 = 180°$ (trans), $\chi^2 = +25°$, and $\chi^1 = -60°$ (gauche (−)) $\chi^2 = -25°$.[20] C^{α}-Substitution drastically reduces the conformational space available to the peptide backbone, and the torsional angles at the 5-position are limited to values around $\varphi = -50°$, $\psi = -50°$, and $\varphi = +50°$, $\psi = +50°$.[20, 21]

Heteronuclear NMR experiments on bioactive 5-OH-Aic peptide (peptide **3a**) is characterized by the presence of very weak connectivity between the $^{\beta}$H proton at 3.13 ppm and the corresponding carboxylic carbon (172 ppm) (data not shown), suggesting that the 5-OH-Aic residue adopts a twisted side chain conformation where this proton is near to an antiperiplanar orientation with respect to the carboxyl carbon. Nevertheless, the absence of ROE contacts between $^{\beta}$H-NH protons does not permit the assignment of diasterotopic $^{\beta}$H protons and consequently the assignment of gauche (+) or trans side chain orientation of the phosphorylatable residue (Table S4).

The finding that the phosphorylation assays performed with the derivatives containing Hat enantiomers (peptides **4a** and **4b**) show that the bioactive analogue corresponds to the second eluted peptide (peptide **4b**) (Table 2) prompted us to analyze the conformation of both Hat isomers. The NMR data, which are summarized in Table S4, permit the assignment of the possible rotamer around the Cα-to-Cβ (C2-to-C1) bond and also the stereospecific assignments of H^{β} protons. Both Hat residues of peptides **4a** and **4b** exhibit a preference for the gauche (−) rotamer in DMSO solution.

The tetrahydroisoquinoline structure (6-OH-Tic) results in spatial freezing of the aromatic ring by a methylene group through its α-nitrogen atom, yielding a gauche (+) or gauche (−) orientation of the side chain, respectively.[22] The φ angle is limited to values around 90°. The 6-OH-Tic residue gives rise to the presence of trans and cis isomers around the Glu-6-OH-Tic amide bond, as shown by the two resonance sets in 1D- and 2D-NMR spectra (Table S1). Diagnostic NMR data of the presence of this isomerism are the ROE connectivity between $C^{\alpha}_{Glu4}H-C^{\epsilon}_{6-OH-Tic5}H$ and between $C^{\alpha}_{Glu4}H-C^{\alpha}_{6-OH-Tic5}H$, in the ROESY spectrum, characteristic of the trans and the cis conformer, respectively.[22] Weighted populations of the two conformers were determined by measuring the relative volumes of the cis and trans Glu4 NH and 6-OH-Tic $C^{\delta}H$ resonances. A cis/trans population ratio of 1/9 was determined for this peptide. In the most abundant trans isomer, the $^3J_{\alpha\beta}$ coupling constants and the NOE ($^{\alpha}$H−$^{\beta 2,3}$H) contacts are consistent with a gauche (+) side chain conformation, whereas the stereospecific assignment of the β protons was possible using the $^3J_{C'H\beta}$ coupling constants (Table S4).

Peptide Phosphorylation by Different Tyrosine Kinases. The synthetic peptides were tested as potential substrates for the nonreceptor tyrosine kinases Syk and Csk and the two tyrosine kinases Lyn and Fgr belonging to the Src family. Time courses of peptide phosphorylation were run with identical units

of the different kinases determined toward the common substrate poly-Glu_4Tyr. As expected,[3] the very acidic parent peptide EDDE-**Tyr**-EEV (peptide **1**) was an excellent substrate for Syk tyrosine kinase in assays containing 40 μM peptide (Figure 3A). In contrast, 400 μM substrate concentration was used to reach an adequate peptide phosphorylation catalyzed by the other PTKs (Figures 3B, C, and D). These preliminary experiments showed that EDDE-**Tyr**-EEV on one hand could be used as a model substrate to study the effect of conformational constraints introduced in Tyr5 toward the different PTK activities; on the other hand, the synthetic peptides containing the tyrosine derivatives could be analyzed as potential substrates more stringently selective for Syk than the parent peptide. The time courses of peptide phosphorylation show that Syk kinase well tolerated tyrosine derivatives, since the enzyme phosphorylated L-β-Me-Tyr, **a**-5-OH-Aic, and **b**-Hat analogues (peptides **2a**, **3a**, and **4b** of Table 1, respectively) similarly to the natural residue Tyr5 (Figure 3A). While L-mTyr (peptide **6**) and D-β-Me-Tyr (peptide **2b**) are phosphorylated less efficiently, the other derivatives are almost unaffected by Syk (Figure 3A). Interestingly, the tyrosine-constrained analogues are not recognized as phosphoacceptors by the other nonreceptor PTKs with the only exception of L-β-Me-Tyr, which is phosphorylated to a low extent (Figures 3B, C, and D).

To check whether the failure of Csk and Src kinases to phosphorylate the EDDE-**Tyr**-EEV derivatives was due to the inability of enzyme to either interact with the peptides or catalyze the phosphate transfer to the tyrosine constrained analogues, competition experiments were performed. Peptide derivatives were tested as potential inhibitors of the different PTK activities tested on DRVYIHPF-R (angiotensin II containing an additional Arg residue at the C-terminus), a promiscuous peptide substrate for many tyrosine kinases.[5] The phosphorylation of angiotensin is inhibited by several derivatives in a dose dependent manner when Syk is the catalyst (Figure 4). The inhibitory potency of the different analogues is proportional to their ability to act as phosphoacceptors for the tyrosine kinase with the highest inhibitory effect displayed by **b**-Hat > L-β-Me-Tyr > L-mTyr > **a**-5-OH-Aic containing peptide. The finding that the unnatural aromatic alcohols, which are poorly recognized by Syk (Figure 3A), do not inhibit the kinase activity (Figure 4) demonstrates that the replacement of Tyr by these analogues abrogates the ability of the related peptides to be recognized by the enzyme catalytic site.

Consistent with this hypothesis, the kinase activity tested toward angiotensin-R is completely unaffected by the peptide derivatives when the phosphorylating kinases are Csk, Lyn, or c-Fgr with the only exception of L-β-Me-Tyr, which, as expected, when added at high concentration slightly inhibits Lyn and Fgr activities (data not shown).

The influence of the different tyrosine constrained analogues on the Syk phosphorylation mechanism was better analyzed by kinetic experiments (Table 2). The kinetic constants demonstrate that the efficient phosphorylation of L-β-MeTyr, **b**-Hat, and L-mTyr derivatives by the tyrosine kinase is accounted for by both the K_m values (6.5, 3.6, and 5.5 μM, respectively), which are very low and similar to that of the tyrosine containing peptide (3.6 μM), and K_{cat}, whose values, albeit lower than that of the parent peptide, remained nevertheless very favorable. In the case of **a**-5-OH-Aic derivative, the K_{cat} value is similar to that of EDDE-**Tyr**-EEV, while the K_m is about 5-fold higher than that of the parent peptide.

Kinetic experiments (Table 2) also show that the low efficiencies displayed with D-β-MeTyr, **a**-Hat, and **b**-5-OH-Aic derivatives are due to a reduced Syk affinity as judged by the K_m values, which are more than 10-fold higher than that found with peptides containing their respective enantiomers. *m*-Tyrosine (L-mTyr) is phosphorylated by Syk with higher affinity (5.5 μM) and 3-fold lower efficiency than that of L-Tyr. The analogue containing L-α-methyl-tyrosine is a poor substrate of Syk, displaying more than 90-fold lower efficiency than that of the parent peptide.

Discussion

Present data show that, at variance with all the other nonreceptor PTKs tested, Syk tolerates the conformational restriction of both the target residue side chain and the peptide−substrate backbone. Indeed this tyrosine kinase accepts the replacement of the phosphorylatable tyrosine by the conformationally constrained analogues L-β-MeTyr and **b**-Hat, whose phosphorylation efficiencies, albeit lower than that of the natural residue, remain in the same range. Syk recognizes also **a**-5-OH-Aic containing peptide with a phosphorylation efficiency 5-fold lower than that displayed by the standard octapeptide. This behavior likely reflects unique features of Syk substrate binding domain since the constrained derivatives are not phosphorylated by Csk, Lyn, and Fgr and also fail to compete against the phosphorylation of a common substrate by these tyrosine kinases. To highlight the structural bases of such unusual site recognition of Syk, we analyzed the kinase three-dimensional structure, which has been recently solved.[23] The crystal shows that several basic residues are present within the substrate-binding site consistent with the strict consensus sequence of Syk, which requires acidic residues surrounding the target tyrosine.[4]

To better highlight the interactions between EDDE-**Tyr**-EEV and the Syk catalytic domain, an in silico model was performed (Figure 1S, Supporting Information), in which both peptide design and its binding to Syk structure were based on the crystal of the complex between insulin receptor kinase and the peptide IRS727.[24] The model shows the crucial enzyme/substrate contacts occurring through hydrogen bonds. In particular, Glu1 of the peptide interacts with Lys458 (numbering of Syk full length), Asp3 with Lys537 and Lys571, Glu4 with Arg498 and Lys571, Glu6 with Lys533 and Lys537, and Glu7 with Lys533. The phenolic group of the phosphorylatable Tyr5 makes hydrophobic contacts with Pro535, a conserved catalytic residue characteristic of the tyrosine kinase active site.[25] Tyr5 hydroxyl group is oriented in an optimal position to interact with Asp494, which is thought to play the role of catalytic base during the phosphoryl transfer.[26] The strong interactions occurring between Syk catalytic domain and its peptide substrate could be hypothesized to highly favor the transfer of the phosphate to the tyrosine constrained analogues, at variance with Csk and Src-related kinases, which do not display a stringent substrate consensus sequence and thus do not phosphorylate the tyrosine derivatives. However, we have previously found that Syk phosphorylates the constrained tyrosine derivative L-Htc also when it is included in a sequence less favorable as a substrate, while Lyn and Fgr tyrosine kinases are unable to recognize this derivative substituted for tyrosine in a peptide reproducing the sequence containing their own autophosphorylation sites.[3] This finding on one hand validates the results obtained with Csk and Src-like kinases using the peptide EDDE-**Tyr**-EEV, which contains the consensus sequence for Syk, and on the other hand supports the present data showing that, among the tested tyrosine kinases, only the Syk catalytic domain exhibits the property to easily interact with different unnatural aromatic alcohols.

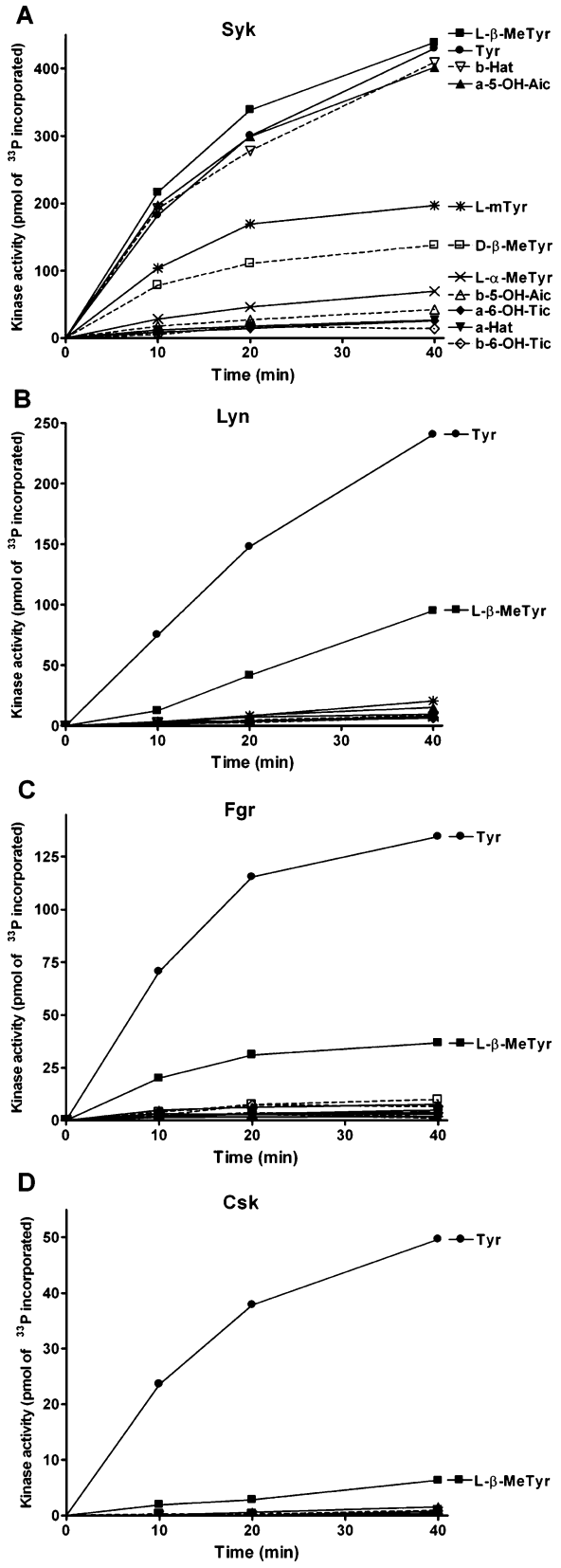

Figure 3. Time courses of peptide phosphorylation catalyzed by Syk (A), Lyn (B), Fgr (C), and Csk (D) tyrosine kinases. Technical details are reported in the Experimental Section. Peptide concentration was 40 μM in assays containing Syk and 400 μM in assays containing the other tyrosine kinases. Peptide 5-position contains Tyr (●), L-β-MeTyr (■), D-β-MeTyr (□), **a**-5-OH-Aic (▲), **b**-5-OH-Aic (△), **a**-Hat (▼), **b**-Hat (▽), **a**-6-OH-Tic (◆), **b**-6-OH-Tic (◇), L-mTyr (∗) and L-α-MeTyr (×). Data are the means of four separate experiments; the SEM values were always less than 18%.

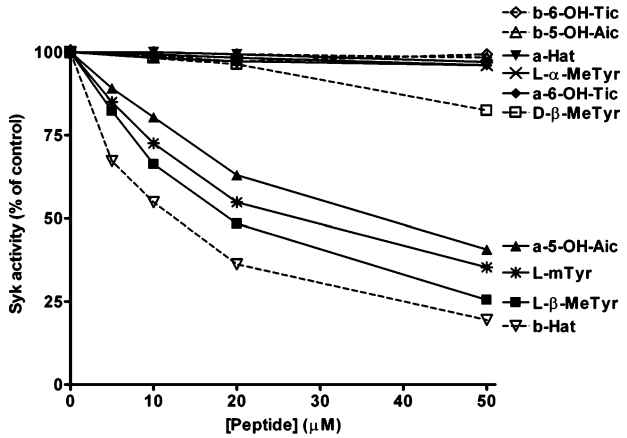

Figure 4. Effect of peptides containing tyrosine analogues on angiotensin-R phosphorylation catalyzed by Syk tyrosine kinase. The indicated peptide derivatives were used as inhibitors of Syk activity tested toward 1.5 mM angiotensin-R. Enzyme activity on angiotensin-R was quantified by phosphocellulose papers, which did not bind the acidic derivatives as detailed in Experimental Section. Data are means of four separate experiments. SEM values were always less than 16%.

In the overwhelming body of literature on tyrosine kinase substrate specificity only few studies analyzed the topography of the phosphorylatable tyrosine residue. In the X-ray structure of the insulin receptor kinase catalytic domain bound to a peptide substrate, the tyrosine was found in the gauche (−) conformation,[27] whereas in the complex of Lyn with a peptide, the χ^1 angle of the tyrosyl residue was 22.6°, between cis and gauche (+), as judged by transferred NOE.[28] The in silico modeling of EDDE-**Tyr**-EEV interacting with the Syk catalytic domain suggests that the mechanism of peptide phosphorylation is favored by a gauche (−) tyrosine conformation (Figure 1S).

To better analyze the effect of the phosphorylatable residue side chain orientation on the process of phosphorylation catalyzed by Syk, we compared the ability of the kinase to recognize different conformational constrained tyrosine analogues.

The good phosphorylation efficiency displayed by Syk with L-β-MeTyr5 derivative as compared to the parent peptide (efficiency 10.12 and 19.47 min^{-1} μM^{-1}, respectively) is consistent with NMR and in silico analyses. NMR investigation of the side chain constrained L-β-MeTyr5 residue showed that this derivative exhibits a preference for the gauche (−) side chain conformation in DMSO solution (Table S4), with an absolute erythro (2*S*,3*S*) configuration. The in silico modeling of Syk with L-β-MeTyr containing peptide confirms that the optimal orientation for interacting with the nucleotide γ-phosphate group is consistent only with the gauche (−) orientation and (2*S*,3*S*) configuration of the analogue. The L-β-MeTyr (2*S*,3*R*) configuration is less favorable due to the steric hindrance caused by the methyl group (not shown). It is interesting to note that L-β-MeTyr5 is the only derivative which can be phosphorylated, albeit to a low extent, by the other nonreceptor tyrosine kinases, which are unable to recognize the other constrained residues, demonstrating their stringent specificity for the natural tyrosyl residue.

The modeling of **a**-5-OH-Aic derivative (peptide **3a**) in complex with Syk suggests that the side chain conformational restriction of the bicyclic structure induces an orientation of the hydroxyl group still compatible with the attack of the nucleotide γ-phosphate (data not shown). The modeling is supported by the kinetic constants showing that the peptide containing the **a**-enantiomer of 5-OH-Aic is phosphorylated by

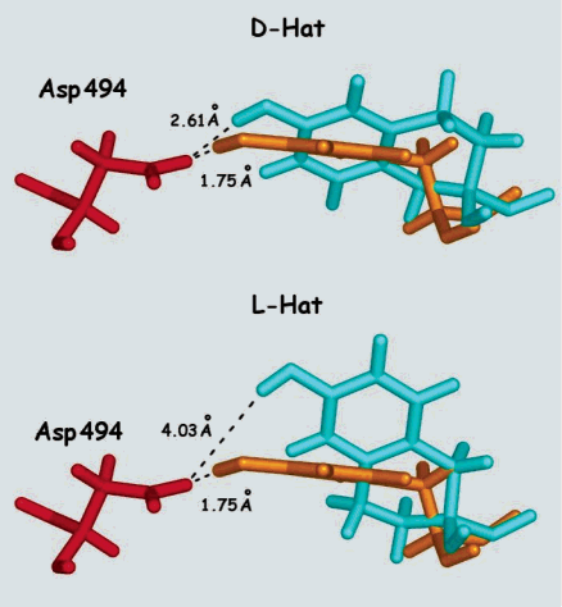

Figure 5. Orientation of Hat enantiomers docked to Syk catalytic domain. The modeling of the Hat containing peptides interacting with Syk catalytic domain (PDB code: 1XBA)[23] was based on the crystal of the complex between insulin receptor kinase and the peptide IRS727 (PDB code: 1GAG).[24] The energy minimization of the Syk−peptide complex was performed with the program OPTIMIZE (TINKER Package)[29] and Amber99 force field until the energy reached a minimum. The distances (dashed lines) between the peptide Tyr5, D-Hat5, or L-Hat5 and Syk Asp494 are shown. Tyr is in orange, D- and L-Hat are in cyan, and Asp494 is in red.

Syk with about 5-fold higher K_m and similar K_{cat} compared to the tyrosine residue.

Peptide containing the Hat **b**-conformation displays with Syk more favorable kinetic constants than 5-OH-Aic **a**-conformation (Table 2), consistent with the higher flexibility of the Hat analogue compared to the other bicyclic derivative. Moreover, both NMR and modeling analyses of **b**-Hat suggest a gauche (−) side chain orientation, which is a positive determinant for Syk phosphorylation (Figure 5). **b**-Hat containing peptide is recognized by Syk with a very low K_m value, suggesting that it interacts with the kinase catalytic domain similarly to tyrosine residue. It is interesting to note that, in the case of β-MeTyr, 5-OH-Aic (Table 2), and Htc,[2] Syk easily phosphorylates the **a**-enantiomers, which display the L-conformation according to NMR and/or modeling analyses. On the contrary, the bioactive Hat residue is the **b**-enantiomer (Table 2), suggesting that the kinase recognizes the D-conformation of this constrained analogue. This hypothesis is confirmed by the modeling of Figure 5, which shows the orientation of the hydroxyl group of Hat enantiomers relative to Syk Asp494, a residue conserved in the catalytic domain of all the protein kinases which plays a role during the phosphoryl transfer.[26]

The model shows that the hydroxyl group of the D-Hat conformation is indeed oriented in a near-optimal position (2.61 Å) for the attack of the nucleotide γ-phosphate group, while the bicycle rotation of the L-Hat enantiomer moves the hydroxyl group away from this alignment (4.03 Å) (Figure 5).

Our phosphorylation data confirm the influence of the chirality in substrate recruitment also in the case of unnatural and conformationally restricted amino acids, since the kinase active site accepts only one of the enantiomers, being L or D tyrosine derivatives, but poorly recognizes the other. In fact, the poorly phosphorylated enantiomers D-β-MeTyr, D-5-OH-Aic, and L-Hat interact with Syk with more than 10-fold lower affinity and only slightly interfere with the Syk-mediated phosphorylation of angiotensin (Figure 4), indicating that the wrong conformation hardly accommodates into the active site of Syk under standard conditions.

The finding that the peptides containing 6-OH-Tic enantiomers do not interact with the Syk catalytic domain (Figures 3 and 4 and Table 2) must be ascribed to the presence of the hydroxyl group in position 6, since we have previously shown that its isomer Htc (7-OH-Tic) is a suitable tyrosine derivative for Syk-catalyzed phosphorylation.[3] On the other hand, L-mTyr containing peptide (peptide **6**) is phosphorylated by Syk with a K_m value which is slightly higher and K_{cat} value 2-fold lower than those found with the tyrosine residue. These results suggest that the presence of the hydroxyl group at the meta position of the ring slightly influences the docking of the peptide to the Syk catalytic site, while it affects the following transfer of the nucleotide γ-phosphate group to the substrate, as expected due to the less favorable alignment of the L-mTyr hydroxyl group with Syk Asp494, which assists in the phosphoryl transfer. The good phosphorylation efficiency of L-mTyr containing peptide suggests that the inability of the 6-OH-Tic derivative to interact with the catalytic domain of Syk is related to the simultaneous presence of two tyrosine side chain modifications: its introduction in a cyclic tetrahydroisoquinoline structure and the shift of the phosphorylatable hydroxyl group.

The backbone torsion angles of the α-MeTyr derivative (peptide **7**) as well as those of 5-OH-Aic and Hat can only assume values around $\varphi = -50°$, $\psi = -50°$, and $\varphi = +50°$, $\psi = +50°$.[20,21,30] The modeling of the interaction between L-α-MeTyr and Syk shows that when a methyl group is present at Tyr5 α−carbon, the approach of this tyrosine derivative to the Syk catalytic site is inhibited by the steric hindrance of the methyl group against the enzyme Lys533 (data not shown). As a consequence L-α-MeTyr is phosphorylated with an efficiency more than 90-fold lower that that of the tyrosine residue (Table 2). These results, compared to that obtained with the cyclic C^{α}-tetrasubstituted analogues 5-OH-Aic and Hat, suggest that the phosphorylation of the aromatic hydroxyl group is less influenced by the backbone conformation around the tyrosine residue than by the flexibility and the steric bulk of the C^{α}-substitution, which strongly affects the interaction with the Syk catalytic domain.

Conclusions

Our data highlight the peculiarity of the tyrosine kinase Syk to recognize constrained tyrosine analogues as phosphorylatable residues. In particular, L-β-MeTyr (peptide **2a**) and D-Hat (peptide **4b**) containing peptides are phosphorylated with efficiencies not far from that found for the parent peptide. The advantages of using these peptidomimetics as compared to the standard octapeptide are as follows: (i) their absolute Syk selectivity, since they contain both the consensus sequence specific for Syk and as phosphorylatable residue a Tyr derivative, which is recognized only by this tyrosine kinase; and (ii) the potentially increased resistance to proteolysis, since conformationally constrained amino acids have been successfully used in the design of metabolically stable inhibitors.[31,32]

The ability of these two peptide derivatives to reveal the activity of Syk among other nonreceptor protein tyrosine kinases, especially those of the Src family, makes them valuable tools for in vitro and in cell studies aimed at the identification of Syk signaling pathways and their biological role. In particular, Src-like and Syk PTKs are widely expressed in hematopoietic cells, where they are involved in coupling membrane immu-

noreceptors to downstream signaling events that mediate several cellular responses including proliferation, differentiation, apoptosis, and malignancy.[33−36] In addition, L-β-MeTyr and D-Hat peptidomimetics provide structural bases for the design of peptide-ATP bisubstrate analogues,[24] to analyze as inhibitors specifically competing with Syk tyrosine kinase.

Experimental Section

General Methods for Peptide Synthesis and Purification. Racemic β-MeTyr, 5-OH-Aic, Hat and 6-OH-Tic were synthesized as previously reported[7−10] and then converted to the corresponding Fmoc (9-fluorenylmethoxycarbonyl) derivative by reaction with Fmoc-OSu (9-fluorenylmethoxycarbonyl succinimido ester).[12] L-mTyr was synthesized by reaction of *m*-methoxybenzyl bromide with the Schiff base prepared from benzophenone imine and glycine ethyl ester.[11] After conversion of the Schiff base into the corresponding Boc derivative, the racemic mixture was resolved by enzymatic digestion with Carlsberg subtilisin,[37] and then the Boc-L-mTyr(Me)-OH was hydrolyzed in 6 N HCl and converted into the corresponding Fmoc derivative by reaction with Fmoc-OSu.

Fmoc-protected amino acids and preloaded Wang resins were purchased from Calbiochem-Novabiochem (Läufelfingen, Switzerland). HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate), HOBt (1-hydroxybenzotriazole), DIEA (*N*,*N*-diisopropylethylamine), and NMP (*N*-methyl pyrrolidone) were obtained from Applied Biosystems-Perkin Elmer (Foster City, CA), whereas HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate) was obtained from PerSeptive Biosystems (Foster City, CA).

Peptides were synthesized by manual solid phase using Fmoc chemistry in 0.1 mmol scale. HBTU/HOBt activation employed a 3-fold molar excess (0.4 mmol) of Fmoc-amino acids in DMF (*N*,*N*-dimethylformamide) solution for each coupling cycle unless otherwise stated. Coupling of Fmoc-Glu(OtBu)-OH to the amino groups of tyrosine constraint analogues was performed using HATU following the standard procedure with the exception that a 1:1 mixture of HATU and Fmoc derivative was used, and HOBt solution was not added.[13] Coupling times were 40 min. Deprotection was performed with 20% piperidine. Coupling yields were monitored on aliquots of peptide-resin either by Kaiser test for the amino groups or by evaluation of Fmoc displacement.[38] Cleavage from the resin and deprotection were performed by treatment with TFA−anisole−triisopropylsilane−H_2O (95:2.5:2.0:0.5 v/v) (45 min).

Peptides were purified by preparative reversed-phase HPLC using a Shimadzu LC-8 (Shimazdu, Kyoto, Japan) system with a Vydac 218TP1022, 10 μm, 250 × 22 mm column (The Separation Group, Hesperia, CA). The column was perfused at a flow rate of 12 mL/min with a mobile phase containing solvent A (0.05% TFA in water) and a linear gradient from 10% to 30% of solvent B (0.05% TFA in acetonitrile/water, 9:1 by volume) in 40 min. The fractions containing the desired product were collected and lyophilized to constant weight in the presence of 0.01 N HCl.

Analytical HPLC analyses were performed on a Shimadzu liquid chromatography LC-10 instrument fitted with a Jupiter C18, 10 μm, 250 × 4.6 mm column (Phenomenex, Torrance, CA) using the above solvent system (solvents A and B), flow rate of 1 mL/min, detection at 216 nm. All peptides showed less than 1% of impurities (Table 1).

Molecular weights of compounds were determined by ESI-MS on a Mariner (PerSeptive Biosystem) mass spectrometer instrument. The mass was assigned using a mixture of neurotensin, angiotensin, and bradykinin at a concentration of 1 pmol/μL as external standard. In addition, to confirm the correct composition of amino acids in synthetic peptides the acid hydrolyses were carried out in azeotropic hydrochloric acid containing 0.25% phenol for 22 h at 110 °C in sealed evacuated vials. The amino acid compositions of the hydrolysates were determined with a Carlo Erba 3A30 amino acid analyzer.

Thin-layer chromatography was carried out on silica gel Polygram SIL G/UV254 (Macherey-Nagel, Düren, Germany) using the following eluent system: (a) *n*-butanol/acetic acid/water (60:20:20); (b) methanol/chloroform (15:85); (c) cyclohexane/chloroform/acetic acid/ethanol (45:45:10:10); (d) 2-butanol/ethyl acetate/water (14:12:5).

NMR Experiments. NMR samples were prepared by dissolving appropriate amounts of the linear octapeptides in DMSO-d_6 (100% isotopic purity, Aldrich, Milwaukee, WI) to make approximately 3 mM solutions.

All spectra were run on a Bruker DRX-400 instrument equipped with an SG-Indy data system, operating at 400 MHz for ^{1}H. Proton and carbon chemical shifts, in parts per million (ppm), are referenced to the residual ^{1}H-DMSO solvent signal (δ = 2.49 ppm) and to the ^{13}C solvent signal (δ = 39.5 ppm), respectively. All NMR experiments have been carried out at a temperature of 298 K, using the BVT2000 temperature control unit. Sample temperature was accurate to ±1 K.

One-dimensional (1D) NMR spectra were acquired using typically 16−32 scans with 32K data size. For the two-dimensional (2D) experiments, pulse programs of the standard Bruker library were used. All 2D experiments were acquired in the phase-sensitive mode, with quadrature detection in both dimensions, by use of the time proportional phase increments (TPPI).[39] Typically 512 experiments of 48 scans each were performed: relaxation delay 1 s; size 2K; 6024 Hz spectral width in F2; zero filling to 1K in F1; square cosine or Gaussian multiplication was used in both dimensions before the Fourier transformation. Mixing time of 75 ms was used for TOCSY.[40] ROESY[41] experiments were run at mixing times of 350 ms. NOESY[42] experiments were run at mixing times ranging from 200 to 350 ms. Coupling constants were determined by 1D and by exclusive COSY (E.COSY)[43] ^{1}H NMR experiments. All spectra were run at 298 K.

The HMBC[44] experiment was used for the assignment of carbon resonances as well as for the qualitatively evaluation of long range $^3J_{C'H\beta}$ coupling constants. An HSQMBC[45] experiment was used for the evaluation of long range heteronuclear coupling costants. Heteronuclear spectra were obtained by recording 240 experiments of 600 transients each. A relaxation delay of 1.0 s was allowed after each acquisition. The spectral width was 5430 and 16 000 Hz in the proton and the carbon dimension, respectively.

Purification of Tyrosine Kinases. Syk, Csk, Lyn, and c-Fgr tyrosine kinases were purified to near homogeneity from rat spleen as previously described.[46−48] One unit was defined as the amount of kinase transferring 1 pmol of phosphate per min to the random polymer polyGlu$_4$Tyr (0.1 mg/mL) under standard conditions.

Phosphorylation Assays. Phosphorylation assays were performed by incubating at 30 °C 40 μL of a medium containing 50 mM Tris/HCl, pH 7.5, 5 mM $MnCl_2$ (Fgr activity was assayed in the presence of 5 mM $MgCl_2$ instead of $MnCl_2$), 20 μM [γ^{33}P]-ATP (specific activity 1000 cpm/pmol), the indicated amounts of peptide substrate, and 10 units of enzyme. Reactions were terminated by addition of 1 mL of 1 N HCl, and labeled phosphopeptides were quantified as previously described.[49] In brief, samples were heated for 15 min at 100 °C in order to hydrolyze [γ^{33}P]ATP into ^{33}Pi, which was removed by conversion into a phosphomolybdic complex and extraction with isobutanol−toluene. The radioactivity due to phosphopeptides, present in the aqueous phase, was then measured in a scintillation counter.

To quantify the ^{33}P incorporation into angiotensin-R, which contains three positively charged residues, the phosphocellulose paper procedure was applied.[50] The highly acidic derivatives of EDDE-**Tyr**-EEV peptide, used as inhibitors, could not bind to phosphocellulose paper.

K_m and K_{cat} values were calculated by GraphPad PRISM-4 Software.

Acknowledgment. This work was supported by National Research Council (CNR) of Italy, Italian Association for Cancer Research, Italian Ministry for University and Research (PRIN 2003), and European Union (Integrated Project Prokinase LSHB-CT-2004-503467). The authors are grateful to Mr. G.

Tasinato and Mr. U. Anselmi for skillful technical assistance and for performing amino acid analyses. V.M. is a Research Assistant of the Fund for Scientific Research-Flanders (Belgium).

Supporting Information Available: NMR data (Tables S1−S4) of octapeptides and Figure 1S depicting modeling of the interactions occurring between the Syk catalytic domain and the EDDE-**Tyr**-EEV peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM051080Q