Research Article

Journal of PeptideScience

Received: 7 May 2009 Revised: 14 July 2009 Accepted: 15 July 2009 Published online in Wiley Interscience: 27 August 2009

(www.interscience.com) DOI 10.1002/psc.1173

Switching between low and high affinity for the Syk tandem SH2 domain by irradiation of azobenzene containing ITAM peptidomimetics

Joeri Kuil,[a‡] Loek T.M. van Wandelen,[a] Nico J. de Mol[a]* and Rob M.J. Liskamp[a]

Spleen tyrosine kinase (Syk) plays an essential role in IgE receptor signaling (FcεRI), which leads to mast cell degranulation. Divalent binding of the tandem SH2 domain (tSH2) of Syk to the intracellular ITAM motif of FcεRI activates the kinase domain of Syk, and thereby initiates cell degranulation. The inter SH2 domain distance in Syk tSH2 might be important for Syk kinase activation. In this study, photoswitchable ITAM peptidomimetics containing an azobenzene moiety were synthesized. Irradiation of these constructs changes the distance between the two SH2 binding epitopes and therefore, they may be used as photoswitches. The affinity of the *cis*- and *trans*-isomer for tSH2 was assayed with SPR. The ITAM peptidomimetic with the smallest linker displayed the largest difference in affinity between the two isomers (at least 100-fold), and the affinity of the *cis*-isomer was comparable to monovalent binding. The ITAM mimics with larger photoswitchable linkers displayed modest differences. These results indicate that Syk tSH2 is able to adapt the inter SH2 domain distance to ligands larger than native ITAM, but not to smaller ones. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: high affinity IgE receptor (FcεRI); Spleen tyrosine kinase (Syk); tandem SH2; photoswitch; immunoreceptor Tyrosine-based activation motif (ITAM); surface plasmon resonance (SPR); peptidomimetics; azobenzene

Introduction

The incorporation of photoswitches (in particular, azobenzene) into peptides for the modulation of the conformation and/or bioactivity has been given increasing attention [1–5]. Azobenzene is frequently used because of its fast and fully reversible photoisomerization. One noteworthy disadvantage of azobenzene is that 100% *cis*, unlike 100% *trans*, cannot be obtained.

A particularly interesting system for using the azobenzene photoswitch is the divalent interaction of ITAM and Syk tandem SH2 domain. ITAM (Immunoreceptor Tyrosine-based Activation Motif) is present, among others, in the β- and γ-chain of the multimeric ($\alpha\beta\gamma_2$) high affinity IgE receptor (FcεRI) [6–8]. Once this receptor is stimulated by IgE and antigen binding, γ-ITAM is diphosphorylated (γ-dpITAM). After that, the Spleen tyrosine kinase (Syk) is recruited to the membrane by binding with its two SH2 domains (tandem SH2 domain, tSH2) to γ-dpITAM. This results in a conformational change and activation of the kinase domain of Syk [9]. Syk activation eventually leads to mast cell degranulation and overstimulation of this cascade leads to allergic reactions. Because the ITAM–Syk tSH2 interaction is essential for this signaling pathway [10,11], inhibitors of this interaction are potential anti-allergic agents.

The ITAM sequence consists of Tyr-Xxx-Xxx-(Leu/Ile)-$(Xxx)_{n=6-8}$-Tyr-Xxx-Xxx-(Leu/Ile), in which Xxx can be any amino acid [12]. The underlined residues comprise the binding epitopes for Syk tSH2, when tyrosine is phosphorylated. The intervening residues $(Xxx)_{n=6-8}$ (in γ-ITAM $n = 7$) are not essential for binding and can be replaced by a rigid linker without significant loss of binding affinity [13].

In a previous study, we showed that the intervening amino acids can also be replaced by the (4-aminomethyl)phenylazobenzoic acid (AMPB) photoswitch and two additional glycine residues with largely preservation of the binding affinity when in *trans*-configuration [14]. The binding affinity of the photostationary state containing a maximal amount of *cis*-isomer was two times lower. In this photostationary state still 40% *trans* was present; therefore, the intrinsic affinity of the *cis*-isomer was even lower [14]. Although the difference in binding affinity was significant, a larger difference is needed for applicability in a cellular system, ideally switching from an inactive to an active divalent binding ligand. Therefore, in the present study, the design of new photoswitchable ligands for Syk tSH2 was aimed at optimizing the difference in binding affinity between the *cis*- and *trans*-isomer. To accomplish this, two strategies were explored. On the one hand, the linker in the previously reported ITAM mimic was shortened and rigidified. On the other hand, also two photoswitchable ligands were designed

* *Correspondence to: Nico J. de Mol, Medicinal Chemistry and Chemical Biology, Utrecht University, Utrecht, The Netherlands. E-mail: n.j.demol@uu.nl*

a *Medicinal Chemistry and Chemical Biology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, Utrecht, The Netherlands*

‡ *Present address: Netherlands Cancer Institute, Plesmanlaan 121, 1066, Amsterdam, The Netherlands.*

to bind Syk tSH2 with the highest affinity in the *cis*-configuration. This was done because in the photostationary state with maximal *cis*, always a significant amount of *trans* will be present. Therefore, when *trans* is the active isomer, the difference between *trans* and the 'inactive' state of maximal *cis* will be limited by the presence of *trans*. This problem is not present when the *cis*-isomer binds with the highest affinity, because it is possible to obtain 100% *trans*-isomer.

Materials and Methods

Molecular Simulations

For all calculations YASARA (http://www.yasara.org) was used. PDB files of the compounds were made using ChemDraw and Chem3D. These file were loaded in YASARA and a simulation cell, in which each axis was extended 5.0 Å from the molecule, was defined. The Amber99 forcefield was used and the temperature control was step-10 annealing, starting from 298 K and at every 10 simulation steps the velocity of all atoms was reduced to 90%. Within approximately 2500 fs the temperature of 0 K was reached and the atoms almost did not move anymore. To be sure, the simulation was continued until 10 000 fs was reached. All linkers were modeled as their *N*-terminal acetamide and *C*-terminal amide derivatives. All distances were measured from the *N*-terminal nitrogen atom to the *C*-terminal carbon atom after the simulation of 10 000 fs.

Synthesis

Chemicals were obtained from commercial sources and used without further purification. Solvents, which were used for the solid-phase peptide synthesis, were stored over 4 Å molsieves, except for MeOH, which was stored over 3 Å molsieves. The reactions were performed at room temperature unless stated otherwise. The syntheses of peptidomimetic **1** and the building blocks Fmoc-AMPB-OH and Alloc-4-(3-aminoprop-1-ynyl)benzoic acid were described earlier [13,14].

Peptidomimetics **2**–**4** were assembled on Tentagel®-Rink-NH-Fmoc resin (each 625 mg, 0.15 M, loading 0.24 mM/g) using standard Fmoc/tBu chemistry. The Fmoc protecting group was removed using 20% piperidine in NMP (3 × 8 min) followed by washing steps with NMP (3 × 2 min), CH_2Cl_2 (3 × 2 min) and NMP (3 × 2 min). The Alloc protecting group was removed using 0.25 equivalents of $Pd(PPh_3)_4$ and 10 equivalents of anilinium *p*-toluene sulfinate in MeOH/THF (1 : 1) (2 × 60 min). After Alloc deprotection, the resin was washed with 0.1% sodium diethyldithiocarbamate in NMP (1 × 2 min), NMP (3 × 2 min), CH_2Cl_2 (3 × 2 min) and NMP (3 × 2 min). The amino acid coupling mixtures were prepared by dissolving four equivalents of amino acid, four equivalents of HOBt and HBTU and eight equivalents of DiPEA in NMP, and coupled during a coupling time of 60 min. The resin was washed with NMP (3 × 1 min) and CH_2Cl_2 (3 × 1 min) after every coupling step. The coupling steps and deprotection steps were monitored using the Kaisertest [15]. When the Fmoc deprotection was not complete, the deprotection steps were repeated. Fmoc-Tyr(OP(OBn)OH)-OH was coupled overnight using two equivalents of amino acid, two equivalents of the coupling reagents HOBt and HBTU, and five equivalents of DiPEA. Alloc-4-(3-aminoprop-1-ynyl)benzoic acid was also coupled overnight using two equivalents of amino acid and two equivalents of the coupling reagents HOBt and HBTU, and four equivalents of DiPEA. After the first Fmoc–Tyr(OP(OBn)OH)-OH coupling, an extra washing step (2 × 10 min) with a mixture of 1 M TFA/1.1 M DiPEA in NMP was performed after each Fmoc deprotection step. This was done to replace the piperidine counter ion of Tyr(OP(OBn)OH) for DiPEA. When all the coupling steps were completed, the peptides were acetylated using a capping solution of Ac_2O (4.72 ml, 42.7 mM), DiPEA (2.18 ml, 22.8 mM) and HOBt (0.23 g, 1.7 mM) in 100 ml NMP for 2 × 20 min. The peptides were cleaved from the resin, and the side chains were deprotected with TFA/H_2O/TIS (95/3.5/1.5) for 3h. The resin was removed from the solution by filtration and the peptides were precipitated with MTBE/hexane 1 : 1 v/v at −20 °C and lyophilized from CH_3CN/H_2O 1 : 1 v/v yielding 181.8 mg of mimic **2**, 163.8 mg of **3** and 110.2 mg of **4**. The peptides were purified by preparative HPLC, and analyzed by analytical HPLC and mass spectrometry. For the preparative HPLC, a Gilson system with a UV detector operating at 220 and 254 nm was used. An Alltech Prosphere C18 300Å 10 µm (250 × 22 mm) column and a gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95 : 5) to 100% buffer B (0.1% TFA in H_2O/CH_3CN 5 : 95) in 60 min were used for **3** and **4** and an Alltech Alltima C8 100Å 10 µm (250 × 22 mm) column and a gradient of 100% buffer A to 100% buffer B in 100 min were used for **2**. Analytical HPLC was measured on a Shimadzu HPLC system with a UV detector operating at 220 and 254 nm. For the analytical HPLC, a gradient from 100% buffer A (15 mM TEA in H_2O titrated at pH 6 with 85% H_3PO_4) to 100% buffer B (buffer A/CH_3CN 1 : 9) in 20 min was used. An Alltech Prosphere C18 300Å 5 µm (250 × 4.6 mm) column was used for **3** and **4**, and an Alltech Adsorbosphere XL C8 90Å 5 µm (250 × 4.6 mm) column was used for **2**. The fractions containing pure product were lyophilized yielding orange fluffy solids. For **2**, 90 mg was purified yielding 27.2 mg of pure product. For **3**, 50 mg was purified yielding 1.1 mg of pure product. For **4**, 50 mg was purified yielding 1.0 mg of pure product.

^1H NMR spectra of compound **2** were recorded on a Varian Inova 500 MHz spectrometer in H_2O/D_2O 9 : 1, using dioxane as an internal reference.

HRMS (ESI) of **2**: $[M+H]^+$ calculated 1397.5220, found 1397.4916; $[M+Na]^+$ calculated 1419.5040, found 1419.4063; $[M+2H]^{2+}$ calculated 699.2649, found 699.2689; $[M+H+Na]^{2+}$ calculated 710.2559, found 710.2312; $[M+2Na]^{2+}$ calculated 721.2469, found 721.2332.

LC-MS (ESI) of **3**: $[M+H]^+$ calculated 1711.63, found 1711.63; $[M+Na]^+$ calculated 1733.61, found 1733.71.

LC-MS (ESI) of **4**: $[M+H]^+$ calculated 1825.67, found 1825.54.

Photoisomerization

Photoisomerization was performed using a visible light emitting lamp (Schott/Paes KL-1500) in combination with a glass plate to filter the UV light or a 6 W 366 nm handheld TLC lamp (Konrad Benda) [16,17]. During the real-time SPR analysis of *cis*–*trans* isomerization a 1 W/cm^2 365 nm UV LED lamp (LED-100, Lightning Enterprises, Limington, USA) was used instead of the TLC lamp, because the TLC lamp did not fit into the SPR apparatus.

UV-VIS spectra were recorded with an UV1 spectrophotometer (Thermo Electron Corporation).

Syk Protein Expression

Fusion clones of the gluthathione S-transferase (GST) Syk tSH2 domain were kindly provided by Prof. Gabriel Waksman (Washington University, St. Louis, MI) [18]. The Escherichia coli

Journal of PeptideScience

strain BL21 contained the pGEX-KT vector with amino acids 10–273 of human Syk, enabling thrombin cleavage of the GST moiety. Isolation procedures were previously described [19].

Surface Plasmon Resonance Assays

SPR measurements were performed on a double channel IBISII SPR instrument (Eco Chemie, Utrecht, The Netherlands) equipped with a CM 5 sensor chip (GE Healthcare, Biacore, Uppsala, Sweden).

The sensor chip was immobilized with the native ITAM peptide as was described earlier [20]. The affinity of Syk tSH2 for the immobilized ITAM peptide (K_C) was determined by addition of Syk tSH2 in a concentration range of 0–100 nM in HBS buffer. The K_C value was calculated by fitting the equilibrium signals to a Langmuir binding isotherm.

Stock solutions of the peptide mimics **2**–**4** with a concentration of 1 mM in HBS buffer were prepared. A series of samples was prepared in HBS buffer containing 25 nM Syk tSH2 and a photoswitchable peptide mimic in various concentrations. First, competition experiments were performed with the *trans*-isomer. For this, 240 µl of a sample was irradiated with visible light for conversion to the *trans*-isomer. Then, half of the sample was injected into the SPR apparatus. The other half of the sample was irradiated with UV light of 366 nm to obtain the maximum percentage of *cis*-isomer, and subjected to the SPR assay under strict exclusion of light. K_D values have been derived from the competition experiments as was earlier described [21].

The intrinsic affinity of the *cis*-isomer ($K_{A,cis} = 1/K_{D,cis}$) was derived by applying Eqn 1, in which $K_{A,obs}$ is the observed association constant of a sample with maximal amount of *cis*-isomer, f is the fraction of compound in *cis*-configuration and $K_{A,trans}$ is the affinity assayed for the pure *trans*-isomer.

$$K_{A,obs} = f \cdot K_{A,cis} + (1 - f)K_{A,trans} \quad (1)$$

To monitor the effect of isomerization of compound **2** on binding in real time, experiments were performed with *in situ* irradiation in the SPR instrument. A solution containing 25 nM Syk tSH2 and 750 nM compound **2** in HBS buffer was prepared, and the sensograms of *trans*-**2** and *cis*-**2** were recorded. Then a mixture of Syk tSH2 and *trans*-**2** was injected again, and after 400 s the sample in the SPR cuvette was irradiated with UV light and the change in SPR signal was continuously recorded. Lastly, 25 nM Syk tSH2 and 750 nM *cis*-**2** was injected into the SPR cuvette under exclusion of light. After 400 s the sample in the SPR cuvette was irradiated with visible light for conversion to the *trans*-isomer, while the change in SPR signal was recorded.

Results and Discussion

Design

In the crystal structure of Syk tSH2 complexed with ITAM, the length of the seven intervening residues in ITAM, which are not essential for binding, was found to be 14.1–16.4 Å [18]. To approach this range, in our previous study two glycine residues were added to AMPB to obtain a linker of 18.6 Å in *trans*-configuration (compound **1**, Figure 1 and Table 1) [14]. The binding affinity of the *trans*-isomer of compound **1** for Syk tSH2 was 10-fold less than that of native γ-ITAM (K_D = 65 nM and 5.6 nM, respectively) [14,19]. The linker in *cis*-configuration (7.2 Å) was still long enough to bind divalently to both SH2 domains of Syk tSH2, resulting in an affinity (K_D 100% *cis* was calculated to be 860 nM) which is significantly better than that of a monovalent interaction (K_D = 27 µM) [14,22].

Now, a series of ITAM mimics (**2**–**4**) with large differences in linker lengths was designed, aiming at enlarging the difference in binding affinity between the *cis*- and *trans*-isomer (Figure 1 and Table 1).

ITAM mimic **2**, like **1**, was designed to bind Syk tSH2 with high affinity in the *trans*-configuration. Compound **2** has a linker lacking the two glycine residues, i.e. AMPB, which makes it smaller and more rigid than compound **1**. According to literature data, AMPB is 12.0 Å in *trans*-configuration and 6.2 Å in *cis*-configuration [16,17]. To validate the molecular simulation experiments, used for estimating the linker lengths of the other photoswitchable peptides, the linker of **2** in both configurations, i.e. *trans*-AMPB and *cis*-AMPB, was minimized using simulated annealing, which yielded similar distances (12.3 Å and 6.1 Å, respectively).

For compounds **3** and **4** the other strategy was applied: they were designed to bind Syk tSH2 with high affinity in the *cis*-configuration. In compound **3** a rigid amino propynyl benzoic acid building block was introduced on either side of AMBP, and in compound **4** also a glycine residue was added to each side of the linker. The rigidity of the building blocks should keep the two SH2 epitopes at distance. The length of the linker in *cis*-configuration was estimated for compound **3** to be 11.0 Å and for compound **4** to be 17.8 Å. Both these numbers are at the range of 14.1–16.4 Å found in the crystal structure. The linkers of **3** and **4** in *trans*-configuration, which were estimated to be respectively 23.4 Å and 30.2 Å, are well out of this range.

Synthesis and Switching of ITAM Mimics

The synthesis of the Fmoc-AMPB-OH building block, as well as the synthesis of the rigid aminopropynylbenzoic acid building block have been earlier described [13,14]. The peptides were assembled on the solid phase using Fmoc peptide chemistry. The orange solids obtained after the syntheses were subjected to preparative HPLC. The yields for the compounds **3** and **4** were low, due to uncompleted Alloc deprotections after incorporation of the alkyne containing rigid building blocks and their low solubility in aqueous solutions, which made the purification by HPLC troublesome. Nevertheless, sufficient amounts of all peptides were obtained.

Then UV-VIS spectra of the peptides were recorded by dissolving a small amount of peptide in water. First the spectrum of 100% *trans* was measured. After this, the sample was irradiated with 366 nm light for 120 s to obtain the photostationary state with maximal amount of *cis*-isomer, and the spectrum was immediately recorded (Figure 2). The peak at 335 nm, caused by *trans* $\pi-\pi^*$ transitions in AMPB, dropped after UV irradiation in the spectra of all the photoswitchable peptidomimetics. In the spectra of compounds **3** and **4** also a peak at 268 nm was present, which was caused by the rigid building block.

To establish the percentage *cis*-isomer present in the photostationary state with maximal *cis*, compound **2** was irradiated with UV light and an analytical HPLC was directly measured with a UV detector operating at the isosbestic point of 287 nm (see Supporting Information). By comparison of the obtained HPLC trace with the HPLC trace made after purification, which contained mostly *trans*-**2**, it was clear that the first peak could be assigned to *cis*-**2** and the second peak to *trans*-**2**. From the integration of both peaks, it could be calculated that 66% *cis*-isomer was present after UV irradiation. A close value of 64% *cis* is obtained from the amide signals in the ^{1}H-NMR spectra (see Supporting Information). A

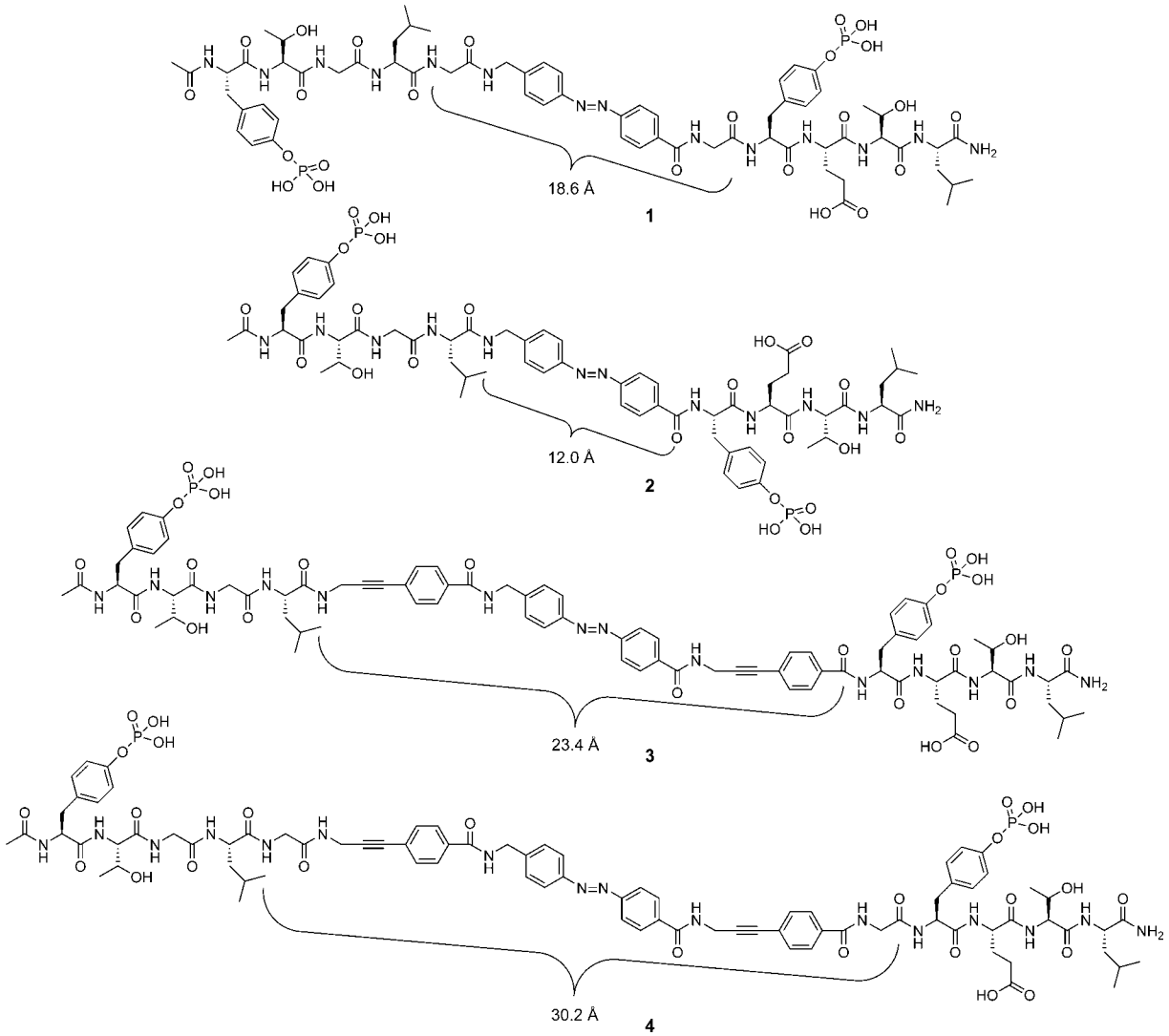

Figure 1. Photoswitchable ITAM mimics. Compound **1** was previously prepared and assayed. Peptidomimetic **2** was newly designed and possesses a smaller and more rigid linker than **1**. Compounds **3** and **4** were newly designed to obtain a higher affinity for the *cis*-isomer. The indicated distances are between the SH2 binding epitopes when azobenzene is in the *trans*-configuration.

Table 1. The distances between the SH2 binding epitopes, measured from the *N*-terminal nitrogen atom to the *C*-terminal carbon atom of the linkers, in the *cis*- and *trans*-configuration

Compound	Distance between SH2 binding epitopes (Å)	
	trans	*cis*
1	18.6	7.2
2	12.0	6.2
3	23.4	11.0
4	30.2	17.8
native ITAM	14.1–16.4	

The distances in the linker in compound **2**, which is AMPB, were obtained from literature [16,17]. The distances in the other photoswitchable compounds were estimated from simulated annealing. The distance in native ITAM was obtained from the crystal structure [18].

similar value was previously found for compound **1** (60%) and this value was also reported by others [23]. The amount of *cis* present after UV irradiation for compounds **3** and **4** was assumed to be approximately the same as for compound **2**.

Binding Studies

The interaction of the peptidomimetics with Syk tSH2 was assayed with surface plasmon resonance (SPR). The sensor chip was immobilized with native ITAM as was described earlier [20]. Then the affinity of Syk tSH2 for the immobilized ITAM peptide determined by the addition of different concentrations Syk tSH2 and the equilibrium signal was fitted to a Langmuir binding isotherm. The K_D value for binding to the chip (K_C) was found to be 7.8 nM, which is comparable to the 5.6 nM affinity found earlier [19]. Then concentration ranges of the photoswitchable peptidomimetics in the presence of 25 nM Syk tSH2 were made for SPR competition experiments. Prior to addition, the samples were irradiated with visible light or UV light (366 nm) to obtain the *trans*-isomer or the maximal amount of *cis*-isomer, respectively. After injection, the binding of tSH2 to the sensor chip was measured. From the obtained inhibition curves, the

Journal of PeptideScience

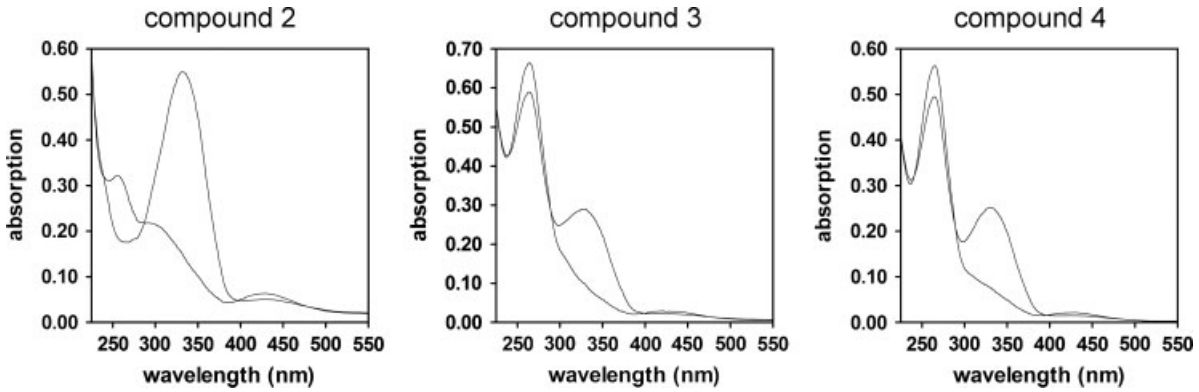

Figure 2. UV-VIS spectra of compounds **2**–**4** in water. The photoswitchable ITAM mimics **2**–**4** had a maximum at approximately 335 nm in *trans*-configuration. In the photostationary state with maximal amount of *cis*-isomer, this maximum was substantially decreased (lower curves).

Table 2. Affinities of the *cis*- and *trans*-isomer of the photoswitchable ITAM mimics for Syk tSH2 obtained from SPR competition experiments

Compound	*trans*	*cis*	
	$K_{D,trans}$ (nM)	$K_{D,obs}$ (nM)	$K_{D,cis}$ (nM)
1 [14]	65 ± 8	146 ± 11	860
2	91 ± 10	332 ± 44	>10 000
3	196 ± 35	161 ± 26	147
4	77 ± 13	56 ± 5	49

$K_{D,obs}$ is the dissociation constant of the photostationary state with maximal *cis*-isomer. $K_{D,cis}$ for compounds **2**–**4** was calculated with Eqn 1, assuming that the *cis* content of the UV irradiated samples was 66%, based on the percentage experimentally obtained for **2**.

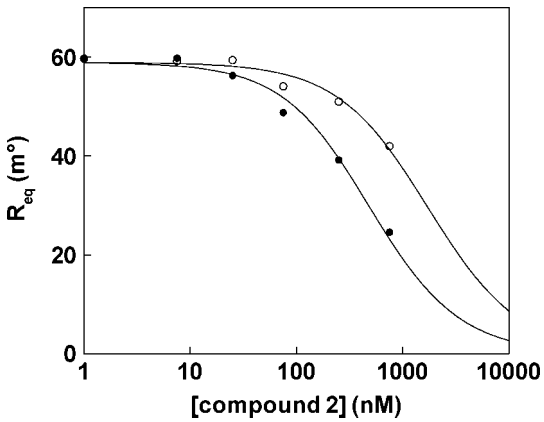

Figure 3. Inhibition curves of *cis*-**2** (○) and *trans*-**2** (●). The curves are fits according to a described model to obtain KD in solution [21].

thermodynamic dissociation constants for the interaction in solution (K_D) were obtained by fitting to a competition model (Table 2) [14,21].

As an example, the inhibition curves of both isomers of compound **2** are shown in Figure 3. The *trans*-isomer of compound **2** had a slightly lower affinity compared to compound **1** (Table 2). This was expected because the linker in compound **2** is considerably shorter than the native linker. Interestingly, the difference in binding affinity between the *cis*- and *trans*-isomer is substantially larger for compound **2**. Actually, the threefold decrease in affinity between the photostationary state with maximal *cis*-isomer and the *trans*-isomer can be accounted for by the presence of approximately one-third of *trans*-isomer. Therefore, the actual K_D value of 100% *cis*-**2** is much higher than *trans*-**2**, and can be estimated to be in the micromolar range or higher, indicating that *cis*-**2** was not interacting divalently. The affinity of a monovalent interaction amounts 27 μM [22]. Thus, the difference in affinity between the *cis*- and *trans*-isomer of compound **2** is at least 100-fold.

In contrast to the large difference in affinity between *cis*-**2** and *trans*-**2**, the compounds **3** and **4**, containing extra rigid units, displayed much less differences. *Cis*-**3** did have a somewhat higher affinity for Syk tSH2 than *trans*-**3**, as designed, but the difference was rather small. Also for compound **4** the difference was very modest. Apparently Syk tSH2 is flexible enough to adapt to both isomers of compounds **3** and **4**. This shows that a great variety of inter SH2 domain distances is possible, while retaining binding affinity.

In addition, the similar affinity of *cis*-**4** (49 nM) and *trans*-**1** (65 nM) corresponds to the similar linker length, i.e. 17.8 Å and 18.6 Å, respectively.

Compound **2** was used to investigate the ability to isomerize during SPR experiments (Figure 4). First sensograms were recorded of 25 nM Syk tSH2 with either 750 nM *trans*-**2** or *cis*-**2**. After that, *trans*-**2** was injected and when equilibrium was reached, the SPR measuring cell was irradiated with 365 nm light. Due to the UV irradiation, the stronger binding *trans*-**2** was transformed to the weaker binding *cis*-**2**, which was less capable in competing with the immobilized ITAM for Syk tSH2 binding. Therefore, the SPR signal gradually increased towards the signal of *cis*- **2**.

Also the opposite was performed: the photostationary state (*cis*-**2**) was injected and after equilibrium the sample was irradiated with visible light, causing a decrease in SPR signal towards the signal of *trans*-**2**, displacing more Syk tSH2 from the sensor surface than *cis*- **2**.

These results show that it is possible to manipulate the binding of compound **2** during an experiment. Affinity for Syk tSH2 could be increased and decreased within an acceptable short time.

Conclusions

A series of photoswitchable ITAM mimics was prepared and the binding affinity of the *cis* – and *trans*-isomer for Syk tSH2 was assayed with SPR. The smallest ITAM mimic (compound **2**)

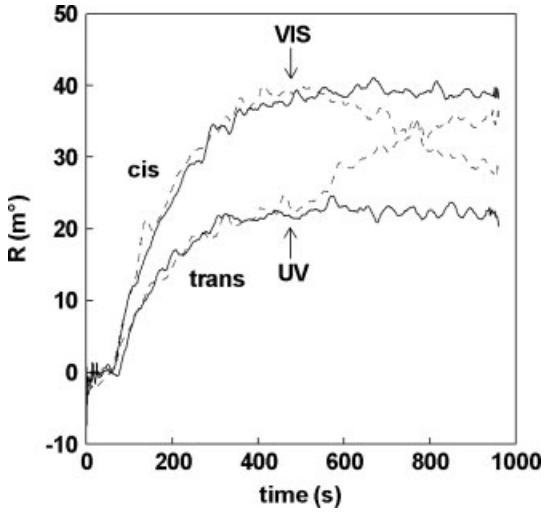

Figure 4. Real-time SPR analysis of the effect of *cis*–*trans* isomerization of compound **2** on the extent of binding to Syk tSH2 by *in situ* UV (of *trans*-**2**) or VIS (of *cis*-**2**) irradiation. These signals are 'noisier' than usually observed, because they are single cell SPR signals without the correction for a reference cell signal.

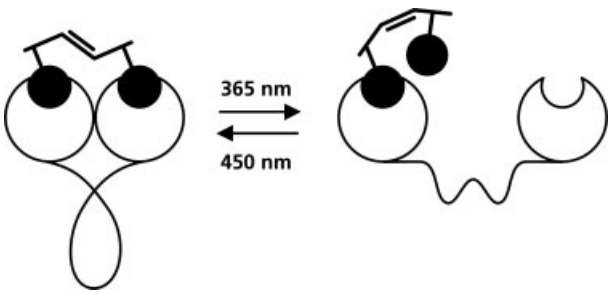

Figure 5. Syk tSH2 binds *trans*-**2** divalently (left) and *cis*-**2** monovalently (right). By irradiation of the sample with UV – or visible light, it is possible to switch between a mono- and divalent ligand for Syk tSH2 within an acceptable short time.

displayed a larger difference in binding affinity between the *cis*- and *trans*-isomer, compared to our previously reported compound **1** [14]. The *cis*-isomer of compound **2** could not bind Syk tSH2 divalently, indicating that the linker length is too small to bridge the smallest inter SH2 domain distance in Syk tSH2 (Figure 5). The affinity of *trans*-**2** for Syk tSH2 was only approximately 10-fold lower than that of native ITAM. However, azobenzene containing compounds with *trans* as the active isomer are of limited use in biological experiments, because 100% *cis* cannot be reached. In other words, when the *trans*-isomer is the active species, the difference in biological activity between the *trans*-isomer and the photostationary state with maximal *cis* is always limited by the amount of *trans* present in the photostationary state with maximal *cis*.

To create azobenzene ligands which are applicable in biological assays, it is desirable that the *cis*-isomer binds the target protein with the highest affinity. This is because it is possible to obtain 100% *trans*-isomer without 'contamination' with the active *cis*-isomer. ITAM mimics **3** and **4** were designed with this strategy in mind. However, the difference in binding affinity between the two isomers of both ITAM mimics was surprisingly small. This result is quite remarkable and can be explained by the relatively large flexibility of Syk tSH2. However, it should be emphasized that the small differences in binding affinity for compounds **3** and **4** does not mean that these are reflected in the bioactivity.

In Syk and in Zap-70, the other member of the Syk family kinases, the inter SH2 domain distance plays probably a key role in kinase activation [9,24,25]. Crystal structures of Zap-70 tSH2 show that the inter SH2 domain distance is larger when there is no ligand present, compared to tSH2 bound to native ITAM [26,27]. In the crystal structure of the inactive full-length Zap-70, the SH2-SH2 linker is in close contact with the kinase domain [24]. Changing the inter SH2 domain distance will strongly effect the conformation of the SH2-SH2 linker [28] (Figure 5) and this could be relevant for kinase activation [24,25]. For Syk kinase no crystal structure of the complete protein is available yet. However, based on the comparable function and architecture of the Syk and Zap-70 kinases, similar activation mechanisms may apply. This could mean that the *trans*-isomer of compound **3** and/or **4** maintains Syk in an inactive state, by keeping the SH2 domains far apart from each other, and that the *cis*-isomer activates kinase activity. To verify this hypothesis, the effect of these compounds on Syk kinase activity should be established in the future.

Apart from the discussion about the bioactivity of compounds **3** and **4**, it can be concluded that the ITAM mimics **3** and **4**, which were designed to bind Syk tSH2 with higher affinity in the *cis*-configuration, did not display large differences in affinity. The reason for this is the large flexibility of Syk tSH2. Nevertheless, for other less flexible proteins, this strategy could yield ligands with a large difference in binding affinity between the *trans*-isomer and the photostationary state with a maximal amount of *cis*-isomer.

Acknowledgements

Esther Pronker is kindly acknowledged for the SPR competition experiments of compounds **3** and **4**. We thank Kees Versluis (Department of Biomolecular Mass Spectrometry, Utrecht University) for recording the high-resolution mass spectrum of compound **2**.

Supporting information

Supporting information may be found in the online version of this article.

References

1 Gorostiza P, Isacoff E. Y. Optical switches for remote and noninvasive control of cell signaling. *Science* 2008; **322**: 395–399.
2 Renner C, Moroder L. Azobenzene as conformational switch in model peptides. *ChemBioChem* 2006; **7**: 868–878.
3 Mayer G, Heckel A. Biologically active molecules with a ''light switch''. *Angew. Chem. Int. Ed.* 2006; **45**: 4900–4921.
4 Ulysse L, Cubillos J, Chmielewski J. Photoregulation of Cyclic Peptide Conformation. *J. Am. Chem. Soc.* 1995; **117**: 8466–8467.
5 Aemissegger A, Hilvert D. Synthesis and application of an azobenzene amino acid as a light-switchable turn element in polypeptides. *Nat. Protoc.* 2007; **2**: 161–167.
6 Singh R, Masuda ES. Spleen tyrosine kinase (Syk) biology, inhibitors and therapeutic applications. *Annu. Rep. Med. Chem.* 2007; **42**: 379–391.
7 Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 2006; **6**: 218–230.
8 Kraft S, Kinet J-P. New developments in Fc epsilon RI regulation, function and inhibition. *Nat. Rev. Immunol.* 2007; **7**: 365–378.
9 Siraganian RP, Zhang J, Suzuki K, Sada K. Protein tyrosine kinase Syk in mast cell signaling. *Mol. Immunol.* 2002; **38**: 1229–1233.

10 Turner M, Schweighoffer E, Colucci F, Di Santo JP, Tybulewicz VL. Tyrosine kinase SYK: essential functions for immunoreceptor signaling. *Immunol. Today* 2000; **21**: 148–154.
11 Zhang J, Swaim WD, Berenstein EH, Siraganian RP. Syk protein tyrosine kinase is essential for the high affinity IgE receptor-induced morphological changes in mast cells. *J. Allergy Clin. Immunol.* 1997; **99**: 379–379.
12 Isakov N. ITIMs and ITAMs – The Yin and Yang of antigen and Fc receptor-linked signaling machinery. *Immunol. Res.* 1997; **16**: 85–100.
13 Dekker FJ, de Mol NJ, Fischer MJE, Liskamp RMJ. Amino propynyl benzoic acid building block in rigid spacers of divalent ligands binding to the Syk SH2 domains with equally high affinity as the natural ligand. *Bioorg. Med. Chem. Lett.* 2003; **13**: 1241–1244.
14 Kuil J, van Wandelen LTM, de Mol NJ, Liskamp RMJ. A photoswitchable ITAM peptidomimetic: synthesis and real time surface plasmon resonance (SPR) analysis of the effects of cis-trans isomerization on binding. *Bioorg. Med. Chem.* 2008; **16**: 1393–1399.
15 Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
16 Park SB, Standaert RF. A photoregulated ligand for the nuclear import receptor karyopherin a. *Bioorg. Med. Chem.* 2001; **9**: 3215–3223.
17 Standaert RF, Park SB. Abc amino acids: design, synthesis, and properties of new photoelastic amino acids. *J. Org. Chem.* 2006; **71**: 7952–7966.
18 Futterer K, Wong J, Grucza RA, Chan AC, Waksman G. Structural basis for syk tyrosine kinase ubiquity in signal transduction pathways revealed by the crystal structure of its regulatory SH2 domains bound to a dually phosphorylated ITAM peptide. *J. Mol. Biol.* 1998; **281**: 523–537.
19 De Mol NJ, Catalina MI, Dekker FJ, Fischer MJE, Heck AJ, Liskamp RMJ. Protein flexibility and ligand rigidity: a thermodynamic and kinetic study of ITAM-based ligand binding to Syk tandem SH2. *ChemBioChem* 2005; **6**: 2261–2270.
20 Dekker FJ, de Mol NJ, van Ameijde J, Fischer MJE, Ruijtenbeek R, Redegeld FAM, Liskamp RMJ. Replacement of the intervening amino acid sequence of a Syk-binding diphosphopeptide by a nonpeptide spacer with preservation of high affinity. *ChemBioChem* 2002; **3**: 238–242.
21 De Mol NJ, Gillies MB, Fischer MJE. Experimental and calculated shift in pK(a) upon binding of phosphotyrosine peptide to the SH2 domain of p56(lck). *Bioorg. Med. Chem.* 2002; **10**: 1477–1482.
22 Ruijtenbeek R, Kruijtzer JAW, van de Wiel W, Fischer MJE, Fluck M, Redegeld FAM, Liskamp RMJ, Nijkamp FP. Peptoid-peptide hybrids that bind Syk SH2 domains involved in signal transduction. *ChemBioChem* 2001; **2**: 171–179.
23 Rovero P, Pegoraro S, Vigano S, Amato C, Vaccari L, Balestreri E, Felicioli R. Solid-phase synthesis and dimerization of an Azobenzene-containing peptide as photoisomerizable proteinase-inhibitor. *Lett. Pept. Sci.* 1995; **2**: 27–32.
24 Deindl S, Kadlecek TA, Brdicka T, Cao X, Weiss A, Kuriyan J. Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. *Cell* 2007; **129**: 735–746.
25 Au-Yeung BB, Deindl S, Hsu LY, Palacios EH, Levin SE, Kuriyan J, Weiss A. The structure, regulation, and function of ZAP-70. *Immunol. Rev.* 2009; **228**: 41–57.
26 Folmer RHA, Geschwindner S, Xue Y. Crystal structure and NMR studies of the apo SH2 domains of ZAP-70: two bikes rather than a tandem. *Biochemistry* 2002; **41**: 14176–14184.
27 Hatada MH, Lu XD, Laird ER, Green J, Morgenstern JP, Lou MZ, Marr CS, Phillips TB, Ram MK, Theriault K, Zoller MJ, Karas JL. Molecular-basis for interaction of the protein-tyrosine kinase Zap-70 with the T-Cell receptor. *Nature* 1995; **377**: 32–38.
28 Catalina MI, Fischer MJE, Dekker FJ, Liskamp RMJ, Heck AJR. Binding of a diphosphorylated-ITAM peptide to spleen tyrosine kinase (Syk) induces distal conformational changes: a hydrogen exchange mass spectrometry study. *J. Am. Soc. Mass. Spectrom.* 2005; **16**: 1039–1051.

