Replacement of the Intervening Amino Acid Sequence of a Syk-Binding Diphosphopeptide by a Nonpeptide Spacer with Preservation of High Affinity

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molecular recognition · peptides · SH2 domains · signal transduction · Syk

Communication within the cell occurs through a dynamic network of interacting proteins that is amazingly complex. The increasing number of signaling proteins that is being discovered underlines this complexity. A family of functional modules often found in these signaling proteins comprises the Src homology-2 (SH2) domains.[1, 2] A special member of this family is the Syk tandem SH2 domain, which is part of a protein tyrosine kinase called Syk. This kinase has a role in signal transduction in various immune cells,[3] amongst which the best-defined is its role in signal transduction in mast cell activation.[4, 5] A crucial event in mast cell activation is binding of the Syk tandem SH2 domain to the diphosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of the γ chain from the high-affinity receptor for immunoglobulin E (FcεRI). This ITAM features the consensus sequence pTyr-Xxx-Xxx-Leu-$(Xxx)_{6\text{-}7}$-pTyr-Xxx-Xxx-Leu (pTyr = phosphotyrosine, Xxx = undefined amino acid residue).

The interaction of the ITAM peptide with the Syk tandem SH2 domain has a divalent character, which is apparent from the low affinity of the domain for monophosphorylated peptides in contrast to its high affinity for the diphosphorylated ITAM peptide.[6–8] This divalent character represents the simplest form of multivalency, a phenomenon that is characterized by multiple simultaneous interactions between ligand and receptor.[9] The structural basis of the divalent interaction between the ITAM peptide and the Syk tandem SH2 domain has been published by Fütterer et al.,[10] who elucidated the crystal structure of the human Syk tandem SH2 domain complexed with the CDε-chain ITAM peptide (Protein databank entry code 1A81). This structure shows that the phosphorylated tetrapeptide sequences (underlined) in the human ITAM peptide pTyr-Glu-Pro-Ile-Arg-Lys-Gly-Gln-Arg-Asp-Leu-pTyr-Ser-Gly-Leu are in tight contact with the Syk protein, whereas the seven intervening amino acids make little contact (Figure 1 A). From this it may be assumed that the intervening amino acids contribute little to the overall binding, which suggests that molecular constructs might be prepared in which the interacting tetrapeptides are connected in a different way, that is, by using a nonpeptide spacer (Figure 1 B).

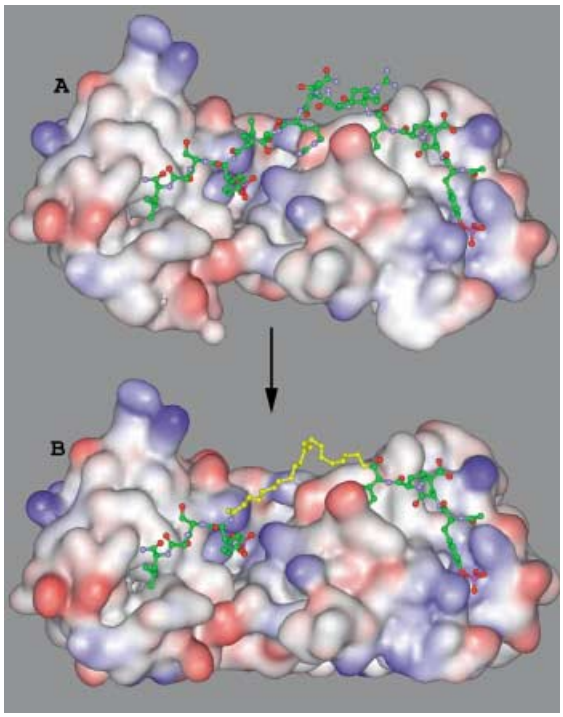

Figure 1. *Crystal structure of the human Syk tandem SH2 domain as published by Fütterer et al.[10] (Protein Databank entry code 1A81). A) The ITAM peptide pTyr-Glu-Pro-Ile-Arg-Lys-Gly-Gln-Arg-Asp-Leu-pTyr-Ser-Gly-Leu complexed with the Syk protein (binding site shown); B) the proposed conversion of the seven intervening amino acids into an oligoethylene glycol spacer.*

Recently, we reported conversion of the monophosphorylated peptide Ac-pTyr-Glu-Thr-Leu-NH_2 (**1**) into a peptoid–peptide hybrid (Scheme 1).[11] Retention of the ability to bind the Syk tandem SH2 domain was demonstrated when the Thr and Leu amino acid residues were converted into the corresponding peptoid residues **2**. However, the affinity of the monophosphorylated peptide for the Syk tandem SH2 domain is modest ($K_d \approx 27$ μM). An attempt was made to link two monophosphorylated tetrapeptides by a nonpeptide spacer in such a way that the affinity of the original diphosphorylated ITAM peptide is approached. To connect the monophosphorylated peptides, an oligoethylene glycol spacer was used, which has been applied successfully as a spacer between interacting parts for other biomolecules.[12–18] This type of spacer is nontoxic, metabolically stable, and hydrophilic, and thus does not give rise to hydro-

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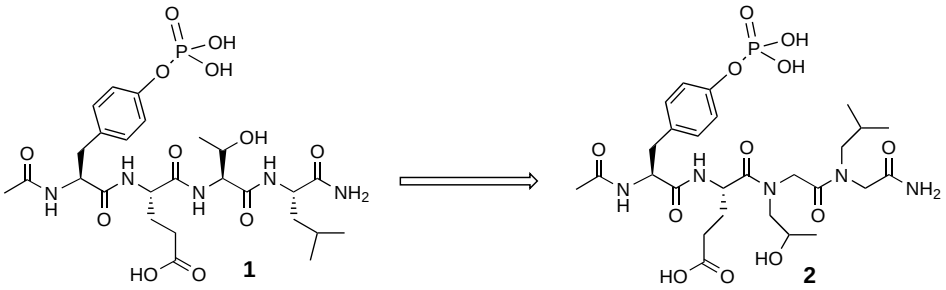


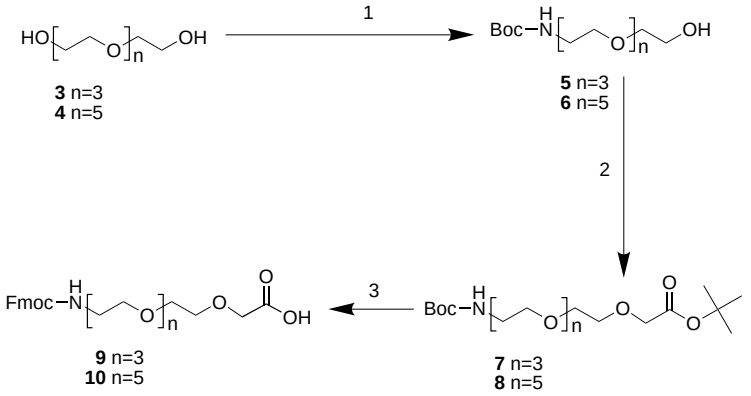
Scheme 1. *Conversion of a peptide into a peptide–peptoid hybrid. For reactions conditions, see Ref. [11] by Ruijtenbeek et al.*

phobic collapse. Here we show that a high-affinity compound can be constructed by linking two relatively weakly interacting monophosphorylated peptides by an oligoethylene glycol spacer. Thus, the intervening amino acid sequence in the original ligand can be completely substituted by a nonpeptide entity.

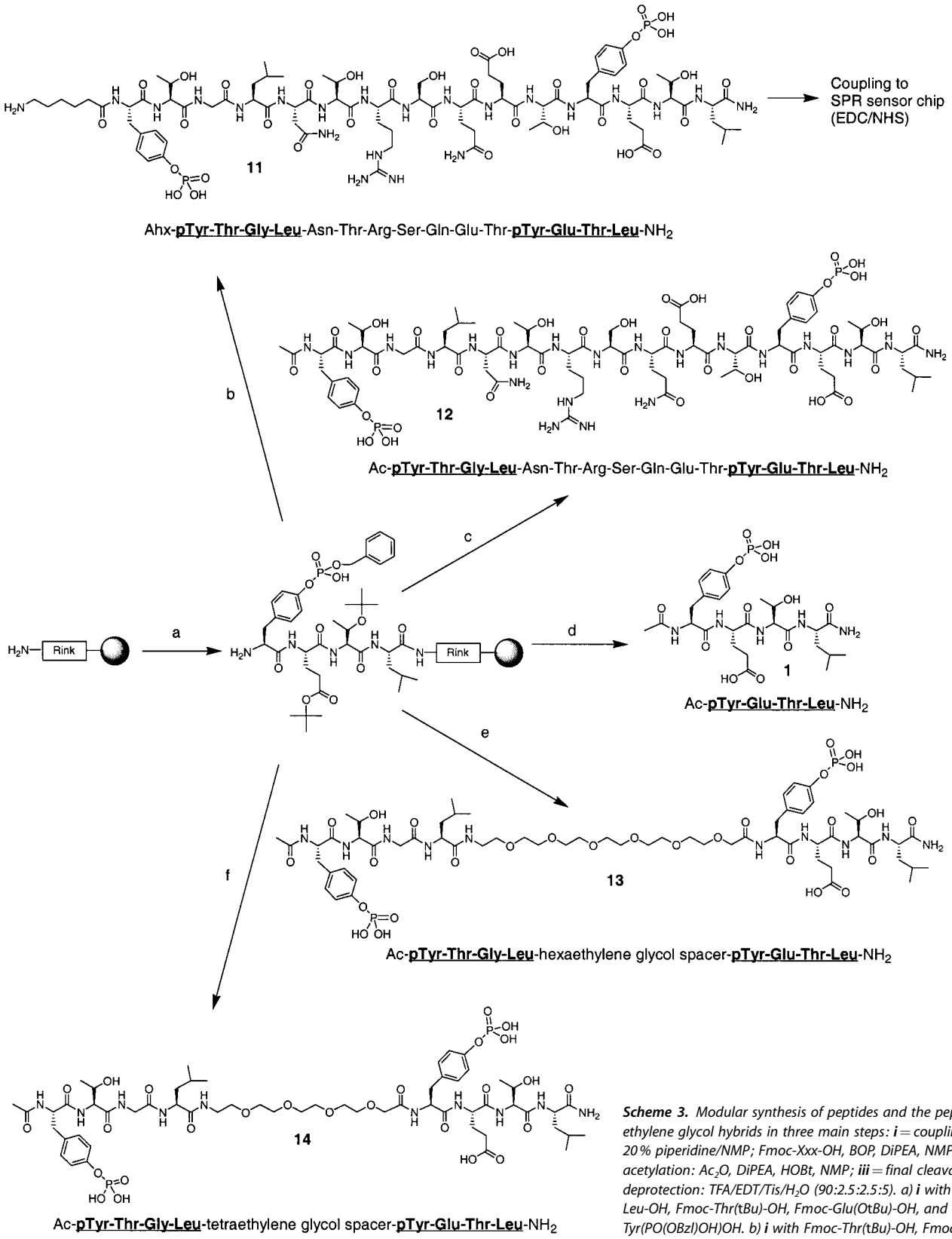
The design of our spacers started with a crystal structure in which one crystallographic unit contains six different copies of the complex consisting of the tandem SH2 domain and the ITAM peptide.[10] The distance between the interacting parts in the six different copies varied from 14.2 – 16.4 Å. One of these copies was more or less arbitrarily selected as a starting point for our design. Spacers of different length were constructed and incorporated between the phosphorylated tetrapeptides by using the MacroModel 7.0 program.[19] These molecular constructs were subjected to energy minimization, while the positions of the phosphorylated tyrosines were retained. The MMFF94 forcefield,[20] as found in MacroModel, was used with water as an implicit solvent in the GB/SA solvation model.[21] A hexaethylene glycol spacer as in molecular construct **13** (Scheme 3) was selected because its length corresponds exactly to the chain of seven intervening amino acids, which comprise a turn. Furthermore, a tetraethylene glycol spacer as in molecular construct **14** was chosen because its length corresponds to the shortest possible distance between the two phosphorylated tetrapeptides.

In order to prepare the required spacers, hexa- and tetraethylene glycol were converted into amino acid superstructures (compounds containing an amino group and a carboxyl moiety; Scheme 2). Monotosylation of tetra- and hexaethylene glycol was realized by using excess (4 equiv) quantities of **3** and **4**, respectively. After conversion into the azide by using sodium azide in DMF, the corresponding amines were obtained by catalytic hydrogenation and subsequently protected with a *tert*-butyloxycarbonyl (Boc) group to give **5** and **6**. An acetate moiety was introduced at the remaining free hydroxy group (**7** and **8**) by using *tert*-butylbromoacetate and sodium hydride in DMF. Cleavage of the protecting groups with hydrochloric acid in ether/dichloromethane followed by reprotection of the amine with a 9-fluorenylmethyloxycarbonyl (Fmoc) group gave compounds **9** and **10**. Next, peptides **1**, **11**, and **12**, as well as the peptide hybrids **13** and **14** were synthesized on the solid phase starting from Fmoc Rink amide resin (Argogel; Scheme 3). Benzotriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), *N*,*N*-diisopropylethylamine (DiPEA), and Fmoc amino acids were used for the couplings, which were monitored with the Kaiser test. The phosphotyrosine residue was incorporated as the monobenzyl-protected building block (Fmoc-Tyr(PO(OBzl)OH)-OH). After cleavage and deprotection, the peptides and peptide hybrids were purified by preparative HPLC. The purity of the peptides was verified by a Shimadzu automated HPLC system with an Alltech Adsorbosphere XL column (C_8 90 Å 5U) and detection at 220 and 254 nm. Elution was performed with a gradient from 100% buffer A (15mM triethylamine/phosphate buffer, pH 6) to 10% buffer A and 90% acetonitrile. Final characterization was performed by high resolution mass spectrometry and NMR spectroscopy. Mass spectrometry analysis was carried out as described in ref. [11] and is reported in Table 1. ^{1}H NMR spectra were recorded on a Varian Inova spectrometer (500MHz) in H_2O/D_2O 9:1 with 4mM peptide and 20 mM phosphate buffer (pH 7).

In order to determine the affinity of the phosphopeptides **1** and **12** and the phosphopeptide hybrids **13** and **14** for the Syk tandem SH2 domain, the tandem SH2 domain of murine Syk was cloned, expressed, and purified as the glutathione S-transferase (GST) fusion protein.[11] This protein was used for a surface plasmon resonance (SPR) assay as described in ref. [11]. In this assay, the peptide featuring the ITAM sequence was extended with an N-terminal 6-aminohexanoic acid (Ahx) moiety to provide a spacer between the SPR sensor chip and the peptide. The peptide Ahx-pTyr-Thr-Gly-Leu-Asn-Thr-Arg-Ser-Gln-Glu-Thr-pTyr-Glu-Thr-Leu-NH_2 was covalently coupled to a Biacore carboxymethyldextran-coated sensor chip (CM5). The dissocia-



Scheme 2. *Synthesis of the ethylene glycol spacers as amino acid building blocks. 1) a. Tosyl-Cl (0.25 equiv), Et_3N (0.5 equiv), CH_2Cl_2; 75 – 80% yield based on Tosyl-Cl; b. NaN_3, DMF; Pd/C, H_2, ethanol/H_2O; Boc_2O, NaOH, dioxane/H_2O; overall yield 75%; 2)* tert*-butylbromoacetate, NaH, DMF; 25%; 3) a. HCl/diethylether, CH_2Cl_2; b. Fmoc-O-Su, Et_3N, CH_3CN/H_2O; overall yield 75%. Tosyl = p-toluenesulfonyl, Su = succinimide.*



Scheme 3. *Modular synthesis of peptides and the peptide-ethylene glycol hybrids in three main steps:* ***i*** *= coupling cycle: 20% piperidine/NMP; Fmoc-Xxx-OH, BOP, DiPEA, NMP;* ***ii*** *= acetylation: Ac_2O, DiPEA, HOBt, NMP;* ***iii*** *= final cleavage and deprotection: TFA/EDT/Tis/H_2O (90:2.5:2.5:5). a)* ***i*** *with Fmoc-Leu-OH, Fmoc-Thr(tBu)-OH, Fmoc-Glu(OtBu)-OH, and Fmoc-Tyr(PO(OBzl)OH)OH. b)* ***i*** *with Fmoc-Thr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, and Fmoc-Tyr(PO(OBzl)OH)-OH, Fmoc-εAhx-OH;* ***iii.*** *c)* ***i*** *with Fmoc-Thr(tBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH, and Fmoc-Tyr(PO(OBzl)OH)-OH;* ***ii; iii.*** *d)* ***ii; iii.*** *e)* ***i*** *with* ***10****, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH and Fmoc-Tyr(PO(OBzl)OH)-OH;* ***ii; iii.*** *f)* ***i*** *with* ***9****, Fmoc-Leu-OH, Fmoc-Gly-OH, Fmoc-Thr(tBu)-OH and Fmoc-Tyr(PO(OBzl)OH)-OH;* ***ii; iii.*** *Bzl = benzyl, Trt = trityl = triphenylmethyl, EDC = 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide, HOBt = 1-hydroxy-1H-benzotriazole, TFA = trifluoroacetic acid, EDT = ethylenedithiothreol, Tis = triisopropylsilane, NHS = N-hydroxysuccinimide, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-S-sulfonyl, tBu = tert-butyl.*

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Table 1. *High-resolution mass measurements of the phosphopeptides and phosphopeptide hybrids.*

Compound	Calculated [$M+2H^+$]	Found [$M+2H^+$]
1	646.250	646.251
11	2048.89	2048.92
12	1977.82	1977.84
13	1482.62	1482.58
14	1394.56	1394.52

tion constant for interaction of the peptide on the chip with the Syk tandem SH2 domain was 6($\pm$1.7) nM ($n = 4$, measured at equilibrium). The affinities of **1**, **12**, **13**, and **14** for the Syk tandem SH2 domain were measured in competition experiments (Table 2, Figure 2; $n = 3$, concentration of Syk tandem SH2 GST = 100 nM). The concentrations of the test compounds that gave 50% inhibition of the binding of the Syk tandem SH2 domain to the chip-coupled peptide (IC_{50} values) were derived by nonlinear curve fitting to a sigmoid function by using the SlideWrite Plus program.

Table 2. *Affinities of experimental compounds for the Syk tandem SH2 domain.*

Compound	IC_{50} [μM]
12	0.38 $\pm$ 0.03
13	1.8 $\pm$ 0.1
14	75 $\pm$ 14
1	598 $\pm$ 45

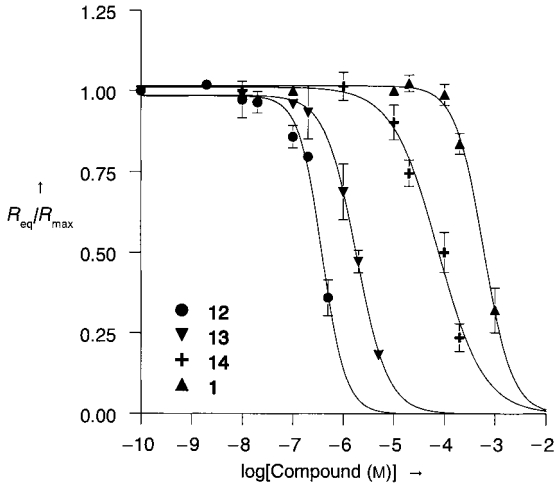

Figure 2. *Affinities of phosphopeptides **1** and **12** and of phosphopeptide hybrids **13** and **14** for the Syk tandem SH2 domain (n = 3).*

The results presented in Table 2 confirm the previously reported 1000-fold difference in binding strengths between the monophosphorylated peptide **1** and diphosphorylated peptide **12**.[6–8] The molecular construct with the tetraethylene glycol spacer **14** showed only a moderately improved affinity for the Syk tandem SH2 domain compared to the monophosphorylated peptide **1**. Favorably, the molecular construct possessing the hexaethylene glycol spacer **13** showed an affinity comparable to the native diphosphorylated ITAM peptide **12**. Apparently the hexaethylene glycol spacer is long enough to position the phosphorylated tetrapeptides properly for interaction with the Syk tandem SH2 domain. Moreover, the high binding affinity of this compound provides unambiguous proof that the seven intervening amino acids do not contribute significantly to binding. In contrast, molecular construct **14**, with the tetraethylene glycol spacer, showed a significantly lower affinity for the Syk tandem SH2 domain as compared to molecular construct **13**. Modeling suggested that the tetraethyleneglycol spacer in molecular construct **14** is long enough to bridge the intervening amino acids. However, to achieve this it has to assume a fully extended conformation; this would require a considerable reduction of flexibility upon binding, resulting in a large entropy loss that is unfavorable for binding.

To our knowledge this is the first time that two interacting phosphopeptides have been linked by a nonpeptide spacer to give rise to a divalent interaction with an affinity that is comparable to that of the native diphosphorylated ITAM peptide. A nonpeptide spacer can clearly substitute the intervening amino acids in the native Syk tandem SH2 domain binding ligand. Furthermore, it shows that multivalency, even in its most simple form as a divalent interaction, is crucial for high affinity.

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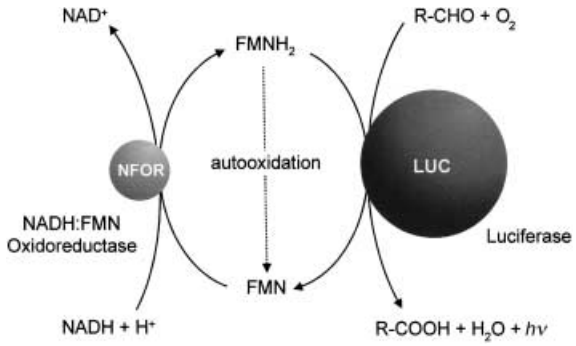

Figure 1. *Schematic representation of the bienzymic reaction cascade catalyzed by NAD(P)H:FMN Oxidoreductase (NFOR) and Luciferase (Luc). R = $CH_3(CH_2)_{10}$.*

DNA-Directed Assembly of Bienzymic Complexes from In Vivo Biotinylated NAD(P)H:FMN Oxidoreductase and Luciferase

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The DNA-directed assembly of proteins offers a promising route to the generation of spatially ordered multienzyme complexes (MECs), which are not accessible by conventional chemical cross-linking or genetic engineering.[1] MECs with several catalytic centers arranged in a spatially defined way are abundant in nature. Mechanistic advantages of MECs are revealed during the multistep catalytic transformation of a substrate since reactions limited by the rate of diffusional transport are accelerated by the immediate proximity of the catalytic centers. Furthermore, the "substrate-channeling" of intermediate products avoids side reactions. Artifical multienzymes would allow the development of novel catalytic systems for enzyme process technology that are capable of regenerating cofactors,[2] as well as multistep chemical transformations;[3–5] they are also useful for exploration of proximity effects in biochemical pathways.

Herein we report the initial steps towards the development of artificial multienzyme complexes through the DNA-directed assembly of two enzymes, NAD(P)H:FMN Oxidoreductase (NFOR) and Luciferase (Luc), which catalyze two consecutive reaction steps (Figure 1). NFOR reduces flavin mononucleotide (FMN) to $FMNH_2$ by using nicotinamide adenine dinucleotide (NADH) as an electron donor. $FMNH_2$ dissociates from NFOR and binds to Luc. In a second step, dodecanal is oxidized by Luc with the aid of molecular oxygen and emits blue-green light.[6] The spontaneous autooxidation of $FMNH_2$ may take place as a competing side reaction.

To facilitate the DNA-directed organization of NFOR and Luc, we chose a modular construction approach, based on the high-affinity biotin – streptavidin coupling system (Figure 2). To avoid damage of the enzymes through chemical biotinylation procedures, and to improve the regioselective coupling of the DNA and the protein moieties, we employed the in vivo biotinylated

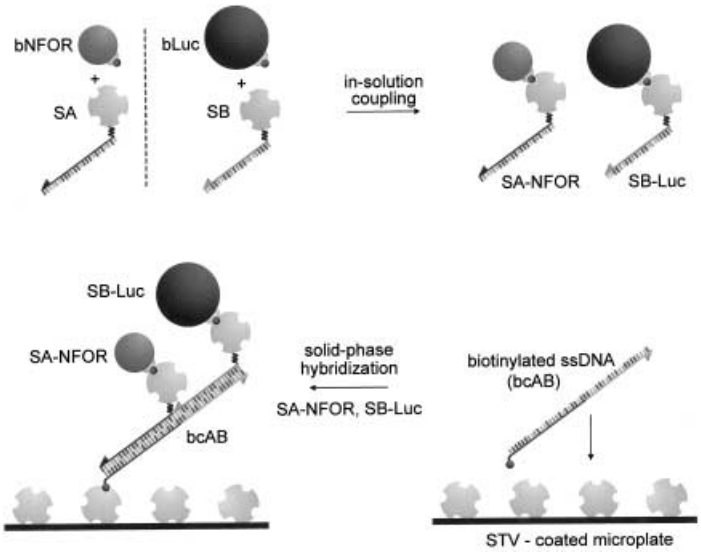

Figure 2. *Schematic representation of the DNA-directed organization of NFOR and Luc. A modular construction approach was chosen which used in-vivo biotinylated recombinant enzymes, bNFOR and bLuc, each with a single biotin group attached at the amino terminus. The biotinylated enzymes were coupled with covalent DNA – streptavidin (STV) conjugates, SA or SB, and the resulting enzyme – DNA conjugates (SA-NFOR and SB-Luc shown) self-assemble on a single-stranded DNA carrier containing complementary sequence stretches (for example, bcAB). The 3′-ends of the DNA fragments are indicated by arrow heads. Note that the schematic drawing of the SA – NFOR and SB – Luc is simplified, since conjugate species of other stoichiometry are also present in the assembly reaction.*

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