Designed, Folded Polypeptide Scaffolds That Combine Key Biosensing Events of Recognition and Reporting

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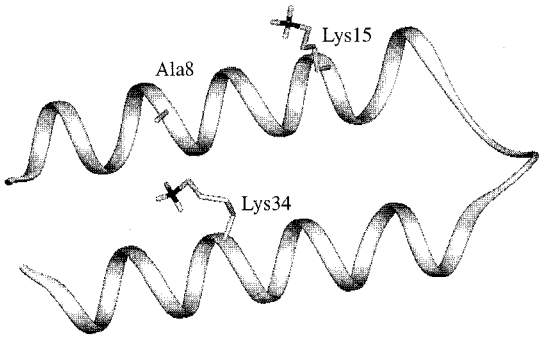
The de novo design of folded polypeptides aims at improving our understanding of protein structure and also provides a platform for the engineering of new proteins with tailored functions.[1−3] Designed, folded polypeptides that undergo pH-controlled, site-selective self-functionalization with ligands[4,5] constitute an excellent toolbox for the construction of various complex molecular systems, e.g., model glycoproteins[6,7] or complex receptors. With a focus on the development of functional units in biosensors, we present in this paper, folded, ligand-modified helix−loop−helix polypeptide scaffolds that connect the key biosensing events of recognition and reporting. The well-characterized interaction between the enzyme human carbonic anhydrase II (HCAII) and its benzenesulfonamide inhibitor[8] (4-carboxybenzenesulfonamide, **Ia**) was selected for a proof of principle demonstration. However, the variety of molecules that can be incorporated in the polypeptides and the ease by which their relative positions can be varied allow for a systematic development of biosensing units for a wide range of receptor−ligand systems.

The design of the peptides KE2 and KE3 (Figure 1) was based on the sequence of LA-42b, a 42-residue polypeptide that folds into a helix−loop−helix motif and dimerizes to form a four-helix bundle in aqueous solution.[6] Out of 42 residues, more than 32 were conserved in the design of KE2 and KE3. The solution structure of LA-42b has been extensively studied by NMR and CD spectroscopy, and because of the sequence similarity with KE2 and KE3, they were assumed to fold into helix−loop−helix dimer motifs as well. KE2 and KE3 were synthesized using solid-phase peptide synthesis and



KE2:

1 15 19
N-A-A-D-L-E-A-A-I-R-H-L-A-E-**K**-L-A-A-R
20 23
-G-P-V-D
42 34 24
G-A-R-A-F-A-E-F-**K**-K-A-L-Q-E-A-L-Q-A-A

KE3:

1 8 15 19
N-A-A-D-L-E-A-**K**-I-R-H-L-A-E-**K**-L-A-A-R
20 23
-G-P-C-D
42 24
G-A-R-A-F-A-E-F-R-R-A-L-Q-E-A-L-Q-A-A

Figure 1. Modeled structure of KE2 and KE3, showing sites of introduction of dansyl (position 15) and benzenesulfonamide (positions 34 and 8, respectively). Only the amino acid side chains in positions involved in functionalization are shown and only those in the sequence of KE2. Amino acid sequences of KE2 and KE3, where lysine residues in bold represent sites of modification, are also shown. The amino terminals of both peptides were acetylated.

identified by mass spectrometry. The MALDI-TOF spectra of KE2 and KE3 with Cys22 protecting group intact showed single peaks at 4446.3 and 4563.2, respectively (calcd 4446.0 and 4563.2). The peptides were designed to allow for a site-specific incorporation of a fluorescent probe at the side chain of Lys15, as well as of a ligand with high affinity for a target protein at the side chains of Lys34 (KE2) or Lys8 (KE3). The side chain of Lys15 was orthogonally protected to allow the coupling of a fluorescent probe on the solid phase. Before cleaving of the peptide from the resin, the Lys15 Alloc protection group was removed by 3 equiv of $Pd(PPh_3)_4$ in a mixture of 9.25 mL of$CHCl_3$, 0.5 mL of AcOH, and 0.25 mL of morpholine. Reaction of the selectively deprotected peptides with 2 equiv of dansyl chloride in the presence of 8 equiv of diisopropylethylamine in DMF provided KE2-P and KE3-P. The MALDI-TOF spectra of KE2-P and KE3-P showed single peaks at 4680.4 and 4868.2, respectively (calcd 4679.4 and 4867.6). The notation -P indicates that a fluorescent probe has been covalently attached, and the notation -PL indicates the attachment of both fluorescent probe and high-affinity ligand. In all experiments Cys22 remained protected.

The incorporation of the benzenesulfonamide ligand was accomplished by reacting the polypeptides with the active ester **Id** in aqueous solution at pH 8. We have previously shown that, after Lys15, Orn34 is the most reactive of all lysine and ornithine residues in LA-42b[9] in terms of its reactivity toward active esters, because it

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(1) DeGrado, W. F.; Summa, C. M.; Pavone, V.; Nastri, F.; Lombardi, A. *Annu. Rev. Biochem.* **1999**, *68*, 779−819.
(2) Micklatcher, C.; Chmielewski, J. *Curr. Opin. Chem. Biol.* **1999**, *3*, 724−729.
(3) Baltzer, L.; Nilsson, H.; Nilsson, J. *Chem. Rev.* **2001**, *101*, 3153−3163.
(4) Broo, K.; Brive, L.; Lundh, A. C.; Ahlberg, P.; Baltzer, L. *J. Am. Chem. Soc.* **1996**, *118*, 8172−8173.
(5) Baltzer, L.; Nilsson, J. *Curr. Opin. Biotechnol.* **2001**, *12*, 355−360.
(6) Andersson, L.; Stenhagen, G.; Baltzer, L. *J. Org. Chem.* **1998**, *63*, 1366−1367.
(7) Andersson, L. K.; Dolphin, G. T.; Kihlberg, J.; Baltzer, L. *J. Chem. Soc. Perkin Trans. 2* **2000**, 459−464.
(8) Eriksson, A. E.; Kylsten, P. M.; Jones, T. A.; Liljas, A. *Proteins* **1988**, *4*, 283−293.

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is situated in a position that forms part of the hydrophobic core and has a selectively depressed pK_a value.[10] Some competition from Lys19 was observed[9,10] and therefore Lys19 was replaced by Arg19 in the sequence of KE2. Lys10 was also replaced by an Arg residue. No competition from Lys33 was expected on the basis of the previous investigation, and it was therefore not removed. In KE3, where Lys8 was introduced, all competing lysines were replaced by Arg residues.

The affinity of HCAII for unprotonated **Ia** in aqueous phosphate buffer at room temperature and pH 6.5 has been reported previously, and the equilibrium dissociation constant K_d was 27 μM.[11] Preliminary affinity measurements by surface plasmon resonance suggested that K_d for KE2-PL modified with **Ia** was in the μM range, and we reasoned that, in order to avoid competition from nonspecific binding of HCAII, a higher affinity ligand than that corresponding to **Ia** was needed. With benzenesulfonamide derivatives bearing alkyl chains of different lengths in the para position, increased affinities toward HCAII as compared to that of **Ia** have been reported.[12] Thus, to increase the affinity of the benzenesulfonamide inhibitor, and to minimize sterical constraints upon HCAII binding that could be introduced by coupling the benzenesulfonamide ligand to the peptide, an aliphatic spacer was introduced. The *N*-hydroxysuccinimidyl ester of 4-carboxybenzenesulfonamide (**Ib**) was reacted with 6-aminohexanoic acid to form **Ic**, which was further activated with *N*-hydroxysuccinimide to form **Id**. **Id** (1.4 equiv) was allowed to react with 1 equiv of KE2-P or KE3-P in 50 mM Tris-HCl buffer at pH 8.0 and room temperature. The modified peptides KE2-PL and KE3-PL were purified by reversed-phase HPLC on a Hichrom C-8-column using 0.1% TFA in 40% aqueous 2-propanol as the eluent and lyophilized. The yield was 55% and 77% for KE2-PL and KE3-PL, respectively. The MALDI-TOF spectra of KE2-PL and KE3-PL showed single peaks at 4977.2 and 5165.0, respectively (calcd 4975.7 and 5163.9).

The biosensing capabilities of KE2-PL and KE3-PL were investigated by recording their fluorescence emission spectra between 450 and 650 nm upon excitation at 335 nm. Spectra were recorded of 1 μM peptide solutions in HBS buffer (10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20) at pH 7.4 and 298 K, in the absence and presence of 50 μM HCAII, with 1 μM solutions of KE2-P and KE3-P as negative controls (Figure 2). Upon addition of HCAII, the fluorescence intensities of KE2-PL and KE3-PL increased by 80% and 60%, respectively, whereas the fluorescence intensities of the control peptides were not affected by HCAII. The observed intensity increases were thus caused by the binding of HCAII to the benzenesulfonamide moiety of the peptide scaffolds, with negligible effects induced by nonspecific protein−peptide interactions. We interpret the intensity increases to arise from a change in the molecular environment of the dansyl group upon binding of HCAII by the polypeptides; it appears that the probe is partially quenched in the unbound peptide, but to a lesser extent when bound to HCAII. The polypeptides KE2-PL and KE3-PL are therefore capable of reporting on the presence of the target protein HCAII. Recognition is ensured by the specificity of the benzenesulfonamide ligand.

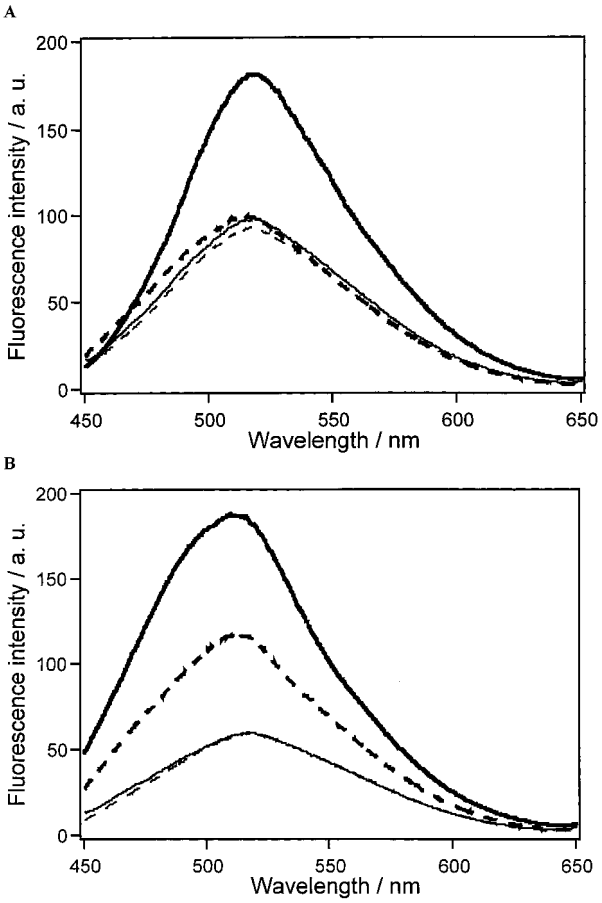


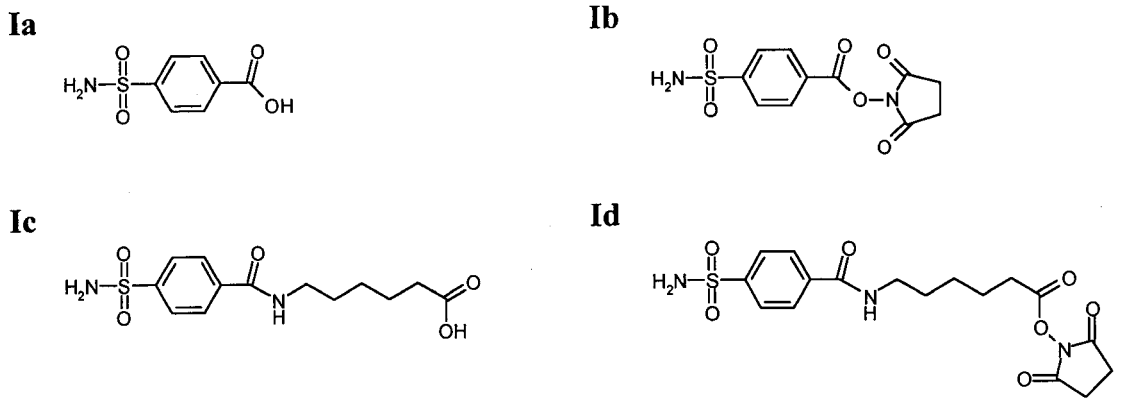
Figure 2. Fluorescence spectra of 1 μM KE2 (A) and KE3 (B) modified with the dansyl fluorescent probe. Bold and thin curves correspond to peptides with and without benzenesulfonamide, respectively (KE2-PL, KE3-PL; KE2-P, KE3-P). Dotted lines represent spectra in the absence of HCAII, and solid lines represent spectra where 50 μM HCAII has been added. The fluorescence of the control peptides KE2-P and KE3-P is not affected by the presence of HCAII, but the fluorescence of KE2-PL is increased by 80%. KE3-PL displays a similar but less pronounced behavior.

Upon titration of 1 μM KE2-PL with 5 nM−50 μM HCAII, a sigmoid curve was obtained (Figure 3). For the bimolecular association between KE2-PL and HCAII, the equilibrium dissociation constant K_d equals the concentration of free HCAII at the inflection point. K_d was estimated to be 0.02 μM from the best fit to the experimental results of an equation describing the dissociation of a bimolecular complex. This result constitutes the proof of principle for functional helix−loop−helix-based biosensor units, since binding results in fluorescence intensity changes. The use of an array of peptides modified with ligands of different affinities makes measurements of analyte concentrations possible, at levels of accuracy limited, in principle, only by the number of different ligand variants available and by the affinity range of those variants.

The CD spectra of 1 μM solutions of KE2-P and KE3-P in 10 mM phosphate buffer at pH 7.5 revealed a higher degree of helical content than that of the template peptide

(9) Andersson, L. K.; Dolphin, G. T.; Baltzer, L. *ChemBioChem* In press
(10) Andersson, L. K.; Caspersson, M.; Baltzer, L. *Chem. Eur. J.* In press
(11) Taylor, P. W.; King, R. W.; Burgen, A. S. V. *Biochemistry* **1970**, *9*, 2638−2645.
(12) King, R. W.; Burgen, A. S. V. *Proc. R. Soc. London B* **1976**, *193*, 107−125.

Scheme 1



LA-42b at comparable concentrations.[7] The mean residue ellipticity $[\theta]_{222}$ was $-19\,100 \pm 1000$ and $-17\,300 \pm 1000$ deg cm^2 dmol^{-1} for 1 μM KE2-P and KE3-P, respectively, and that of 2 μM LA-42b was -8000 ± 1000 deg cm^2 dmol^{-1}. The large difference between the KE-P peptides and LA-42b suggests that the probe has an effect on helix stability, possibly due to interactions between the probe and the hydrophobic core of the peptides, or due to the removal of a positive charge upon probe attachment.[7] Difference CD spectra obtained by subtracting that of 2 μM HCAII from that of 1 μM KE2-PL + 2 μM HCAII showed that the helicity of KE2-PL was unchanged upon binding to HCAII. At these concentrations, and based on a dissociation constant of 0.02 μM, more than 90% of the peptide is bound to HCAII.

There was a significant difference between the fluorescence intensities of KE3-P and KE3-PL in the absence of HCAII. The attachment of a ligand in position 8 close to the probe in position 15 may change the peptide structure enough to decrease the quenching. This hypothesis is supported by the observed differences in helical contents between 1 μM KE3-P and 1 μM KE3-PL, $[\theta]_{222} = -17\,300 \pm 1000$ and $-24\,700 \pm 1000$ deg cm^2 dmol^{-1}, respectively.

The affinity obtained from fluorescence spectroscopy was compared to that measured with surface plasmon resonance, where the interaction between immobilized HCAII and KE2-PL in solution was monitored for a series of peptide concentrations ranging from 40 nM to 10 μM. The steady-state affinity was estimated to be 0.08 μM from curve fitting to a plot of equilibrium responses as a function of peptide concentration using a 1:1 binding model. Slightly nonideal behavior was observed, which might be explained by an influence on the affinity by concentration-dependent supersecondary structure formation of the peptide. The steady-state affinity of HCAII for **Ic** was also determined and K_d was found to be 0.044 μM. The results suggest that the affinity of HCAII for the ligand decreases due to steric effects when the ligand is attached to the peptide. An even longer spacer might be useful to increase the affinity of HCAII for the peptide even further. Lower affinity was observed with the surface plasmon resonance-based assay than with the solution-based fluorescence method. This may reflect steric repulsion and/or orientational restrictions in the presentation of the enzyme active site when HCAII was immobilized. Similar results have been reported previously for the benzenesulfonamide−carbonic anhydrase system.[13]

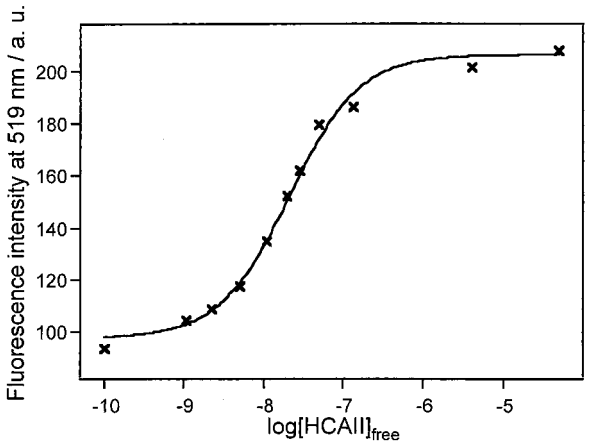


Figure 3. Sigmoid binding curve obtained upon titration of 1 μM KE2-PL with HCAII by plotting the maximum fluorescence intensity versus the logarithm of free HCAII concentration. The affinity of the interaction was estimated to be 0.02 μM from curve fitting using a two-state binding model.

Our results demonstrate the first steps in the successful application of folded helix−loop−helix peptides to the area of biosensing. A peptide that binds to a receptor has been shown to be able to report on this event via a fluorescent probe. Furthermore, this concept has been applied to the determination of an affinity constant. The possibility of conveniently incorporating a wide range of probes and ligands at different relative positions provides an attractive way of optimizing the biosensing conditions, such as sensitivity and fluorescence response, for any target biomacromolecule. As indicated by our results, the structure of the peptide scaffold also plays an important role in sensor performance. The molecular basis for biosensing is now under investigation in our laboratory. A determination of analyte concentration is possible using an array of peptides modified with ligands of different affinities. To simplify the use of such peptide-based biosensor arrays, immobilization strategies for coupling to regenerable surfaces will be developed.

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(13) Mrksich, M.; Grunwell, J. R.; Whitesides, G. M. *J. Am. Chem. Soc.* **1995**, *117*, 12009−12010.

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Supporting Information Available: Sequence of the template peptide LA-42b and figures displaying results from CD spectroscopy and surface plasmon resonance-based affinity analysis. This material is available free of charge via the Internet at http/::pubs.acs.org.

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