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The Binding of Human Carbonic Anhydrase II by Functionalized Folded Polypeptide Receptors

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Summary

Several receptors for human carbonic anhydrase II (HCAII) have been prepared by covalently attaching benzenesulfonamide carboxylates via aliphatic amino-carboxylic acid spacers of variable length to the side chain of a lysine residue in a designed 42 residue helix-loop-helix motif. The sulfonamide group binds to the active site zinc ion of human carbonic anhydrase II located in a 15 Å deep cleft. The dissociation constants of the receptor-HCAII complexes were found to be in the range from low micromolar to better than 20 nM, with the lowest affinities found for spacers with less than five methylene groups and the highest affinity found for the spacer with seven methylene groups. The results suggest that the binding is a cooperative event in which both the sulfonamide residue and the helix-loop-helix motif contribute to the overall affinity.

Introduction

Biomolecular interactions involving proteins are ubiquitous in nature and crucial for the function of the living cell. Protein biosynthesis and signal transduction, for example, depend on the ability of proteins to recognize and bind other proteins with high affinity and specificity. Efficient and specific small-molecule recognition of proteins is a required property of successful pharmaceuticals and a prerequisite for drug action.

The design and characterization of receptors that recognize and bind proteins can be expected to provide fundamental insights into biomolecular recognition phenomena in vivo and to have many applications in chemistry, biology, and biomedicine. Molecules capable of recognition and binding in aqueous solution are difficult to construct because although hydrogen bonds and charge-charge interactions are inherently strong, they are inefficient in water due to the complex solvation equilibria and the high dielectric constant of the solvent [1]. Since potential receptors must be of sufficient complexity to allow multiple interactions with the protein, simultaneously and cooperatively, biomacromolecules are obvious candidates as starting points for receptor design. A designed helix-loop-helix motif was reported previously to recognize and bind the calmodulin binding domain of calcineurin with an apparent dissociation constant of 1 μM [2]. Organic supramolecules capable of organizing several functional groups toward a macromolecular target have been shown to recognize and bind proteins. Calixarene functionalized with peptidic loops was reported to bind chymotrypsin and to disrupt its interactions with protease inhibitors [3]. Protein scaffolds are well suited for the presentation of large, shape-complementary hydrophobic surfaces, and surfaces that are charge complementary, to target proteins. Small, rigid organic residues are well suited to present preorganized hydrogen bond donors and acceptors. The introduction of small organic molecules in protein scaffolds is an attractive approach in the development of protein receptors, provided that synthetic strategies are available for efficient and site-selective modification of the protein scaffold. We reported previously on a helix-loop-helix motif that when functionalized with a spacered benzenesulfonamide ligand was capable of binding HCAII with a dissociation constant of 20 nM [4]. The helix-loop-helix motif was found at low micromolar concentration to be a dimer in aqueous solution but to bind the protein as a monomer [5]. Hydrophobic interactions between scaffold and protein, and the interaction between the sulfonamide group and the active site of HCAII were to a large degree responsible for its high affinity. This approach provides a general strategy for constructing high-affinity binders from moderate ones. In biology, modest binders are abundant, and a strategy for upgrading moderate affinity to high affinity therefore, in principle, provides an efficient route to recognition elements for a large body of relevant biomacromolecules. Given that the scaffolds are readily further funtionalized in several positions, for example by covalently attaching radiometal chelates, fluorophores, handles for immobilization, PEG, active transporters, etc., or combinations thereof, many opportunities are apparent in biotechnological applications. The interactions between the functionalized scaffold and HCAII have now been characterized to provide a platform for the further development of this new class of receptor molecules. The importance of spacer length and charge-charge interactions for the recognition and binding of HCAII has been elucidated, and the binding site has been identified. A key question is whether the interactions between scaffold and protein, and between sulfonamide and protein, are cooperative, since an understanding of how to engineer cooperativity in binding of functionalized protein scaffolds would make it possible to construct new receptors with exceptionally high affinities.

Results and Discussion

The Affinities of the Receptor Molecules

Polypeptides designed to fold were synthesized and conjugated to a series of benzenesulfonamide derivatives, in which the spacer that links the ligand to the

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Ac-NAAD**L**EAA**I**R HLAEK**L**AARG PVDAAQ**L**AEQ LA**K**KFEAFAR AG-OH
1 11 21 31 41
KE2

Ac-NAAD**J**EA**K**IR HLAEK**J**AARG PVDAAQ**J**AEQ LA**R**KFEAFAR AG-NH2
1 11 21 31 41
TA4

Figure 1. Scaffold Sequences

The sequences of the scaffold polypeptides KE2 and TA4. Residues that differ between the sequences are shown in boldface. In both sequences, the ligand was attached to the side chain of Lys-34, and the fluorescent dansyl group was attached to the side chain of Lys-15. In the peptide KE-Q, no ligand was incorporated, and a fluorescent dabcyl group was attached to the side chain of Lys-15. Norleucines are designated as "J."

polypeptide scaffold was systematically varied. The scaffold peptides were KE2 and TA4 (Figure 1), and the incorporated ligands are shown in Table 1. In all conjugates, the spacer was covalently bound to the side chain of Lys-34, and a dansyl fluorescent probe was covalently attached to the side chain of Lys-15. The affinities were measured by fluorometric titration of the receptor with the target protein, HCAII, and by titration of the HCAII receptor complex with acetazolamide in competitive binding experiments. The affinities were found to span approximately three orders of magnitude, with, in general, the longer spacer giving the higher affinity. The sequence KE2 has been described previously [4]: it was designed to fold into a helix-loop-helix motif and dimerize to form a four-helix bundle. It was further designed to allow for the incorporation of one flurophore and one ligand, to form a sensor peptide for screening applications. For this reason, only two lysine residues were allowed in close proximity to the histidine [5]. TA4 was based on the sequence of KE2, with the difference that three substituents were to be incorporated in close proximity to the His residue and one more lysine had to be incorporated. The scaffold sequences differ by five residues. Three leucines in the sequence of KE2, residues 5, 16, and 27, were replaced by norleucines in the design of TA4. One lysine in the sequence of KE2, residue 33, was replaced by an arginine in TA4, to avoid undesirable functionalization, and one alanine in the sequence of KE2, residue 8, was replaced by a lysine in TA4 for the reasons given above. Furthermore, the C terminus of TA4 was capped to form an amide to ensure a total charge of +1, and thus solubility, at around neutral pH, whereas that of KE2 was a free carboxylate, giving a total charge of −1 at the same pH. SPR experiments showed that KE2 had no measurable affinity for HCAII, whereas TA4 bound to the protein with a dissociation constant of ~100 μM (unpublished data). We have recently reported functionalized KE2 polypeptides to be dimers in solution at low micromolar concentration and to bind to the protein as monomers [6], possibly folded into hairpin helix-loop-helix motifs. The mean residue ellipticities reported in Table 1 show that the functionalized scaffolds are highly helical and thus most likely also dimers under the experimental conditions. The difference spectrum in which the ellipticity of the functionalized scaffold peptide has been subtracted from that of its complex with HCAII is compatible with helix formation [4], suggesting that it binds HCAII as a helix-loop-helix motif. If the polypeptide is folded into a helix-loop-helix motif, large hydrophobic surface areas can interact with hydrophobic residues on the surface of HCAII. The large difference in affinity between KE2 and TA4 suggests that interactions between charged residues are also important for binding.

Benzenesulfonamide is a known inhibitor of HCAII with a K_d of 1.5 μM [7]. We reported previously on the affinities of a series of KE2-linked benzenesulfonamide receptors in which the ligands were bound to the scaffold via aliphatic spacers that differed in length due to variation in the number of methylene groups [8]. The affinity was surprisingly invariant to spacer length (Table 1) in the case of peptides containing ligands with between zero and for methylene groups, whereas the introduction of a fifth methylene group to form KE2-D(15)-6 increased the affinity by a factor of 40. We have now increased the range of spacers to determine whether the observed affinity is due to cooperativity between the ligand and the scaffold, and also to elucidate the reason for the affinity variation.

The relatively weak fluorescence intensity of the dansyl probe limited the experimentally accessible range of functionalized scaffold to micromolar concentrations, which is well above the dissociation constants of the tightest binders. Affinities were measured by direct

Table 1. Structures and Affinities of Ligands for HCAII Recognition and Binding

	Benzenesulfonamide Ligand	Corresponding Peptide	K_d/nM A	K_d/nM B	K_d/nM C	$[\Theta]_{222}$ (deg·cm^2·dmol^{-1})[a]
1	$H_2NSO_2C_6H_4CO$-NHS	KE2-D(15)-0	3000			−19 600
2	$H_2NSO_2C_6H_4CONHCH_2CO$-NHS	KE2-D(15)-2	1000			−19 400
3	$H_2NSO_2C_6H_4CONH(CH_2)_3CO$-NHS	KE2-D(15)-4	700			−19 800
4	$H_2NSO_2C_6H_4CONH(CH_2)_4CO$-NHS	KE2-D(15)-5	800			−18 600
5	$H_2NSO_2C_6H_4CONH(CH_2)_5CO$-NHS	KE2-D(15)-6	20	23 ± 2; 28 ± 4	19 ± 2; 13 ± 2	−18 200
6	$H_2NSO_2C_6H_4CONH(CH_2)_6CO$-NHS	KE2-D(15)-7	10			
7	$H_2NSO_2C_6H_4CONH(CH_2)_7CO$-NHS	KE2-D(15)-8	4	29 ± 8; 22 ± 4	6 ± 2; 6 ± 2	
7	$H_2NSO_2C_6H_4CONH(CH_2)_7CO$-NHS	TA4-D(15)-8	70			
8	$H_2NSO_2C_6H_4CONH(CH_2)_{10}CO$-NHS	KE2-D(15)-11		24 ± 4; 22 ± 4	60 ± 16; 53 ± 13	

Values in column A are determined from direct titration of receptor with HCAII. Values in columns B and C are determined from titration of the HCAII-receptor complex with a competitive inhibitor. Duplicate measurements from different batches of HCAII are presented with "best fit" K_d values ± 1 SD. Fluorescence intensities (column B) or wavelength maxima (column C) were used to extract data. Dissociation constants for entries 1–4 as well as ellipticity data from [7].

[a] Mean residue ellipticity at 222 nm of sensor peptides at 1 μM concentration in 100 mM sodium phosphate buffer at pH 7.4.

titration of the sensor peptides with HCAII as well as by titration of the HCAII-peptide complex with a competitive inhibitor, acetazolamide [9]. In the latter case, dissociation constants were determined by plotting both the fluorescence intensities and the fluorescence wavelength maxima versus concentration of acetazolamide. The accuracy of the measurements is shown in Table 1, and the variation in affinity is significant in comparison with the experimental variations. Even when measurements were carried out with different batches of HCAII, the experimental variations were small. In general, wavelength maximum variations are expected to provide more reliable results than intensity variations since intensities may be affected by quenching phenomena that are not linearly related to the dissociation equilibrium. Acetazolamide quenches the fluorescence from the dansyl probe at micromolar concentrations [8], and the largest deviations between the measurement methods were observed in the determination of the highest affinity, that of KE2-D(15)-8, in which the largest concentration of acetazolamide was needed to expel the functionalized polypeptide from the binding site of HCAII. The measured dissociation constants were 5 nM and 26 nM, depending on the method, and were reproducible. We have greater faith in the dissociation constants obtained by direct titration or by competition measurements based on wavelength shifts. Thus, results obtained by measurements of intensity in the presence of acetazolamide will be disregarded in the following sections of this manuscript.

The affinities for HCAII of receptors carrying spacers with five methylene groups or more are all high; dissociation constants of 17, 10, and 5 nM were found for KE2-D(15)-6, KE2-D(15)-7, and KE2-D(15)-8, respectively. The corresponding spacers contain five, six, and seven methylene groups. The highest affinity measured for a functionalized polypeptide is almost three orders of magnitude larger than that of benzenesulfonamide. KE2-D(15)-11 is equipped with a spacer with ten methylene groups and has an affinity for HCAII that is slightly less than that of KE2-D(15)-6. The K_d was found to be 57 nM. The affinity for HCAII thus appears to be at a maximum for spacers with seven methylene groups.

We also measured the affinity of a TA4-based receptor molecule since TA4 by itself was known to bind to HCAII with ~100 μM affinity, in contrast to that of KE2, which was not measurable by SPR. KE2-D(15)-8 and TA4-D(15)-8 (Table 1) both have seven methylene groups in the spacer, and the KE2-based receptor had a K_d of 5 nM. The corresponding TA4-linked receptor had an affinity that was 14-fold less, a result that demonstrated that there was a significant effect from the polypeptide scaffold on the overall affinity. The observation of a weaker affinity for HCAII of the TA4-based receptor than of the KE2-based receptor, in spite of the fact that unfunctionalized TA4 has higher affinity than unfunctionalized KE2, suggests that the scaffolds do not bind in the same way to HCAII when attached to the ligand as when unmodified. It should also be taken into consideration that upon acylation of two lysine residues, the total charges of both the KE2 and TA4 scaffolds are reduced by two, and that this may cause a loss of affinity and alter the binding site in comparison with those of the unsubstituated scaffolds.

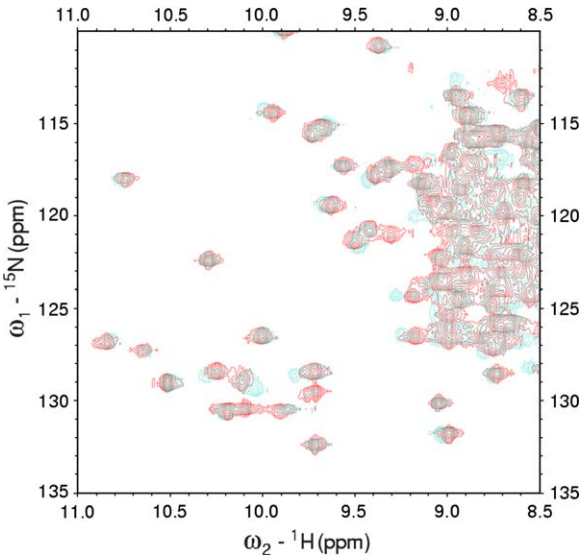

Figure 2. Identification of Binding Site

Overlay of HSQC spectra of 50 μM ^{15}N-labeled HCAII in aqueous solution at pH 7 and of 50 μM ^{15}N-labeled HCAII in the presence of 50 μM KE2-D(15)-6. Red crosspeaks are from free HCAII, blue crosspeaks are from the 1:1 mixture, and some peaks show a lack of overlap due to complexation with the receptor molecule.

NMR Spectroscopic Investigations of Receptor-Protein Complexes

HSQC spectroscopic investigations of ^{15}N-labeled proteins in the presence of small-molecule ligands are commonly used to probe small molecule-protein interactions with the specific goal of searching for weak interactions [10]; "weak" is defined as affinities with dissociation constants in the millimolar to micromolar range. The ^{1}H and ^{15}N NMR spectra have been assigned for HCAII [11], and the interactions between scaffolds and HCAII and functionalized scaffolds and HCAII were studied by recording the HSQC spectra of free HCAII and of mixtures of HCAII and receptor molecules, typically in a 1:1 ratio at 50 μM concentrations by using cryoprobe technology at 600 MHz (Figure 2). Shifts in the ^{1}H or ^{15}N dimensions larger than 10 Hz were considered perturbations since the chemical shifts of the amide proton and nitrogen atoms of HCAII in seven different samples of HCAII under native conditions were ±2.5 Hz in the proton dimension and ±1.5 Hz in the nitrogen dimension (M.L., unpublished data). Several amide resonances were shifted in the presence of the receptor molecules, and from the assigned HCAII spectrum [11], it was possible to identify the affected residues and to obtain information about the site of binding on the HCAII surface.

Unmodified KE2 did not affect the chemical shifts of HCAII, whereas unmodified TA4 did, in agreement with the relative affinities measured by SPR. Due to the weak affinities of the scaffolds for HCAII, these measurements were carried out at 100 μM concentration of HCAII and 200 μM concentration of TA4 and KE2. Among the functionalized scaffolds, no shifts were observed for KE2-D(15)-5, a receptor with a K_d of 800 nM, but strong shifts were observed for KE2-D(15)-6, with a K_d of 17 nM. KE2-D(15)-8 bound even stronger to HCAII than KE2-D(15)-6 (Table 1), but the perturbations of HCAII chemical shifts were less pronounced. In Figure 3, the structure of HCAII

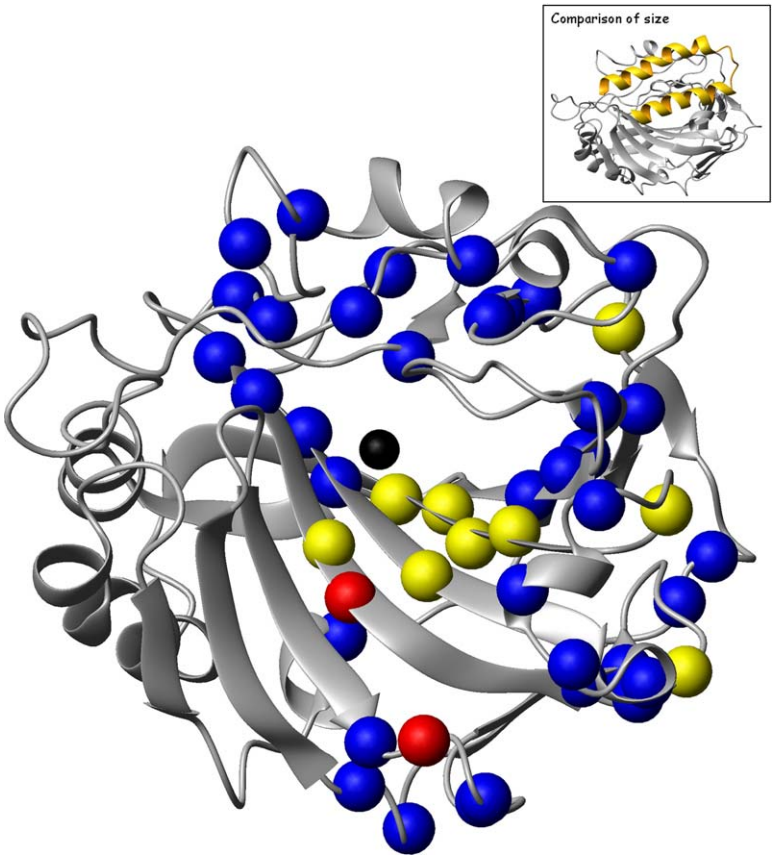

Figure 3. Efficient Binding of HCAII by Functionalized Helix-Loop-Helix Receptors Is Manifested by Strong Perturbations of the HCAII Surface

Illustration of binding of HCAII by KE2-D(15)-6 and by KE2-D(15)-8, in which the amide nitrogens of residues perturbed by complexation are shown in space-filling mode. Yellow nitrogens show residues that are affected by both receptors, residues affected by KE2-D(15)-8, but not by KE2-D(15)-6, are shown in red, and residues affected only by KE2-D(15)-6 are shown in blue. The shorter spacer of KE2-D(15)-6 causes larger perturbations of the target protein than does that of KE2-D(15)-8. A shift is registered if the chemical shift is changed by 10 Hz or more. The figure was prepared by using the program MOLMOL [18]. Inset: As an illustration of the relative sizes of HCAII and the scaffold, a modeled structure of TA4 is compared to that of the crystal structure of HCAII.

is shown with the residues affected in the presence of KE2-D(15)-6 and KE2-D(15)-8 highlighted to illustrate the interaction between the scaffold and HCAII. The residues that are affected are located in the central hydrophobic cluster and on the surface of the protein in the neighborhood of the active site, and they include hydrophobic as well as polar and charged residues. The perturbations on the surface of HCAII covered an area that is large, but they correspond approximately to what would be expected from an interaction between HCAII and a helix-loop-helix motif (Figure 3). The size of the folded helix-loop-helix motif is ~20 × 25 Å, and that of HCAII is 47 × 41 Å [12], which makes the two proteins of comparable size. Thus, it is not surprising that the chemical shift perturbations are found over a large area of HCAII. The receptor with the longest spacer, KE2-D(15)-11, gave rise to severe perturbations of the amide groups (Figure 4) in spite of the fact that its affinity was less than that of other receptor molecules with more than four methylene groups in the spacer; however, in this case, the interaction was found to be more complex.

In the case of the longest spacers, there was a pronounced reduction in the overall intensity of the NMR spectrum, an effect that may be due to further aggregation of the receptor-HCAII complexes. Only 30% of the overall intensity remained in the spectrum of HCAII after the addition of KE2-D(15)-11, an observation that is in agreement with the hypothesis that there is more than one HCAII and possibly more than one receptor in the complex, which has a resulting molecular weight of 70 kDa or more. Under these conditions, the resonances from aggregated proteins would be broadened beyond detection, and only the fraction of protein that remains nonaggregated would be observable. An approximate 30% loss of HCAII intensity was observed in the presence of KE2-D(15)-8.

The observation that KE2-D(15)-5 did not perturb the chemical shifts of the amide residue and showed a weak affinity, whereas KE2-D(15)-6 perturbed the chemical shifts severely and showed high affinity, suggests that the polypeptide scaffold cannot form a helix-loop-helix motif when bound to HCAII if the spacer is too short. While we have no direct structural evidence for this hypothesis, the crystal structure of HCAII [12] shows that the active site is located in a 15 Å deep cleft that appears not to be able to accommodate a folded helix-loop-helix motif, but can possibly accommodate an unordered polypeptide chain. The ligand was attached to the side chain of Lys-34; thus, most of the polypeptide chain, if unordered, can extend from the active site into the solution. There are no chemical shift perturbations for any of the residues in the binding pocket to suggest that the active site has been rearranged upon binding to KE2-D(15)-5 to accommodate the bulky helix-loop-helix motif. It is therefore likely that in the complex formed between HCAII and KE2-D(15)-5 the interaction between the active site zinc ion and the benzenesulfonamide residue dominates, the helix-loop-helix motif is not formed, and the structure of HCAII remains intact. The affinities remain largely constant for the short spacers, which is in agreement with this model. With longer spacers, the scaffold can fold in the complex. In the case of KE2-D(15)-6,

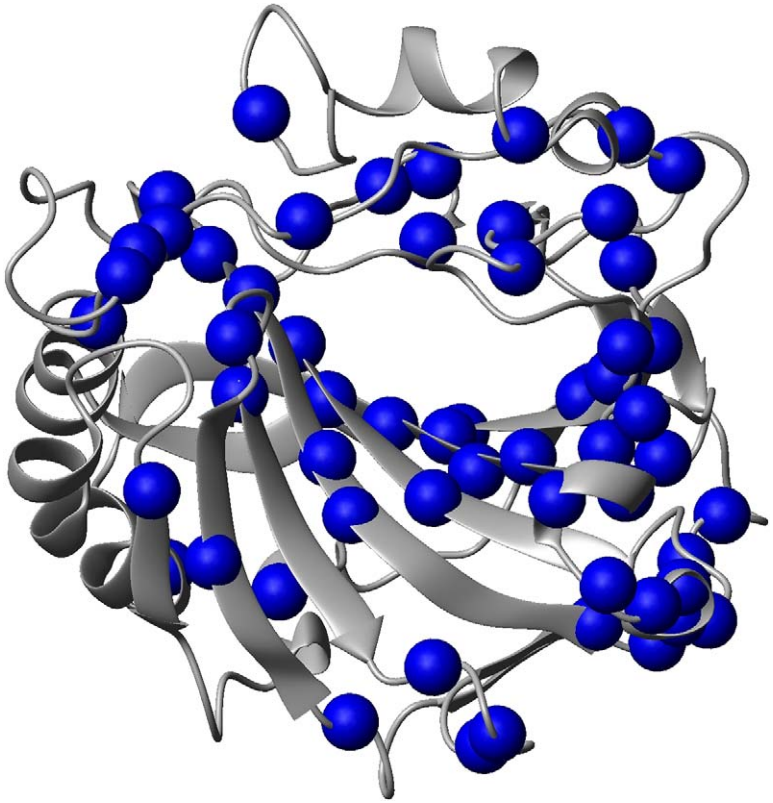

Figure 4. Binding of HCAII by a Long-Spacered Receptor

Illustration of binding of KE2-D(15)-11 to HCAII, in which the amide nitrogens of residues perturbed by complexation are shown in space-filling mode. A shift is registered if the chemical shift is changed by 10 Hz or more. The figure was prepared by using the program MOLMOL [18].

the scaffold can fold under considerable perturbation of the target protein. With an even longer spacer, as in KE2-D(15)-8, the binding to HCAII is strong, but the perturbation is less, possibly because the receptor molecule has a more well-defined interaction with the binding site of HCAII. For the longest spacer studied, the perturbations are again severe, showing that the interactions between scaffold and the hydrophobic patch are also important in this case although the affinity is slightly reduced.

While we have not fully characterized the state of aggregation of the HCAII-receptor complexes with the longest spacers, we have been able to exclude the possibility that ternary complexes are formed in which one HCAII binds two receptor polypeptides. The dissociation constant for the ternary complex between HCAII, KE2-D(15)-6, and the polypeptide KE2-Q was determined and was found to be 286 μM. KE2-Q has the same sequence as KE2, but there is no benzenesulfonamide attached, and the dansyl probe has been replaced by 4-(4-dimethylamino-phenylazo)-benzoate (dabcyl), a known quencher of fluorescence. Under the assumption that KE2-Q forms a heterodimer with KE2-D(15)-6, with a dissociation constant that is similar to that of the homodimer of KE2-D(15)-6, KE2-Q can be used to probe ternary complex formation. The dissociation constants for the ternary complexes with HCAII and KE2-Q were 105 μM and 87 μM for KE2-D(15)-8 and KE2-D(15)-11, respectively. Consequently, under the experimental conditions for affinity measurements, very little was formed of the ternary complexes involving two receptor peptides and one HCAII molecule. Also, the observed loss of NMR intensity upon complexation is only compatible with the formation of a high-molecular weight species, suggesting more than one HCAII per complex.

The Cooperative Binding of HCAII by Functionalized Polypeptide Scaffolds

In the cooperative binding of a protein by a receptor molecule, in which several groups bind simultaneously to specific residues on the protein surface, the overall binding energy amounts to at least the sum of the contributions from each individual interaction. If a protein scaffold with some affinity for a target protein is covalently functionalized with a small molecule that is also capable of binding to the target protein, and if the small molecule is linked in a way that allows both the scaffold and the small molecule to bind simultaneously, the functionalized protein scaffold will show an affinity that is larger than that of the small molecule. It will, in principle, act as a receptor that has a dissociation constant that is at least the product of the two individual dissociation constants, but it may have a dissociation constant that is considerably lower, for entropic reasons. The measured affinities of the polypeptide benzenesulfonamide conjugates with five or more methylene groups in the spacer are all larger than that of benzenesulfonamide itself. The observed affinities suggest that the scaffold and benzenesulfonamide bind cooperatively to HCAII.

The longer and more flexible the spacer, the smaller the entropic advantage, since the level of preorganization is less. The observation of a maximum binding affinity as a function of spacer length is compatible with a cooperative binding behavior. An alternative explanation for the weaker affinity found in the complex between HCAII and the receptor molecule with the longest spacer may, however, be that the preferred binding modes of the benzenesulfonamide group and the scaffold cannot accommodate an unstrained methylene spacer to connect the two. The difference in affinity is less than a factor

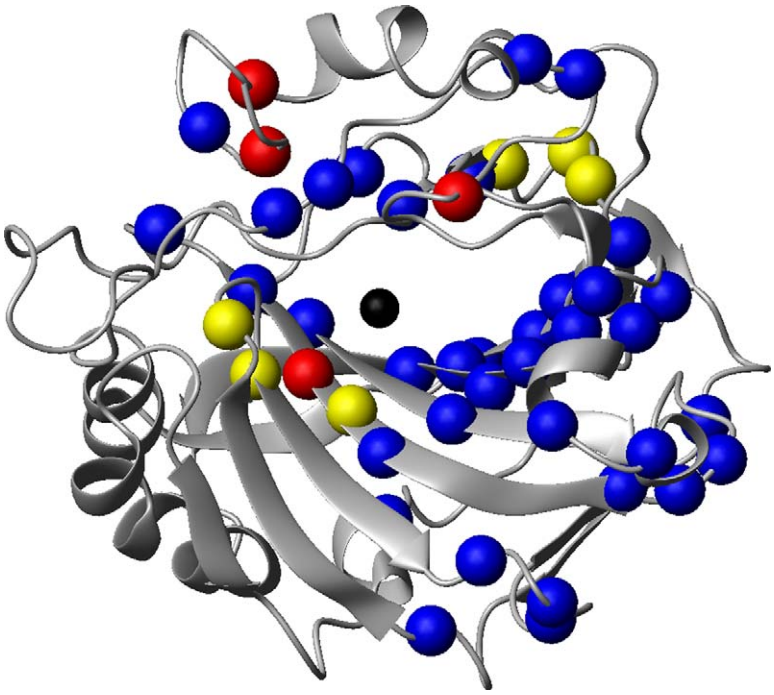

Figure 5. Scaffold Binding Is Not Optimal in TA4-D(15)-8

Illustration of binding of TA4 and of TA4-D(15)-8 to HCAII. The yellow nitrogens are those of residues affected by binding of both receptors, the red nitrogens are those of residues affected only by TA4, and the blue nitrogens are those affected only by TA4-D(15)-8. The binding site of TA4 is more distant from the active site of HCAII than that of TA4-D(15)-8, showing that, in the latter case, the scaffold does not, and probably cannot, bind in the site for which the 100 μM dissociation constant was measured. The Cut-off limit for a chemical shift change is 10 Hz. If a lower cut-off than 10 Hz is used for TA4 to compensate for the weaker binding, it nevertheless shows that the binding site of TA4 deviates significantly from that of TA4-D(15)-8. The figure was prepared by using the program MOLMOL [18].

of ten, and the corresponding difference in free energy is comparable to that found for different conformers of aliphatic residues.

The observation of a perturbed surface area on HCAII shows that in the receptors with long spacers the scaffolds bind to the target protein. The complete lack of observable perturbed residues in the complex formed from HCAII and KE2-D(15)-5 provides strong evidence that, in this case, the scaffold does not bind, and that binding interactions are required for shift perturbations. The difference in binding is reflected in a more than two orders of magnitude difference in affinity (Table 1). The sulfonamide binds to the active site zinc ion in all cases. Although the observed affinities are very similar to those reported for benzenesulfonamide derivatized with aliphatic substituents [13], the NMR spectroscopic results clearly demonstrate that there is an interaction between polypeptide and HCAII, and, therefore, that the binding is cooperative. The difference in binding affinity between that of KE2-D(15)-8 and that of TA4-D(15)-8 strengthens the conclusion that the scaffold participates in binding. The introduction of a methoxycoumarine derivative into position 25 of KE2-D(15)-6 was shown previously to enhance the affinity of the functionalized polypeptide by approximately a factor of four [6].

It was shown by Jain et al. that benzenesulfonamides modified with aliphatic and aromatic substituents exhibited considerably higher affinities for HCAII than the unsubstituated compounds, probably due to hydrophobic interactions in the binding pocket. Affinities in the range of 1–5 nM were obtained in 20 mM phosphate buffer at pH 7.4 [13]. It could be argued that the affinity increase observed for the protein scaffolds in Table 1 is due to similar effects, but the observation of an affinity maximum is not expected from such a binding model, and no evidence was obtained from NMR spectroscopy to support such a conclusion. In no complex were perturbations of residues located in the binding pocket observed, which would be expected if there were interactions between spacer and hydrophobic amino acid residues in analogy with those reported for the benzenesulfonamide derivatives with alifatic spacers [13].

Unmodified KE2 does not bind to HCAII, but when linked to a spacered sulfonamide the scaffold binds to a hydrophobic patch close to the active site. The observation of chemical shift changes in HCAII in the presence of KE2-D(15)-6, but not in the presence of KE2, indicates that the proximity of the scaffold to the protein in the former enhances its affinity for HCAII, most likely for entropic reasons. TA4-D(15)-8 showed a K_d of 70 nM, an order of magnitude larger than that of the KE2-linked receptor, although TA4 by itself binds more strongly to HCAII than KE2. The HSQC spectrum revealed the reasons for the unexpected weak affinity (Figure 5). A comparison between the perturbation pattern induced by TA4 with that induced by TA4-D(15)-8 shows that in the case of the latter the scaffold does not bind at the same site as TA4. It is possible that the spacer restricts the access of the scaffold to its preferred binding site, and that a longer spacer, or an alternative linkage position, would give rise to higher affinity.

Significance

Functionalization of folded polypeptides with ligands of modest affinity for a target protein can serve as a strategy for the design of receptors in which the overall affinity is higher than that of the ligand due to cooperative effects. In addition to providing fundamental insights into the thermodynamics of multidentate receptor molecules that recognize proteins, these receptors are envisioned to have a plethora of applications in drug screening, bioseparation, and protein chip fabrication. These molecules are robust, have high affinity, and should, in principle, have the potential to provide better than nanomolar affinities for proteins, provided

that the optimal relationship between scaffold structure and the ligand linkage site can be identified. From the point of view of receptor design, a spacer that is too short may drastically reduce the overall affinity, whereas a spacer that is too long may give rise to unwanted aggregation and ambiguous results, although the apparent affinity may not be much affected.

Experimental Procedures

Peptide Synthesis and Purification

The peptides were synthesized on a Pioneer automated peptide synthesizer (Applied Biosystems) by using standard fluorenylmethoxycarbonyl (Fmoc) chemistry with O-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, Alexis Biochemicals) and diisopropylethylamine (DIPEA, Applied Biosystems) as activating agents. The synthesis was performed on a 0.1 mmole scale, with a PAL-PEG-PS resin giving a C-terminal amide upon cleavage; a 4-fold excess of amino acid was used in each coupling. The side chains of the amino acids (Calbiochem-Novabiochem AG) were protected by base-stable groups: tert-butyl ester (Asp, Glu), tert-butoxymethyl (Lys), trityl (His, Asn, Gln), and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg). Lys-15 was protected by allyloxycarbonyl (Aloc). The N terminus was capped with 0.3 M acetic anhydride in DMF. Prior to cleavage, the Aloc group of Lys-15 was selectively deprotected by using three equivalents of tetrakis(triphenylphosphine)palladium(0) ($Pd(Ph_3)_4$) dissolved in a mixture of N-methylmorpholine, acetic acid, and trichloromethane (5:10:85, 10 ml per gram of resin). After 2 hr at room temperature, in darkness, the resin was alternately rinsed with 0.5% sodium diethyldithiocarbamate (DEDT) in DMF and 0.5% DIPEA in DMF. The dansyl probe was coupled by adding six equivalents of DIPEA and three equivalents of 5-dimethylamino-naphthalene-1-sulfonyl chloride in DMF. After 1 day at room temperature, the resin was rinsed with DCM and dried under nitrogen. The peptides were cleaved from the resin by treatment with a mixture of trifluoroacetic acid (TFA), water, and triisopropylsilane (95:2.5:2.5 v/v, 10 ml per gram of polymer) for 3 hr at room temperature. After filtration and concentration, the peptide was precipitated by the addition of cold diethyl ether, centrifuged, washed in diethyl ether, and dried under nitrogen. The crude peptides were purified by revers-phase HPLC on a semipreparative C-8 HICHROM column, eluted with 37%–45% isopropanol and 0.1% TFA in water at a flow rate of 10 ml/min. Collected fractions were identified by MALDI-TOF mass spectrometry, concentrated, and lyophilized. The peptides were dissolved to a concentration of 1 mM in 50 mM sodium phosphate (pH 8.0), and Lys-34 was selectively modified with 1.5 equivalents of the benzenesulfonamide derivate. The dabcyl probe used for the quencher experiments was coupled on the solid phase to a peptide with Lys-15 deprotected as described above by mixing for 1 day with three equivalents of the active ester 4-(4-dimethylamino-phenylazo)-benzoic acid benzotriazol-1-yl ester in situ synthesized from 4-(4-dimethylamino-phenylazo)-benzoic acid, diisopropylamine, 1-hydroxybenzotriazol, and benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP). Cleavage and workup are as described above.

Synthesis of Benzenesulfonamide Derivatives

$H_2NO_2SC_6H_4CO$-NHS (1)

4-carboxybenzenesulfonamide (3.70 g, 18.4 mmole) and N-hydroxysuccinimide (NHS, 2.11 g, 18.3 mmole) were dissolved in a mixture of 37 ml dioxane and 15 ml N,N-dimethylformamide (DMF) and were cooled in an ice bath. Dicyclohexylcarbodiimide (3.80 g, 18.4 mmole) was added to the solution, and the reaction mixture was stirred for 10 min and then left overnight at 4ºC. Precipitated N,N-dicyclohexylurea (DCU) was removed by filtration. The solvent was reduced to an oil, and crystallization from 50 ml warm 2-propanol afforded a white solid (24.40 g, 81%) in two crops. Purity was confirmed by ^{1}H-NMR (300 MHz, $CDCl_3$, 25ºC).

General Procedure for the Synthesis of $H_2NO_2SC_6H_4CONH(CH_2)_nCO$-NHS, n = 1, 3, 4, 5, 6, 7 (2–7)

To a 0.2 M solution of (1) in acetone was added one equivalent of nucleophile (glycine, 4-aminobuturic acid, 5-aminopentanoic acid, 6-aminohexanoic acid, 7-aminoheptanoic acid, 8-aminooctanoic acid) as a 0.3 M solution in 50 mM sodium borate (pH 8.5). The reaction mixture was stirred at room temperature for at least 2 hours while the pH was kept between 7.0 and 7.5. The solution was acidified and reduced in vacu. The intermediate carboxylic acid product, $H_2NO_2SC_6H_4CONH(CH_2)_nCOOH$, was washed in cold water and dried. Yields were between 62% and 92%, and purity was confirmed by ^{1}H-NMR. To prepare the NHS esters, one equivalent of the intermediate product was added to 1.1 equivalent of NHS (0.1 M) dissolved in a 1:1 dioxane:DMF mixture at 0ºC. One equivalent of DCC was added to the solution, and the reaction mixture was stirred overnight at 5ºC. DCU was removed by filtration. The solvent was reduced, and crystallization from 2-propanol and hexane afforded a white solid. Purity was confirmed by ^{1}H-NMR, and the yields were between 36% and 67%.

Procedure for the Synthesis of $H_2NO_2SC_6H_4CONH(CH_2)_{10}CO$-NHS (8)

A suspension of 11-aminoundecanoic acid (0.108 g, 0.536 mmole) in acetone (2.4 ml) and 50 mM sodium borate (1.6 ml) was adjusted by the addition of aqueous hydrochloric acid (1.2 M) to pH 9. The suspension was added to (1) (0.160 g, 0.536 mmole). The reaction mixture was stirred at room temperature for 24 hr while the pH was kept between 7.0 and 8.5 by adding aqueous sodium hydroxide (1 M), and the suspension was kept fluent by the addition of a 4 ml water/acetone mixture (2:3). The solution was acidified by addition of aqueous hydrochloric acid (2 ml, 1.2 M), frozen, and lyophilized. The intermediate carboxylic acid product, $H_2NO_2SC_6H_4CONH(CH_2)_{10}COOH$, was washed with water (25 ml) and dried. A white powder was isolated (0.164 g, 80%), and purity was confirmed by ^{1}H-NMR. The NHS ester was prepared from the carboxylic acid (0.159 g, 0.414 mmol) as described in the general procedures, with the exception that crystallization was made from boiling 2-propanol (15 ml). A white powder was isolated (117 mg, 59%), and purity was confirmed by ^{1}H-NMR.

NMR Spectroscopy

The NMR samples were 50 μM uniformly ^{15}N-labeled human carbonic anhydrase II (produced and purified as described by Mårtensson et al. [14]) in 10 mM phosphate buffer (pH 7.4) with a 90:10 ratio of H_2O:D_2O. The temperature was kept at 25ºC during the experiments. The peptides KE2-D(15)-5, KE2-D(15)-6, KE2-D(15)-8, and TA4-D(15)-8 were added to give a 1:1 ratio between protein and peptide and more than 95% complex formation. KE2 and TA4 were added to give a 1:2 ratio between protein and peptide to maximize complex formation that was less than 95% due to weak affinities. The NMR measurements were performed on a Varian Inova Unity 600 MHz NMR spectrometer equipped with a triple resonance cryo-probe. The backbone of the protein was monitored by 2D-HSQC NMR in the sensitivity-enhanced gradient version as described by Kay et al. [15]. The 2D data were processed by using the program nmrPipe [16], and the spectra were analyzed with the program SPARKY [17].

Fluorescence Spectroscopy with Direct Titration

Analyses were carried out on a F-4500 spectrofluorophotometer (Hitachi High-Technologies Corp.) in a 1 cm quartz cuvette. The excitation wavelength was 335 nm, and the emitted fluorescence was measured at 450–650 nm. Excitation and emission slits were 5 nm. Stock solutions of peptides were prepared in distilled water, and the concentration was determined from the absorbance of the dansyl group at 335 nm by using $\varepsilon = 4200\ cm^{-1}M^{-1}$. The stock solutions were diluted by HEPES-buffered saline (10 mM HEPES, 150 mM NaCl [pH 7.4]; from Biacore AB). For affinity determinations, samples of 0.2 μM peptide concentration were titrated with solutions of 0.1, 1, and 10 μM HCAII diluted from a stock solution of 500 μM in HEPES-buffered saline. The concentration of the HCAII stock solution was determined from the absorbance at 280 nm by using $\varepsilon = 54\ 800\ cm^{-1}M^{-1}$. The fluorescence of dansyl at 510 nm was monitored as a function of total protein concentration, and the dissociation constant, K_d, was determined by fitting Equation 1 to the experimental results under the assumption of a 1:1 binding model.

$$F_{obs} = \frac{F_{bound} \cdot [CA] + F_{free} \cdot K_d}{[CA] + K_d}. \quad (1)$$

F_{obs} is the observed fluorescence intensity, F_{bound} is the fluorescence of the peptide bound to HCAII, F_{free} is the fluorescence of

the unbound peptide, and [*CA*] is the concentration of free HCAII. [*CA*] can be derived from Equation 2:

$$[CA] = -\frac{P_{tot} + K_d - CA_{tot}}{2} + \sqrt{\left(\frac{P_{tot} + K_d - CA_{tot}}{2}\right)^2 + K_d \cdot CA_{tot}}, \quad (2)$$

where P_{tot} is the total concentration of peptide, and CA_{tot} is the total concentration of HCAII. Curve fitting was done with Igor Pro 4.03 software (WaveMetrics, Inc.).

Fluorescence Spectroscopy Using Titration with Competitive Inhibitor

Analyses were carried out on a FluoroMax-2 spectrofluorophotometer (Instruments S. A., Inc.) in a 1 cm quartz cuvette. The excitation wavelength was 335 nm, and the emitted fluorescence was measured at 465–545 nm or 475–555 nm. The excitation and emission slits were 2 and 5 nm, respectively. Stock solutions of peptides were prepared as described above. The peptide stock solutions were diluted with HEPES-buffered saline (10 mM HEPES, 150 mM sodium chloride [pH 7.4]; from Biacore AB), and pH was adjusted to 7.4 by the addition of aqueous sodium hydroxide. Stock solutions of HCAII and acetazolamide were prepared in HEPES-buffered saline, and pH was adjusted to 7.4. The concentration of the HCAII stock solution was determined from the absorbance at 280 nm by using ε = 54 800 $cm^{-1}M^{-1}$. For affinity determinations, samples of 2 μM peptide solutions and 1 μM HCAII solutions were titrated with solutions of 4.8, 22, 112, and 800 μM acetazolamide containing 2 μM peptide and 1 μM HCAII prepared by dilution of stock solutions with HEPES-buffered saline. The fluorescence of the dansyl group was monitored as a function of the total acetazolamide concentration, and the dissociation constant, K_d, was determined by fitting Equation 3 to the experimental results under the assumption of a 1:1 binding model:

$$F_{obs} = \frac{F_{bound} \cdot [CAP] + F_{free} \cdot [P]}{[CAP] + [P]}. \quad (3)$$

Depending on the Mode of Data Extraction

F_{obs} is the observed maximum fluorescence intensity or the wavelength of maximum fluorescence, F_{bound} is the fluorescence or the wavelength of maximum fluorescence of the peptide bound to HCAII, and F_{free} is the fluorescence or the wavelength of maximum fluorescence of the unbound peptide. [*CAP*] is the concentration of peptide bound to HCAII, and [*P*] is the concentration free peptide. [*CAP*] and [*P*] can be derived from Equation 4:

$$[CAP]^3 + \frac{K_d \cdot (I_{tot} - P_{tot} - CA_{tot} - K_d) + K_i \cdot (K_d + 2 \cdot P_{tot} + CA_{tot})}{K_d - K_i} \cdot [CAP]^2 + \frac{P_{tot} \cdot [K_d \cdot (CA_{tot} - I_{tot}) - K_i \cdot (K_d + P_{tot} + 2 \cdot CA_{tot})]}{K_d - K_i} \cdot [CAP] + \frac{K_i \cdot CA_{tot} \cdot P_{tot}^2}{K_d - K_i} = 0$$

$$[P] = P_{tot} - [CAP], \quad (4)$$

where P_{tot} is the total concentration of peptide, CA_{tot} is the total concentration of HCAII, I_{tot} is the total concentration of acetazolamide, and K_i is the dissociation constant of the HCAII-acetazolamide complex. The solution to this cubic equation can be found in the Supplemental Data (contained in the article online). Curve fitting was done with Igor Pro 4.03 software (WaveMetrics, Inc.).

Supplemental Data

Supplemental Data including the solution of the cubic equation needed for analysis of the data are available at http://www.chembiol.com/cgi/content/full/12/11/1245/DC1/.

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