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Designed, Functionalized Helix−Loop−Helix Motifs that Bind Human Carbonic Anhydrase II: A New Class of Synthetic Receptor Molecules

Karin Enander, Gunnar T. Dolphin, and Lars Baltzer*

Department of Chemistry-IFM, Linköping University, 581 83 Linköping, Sweden

Received September 30, 2003; E-mail: lars.baltzer@ifm.liu.se

De novo designed proteins offer fundamental insight into protein structure and function and provide, in principle, access to a huge pool of novel biomolecules with tailored functions.[1] The recognition and binding of cofactors,[2] metal ions,[3] and nucleotides,[4] as well as the engineering of catalytic activity,[5] rank highly among recent achievements in functional design. Here, we report on designed, functionalized proteins that recognize and bind the enzyme human carbonic anhydrase II (**HCAII**) based on a concept that can be applied to the recognition of a wide variety of proteins and be envisioned to have a large number of applications in biosensing, bioseparation, and biomedicine.

The incorporation of a benzenesulfonamide derivative (**1**), a known inhibitor of **HCAII**,[6] and a dansyl group (**2**) into a helix−loop−helix motif was shown recently to provide a folded polypeptide capable of binding **HCAII** with a dissociation constant K_D of 20 nM.[7] We have now investigated the nature of the interaction between **HCAII** and the functionalized polypeptide scaffold using circular dichroism and fluorescence spectroscopy. Affinity and specificity were found to originate from the interaction between **1** covalently attached to the side chain of a lysine residue in the polypeptide scaffold and the active site of **HCAII** and from hydrophobic protein−protein interactions between the scaffold and **HCAII**. The overall binding event was found to involve a complex set of equilibria.

Designed polypeptides with sequences similar to those reported here are known to fold into helix−loop−helix motifs that dimerize to form four-helix bundles with dissociation constants typically in the high nM to low μM range.[8] The mean residue ellipticities at 222 nm, $[\theta]_{222}$, range from −16 000 to −25 000 deg cm^2 dmol^{-1} for the dimers, whereas the monomers are largely unstructured with helical contents characterized by mean residue ellipticities of roughly −5000 deg cm^2 dmol^{-1} or less. KE2, Figure 1, was found to be highly helical and thus dimeric in aqueous solution with $[\theta]_{222}$ = −18 600 deg cm^2 dmol^{-1} at 0.1 mM concentration and pH 7. Introduction of **2** (D) and/or a methoxycoumarin group **3** (C) at the side chains of Lys15 and Lys25 using orthogonal protection group chemistry and incorporation of **1** at the side chain of Lys34 by reaction with the corresponding ester in aqueous solution provided peptides KE2-D(15)-5 ($[\theta]_{222}$ = −22 000 deg cm^2 dmol^{-1} at 1 μM), KE2b-C(25)-5 ($[\theta]_{222}$ = −17 600 deg cm^2 dmol^{-1} at 1 μM), and KE2b-D(15)C(25)-5 (**DC**) ($[\theta]_{222}$ = −14 300 deg cm^2 dmol^{-1} at 2 μM). The magnitudes of the mean residue ellipticities showed that the peptides were largely dimeric in the absence of **HCAII** under the experimental conditions.

Fluorescence spectroscopy was used to investigate the binding of **HCAII** by **DC**.[9] As a result of the presence of the dansyl group, the signal from the coumarin residue in the **DC** dimer was significantly quenched in comparison with that in the dimer of KE2b-C(25)-5, Figure 2a. When a 50-fold excess of the photoinactive KE2 was added to a 2 μM solution of **DC** to provide four-helix bundles in which the fraction of **DC** homodimers was

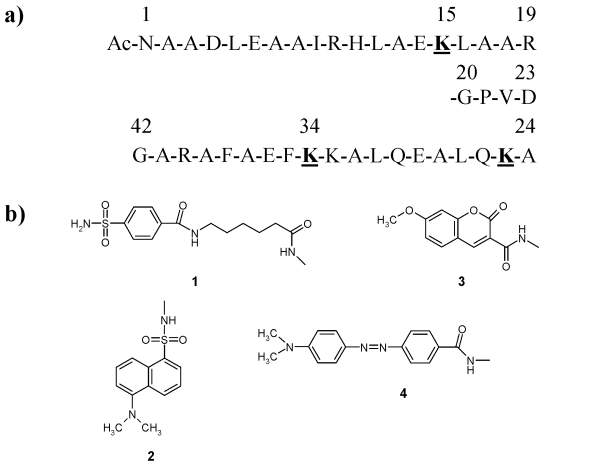

Figure 1. (a) Amino acid sequence of KE2b in which positions for site-selective modifications are highlighted. KE2 is identical to KE2b, with the exception of residue 25, which is Ala in KE2 and Lys in KE2b. (b) Functional residues incorporated in KE2 and KE2b to form KE2-D(15)-5 (**1** and **2**), KE2b-C(25)-5 (**1** and **3**), KE2b-D(15)C(25)-5 (**1**−**3**) and KE2-Q (**4**). (The label 5 refers to the spacer used in **1**.)

negligible, the emission intensities of **DC** at 408 (**3**) and 518 nm (**2**) increased, Figure 2a.

The response of the probes of **DC** to the presence of **HCAII** was similar to that observed in the presence of KE2. The addition of 10 μM **HCAII** to a 2 μM solution of **DC** generated a pronounced light-up effect, Figure 2a, indicating that **DC** bound **HCAII** as a monomer, having expelled one polypeptide subunit from the four-helix bundle upon binding to **HCAII**. The affinity of **DC** for **HCAII** was estimated to be 5 nM from a fluorometic titration. To probe the complexation further, KE2 was modified by the incorporation of a dabsyl group (**4**), capable of quenching the emission from **2** and **3**, at the side chain of Lys34 to form the peptide KE2-Q. In the presence of an excess of KE2-Q, the fluorescence of **DC** was efficiently quenched, Figure 2b. In the presence of KE2-Q *and* **HCAII**, the probes lit up, clearly due to expulsion of KE2-Q upon binding of **DC** to **HCAII** as discussed above. However, at increasingly higher concentrations of KE2-Q the emission again decreased, showing that at low concentrations of **DC**, there is a 1:1 interaction between **DC** and **HCAII** but that complexation of **HCAII**-bound **DC** by KE2-Q becomes significant when KE2-Q is present at high concentrations. From an experiment in which a solution of **DC** (2 μM) and **HCAII** (10 μM) was titrated with KE2-Q, an apparent K_D of 30 μM was obtained for the dissociation of KE2-Q from the ternary complex, a value that is compatible with helix−loop−helix dissociation. The affinity of KE2-D(15)-5 for **HCAII** was found to decrease almost 2 orders of magnitude when measured in the presence of 100 μM TA4,[10] a concentration at which KE2-D(15)-5 is almost saturated with TA4 and can remain bound to **HCAII** only through **1**. The binding of **HCAII** by

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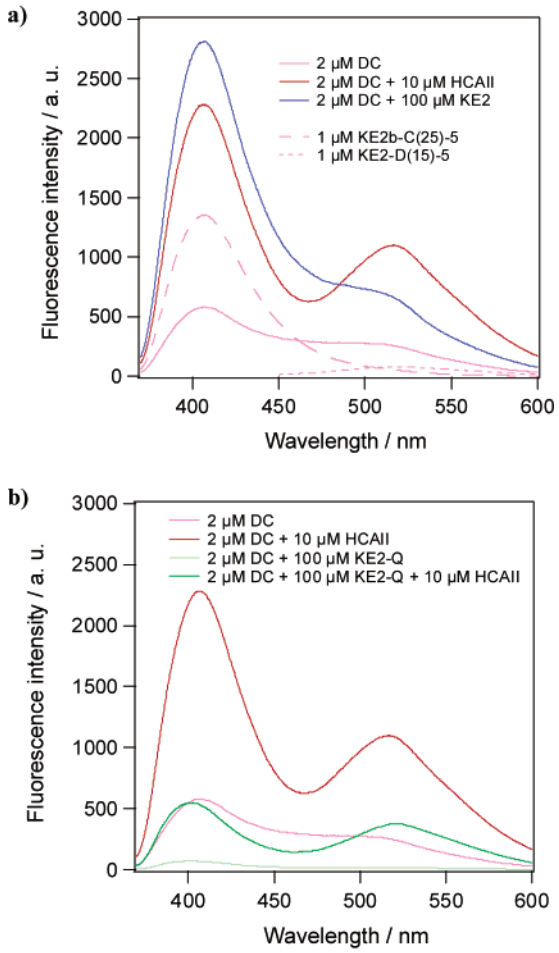


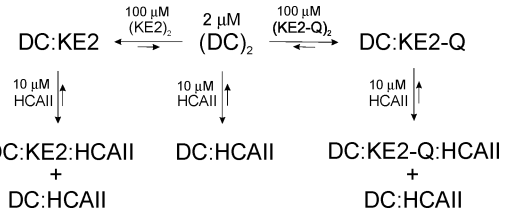
Figure 2. (a) Fluorescence emission spectra of homodimers of KE2-D(15)-5, KE2b-C(25)-5 and **DC**, heterodimers between **DC** and KE2, and **DC** bound to **HCAII**. Emission maxima are observed at 408 nm (methoxycoumarin) and 518 nm (dansyl). (b) Fluorescence emission spectra of **DC**: pure, in the presence of **HCAII**, in the presence of KE2-Q, and in the presence of both **HCAII** and KE2-Q.

polypeptides functionalized with the sulfonamide group thus depends on two interactions, one due to active-site binding of **1** and one due to interactions between the scaffold and **HCAII**. **DC** dimerization is mainly driven by hydrophobic interactions, and successful competition for the **DC** monomer by **HCAII** is therefore probably also hydrophobic in origin.

Thus, at or below μM concentrations, **DC** binds **HCAII** as a monomer and the light-up effect of the methoxycoumarin probe upon binding to **HCAII** is mainly due to homodimer separation. In contrast, the intensity increase of the dansyl probe upon complexation with **HCAII** suggests an effect induced by the enzyme. The complex set of equilibria that describes the protein−protein interactions is illustrated in Scheme 1.

In conclusion, the cooperative binding of **HCAII** by the polypeptide scaffold and the sulfonamide residue accounts for the low observed dissociation constant of 5 nM. The contribution from the scaffold is most likely hydrophobic, whereas that of the sulfonamide is due to its coordination to the zinc ion. This class of molecules that can be constructed in a combinatorial manner combines the properties of biopolymers with those of small organic compounds in the search for new receptors for proteins. Their usefulness in the field of biosensing is evident from the present data, and the intensity enhancements that arise due to disruption of the homodimers upon complexation with the target protein provides a powerful way of improving sensitivities of fluorescence methods for biomolecular identification, quantification, and characterization. Receptor molecules are also of great interest in bioseparation and in biomedicine, and work toward this end is currently underway in our laboratory.

Scheme 1



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(9) All samples were excited at 335 nm at room temperature and pH 7.4. Concentrations of probe-modified peptides were 1 μM (KE2-D(15)-5, KE2b-C(25)-5) or 2 μM (**DC**). Addition of 10 μM **HCAII** saturated the peptides. A surface plasmon resonance-based analysis confirmed binding. Control peptides lacking the ligand **1** did not display any change in fluorescence spectra when **HCAII** was added.

(10) Sequence of TA4 equals that of KE2 with the exception of five amino acids: NAAD**Nle**EA**K**IRHLAEK**Nle**AARGPVDAAQ**Nle**AEQLA**R**K FEAFARAG.

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