Foldamer–Protein Interactions

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Structure of a Complex Formed by a Protein and a Helical Aromatic Oligoamide Foldamer at 2.1 Å Resolution**

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In memory of Frédéric Denonne

Abstract: *In the search of molecules that could recognize sizeable areas of protein surfaces, a series of ten helical aromatic oligoamide foldamers was synthesized on solid phase. The foldamers comprise three to five monomers carrying various proteinogenic side chains, and exist as racemic mixtures of interconverting right-handed and left-handed helices. Functionalization of the foldamers by a nanomolar ligand of human carbonic anhydrase II (HCA) ensured that they would be held in close proximity to the protein surface. Foldamer–protein interactions were screened by circular dichroism (CD). One foldamer displayed intense CD bands indicating that a preferred helix handedness is induced upon interacting with the protein surface. The crystal structure of the complex between this foldamer and HCA could be resolved at 2.1 Å resolution and revealed a number of unanticipated protein–foldamer, foldamer–foldamer, and protein–protein interactions.*

Most proteins operate as complexes or within networks of interactions with other proteins.[1] The recognition of protein surfaces and the inhibition of protein–protein interactions (PPIs) thus offer innumerable opportunities for the development of pharmacological tools or therapeutic agents. Yet they have remained extremely challenging objectives. Indeed, protein surfaces only rarely possess grooves or well-defined active sites that could be targeted by small molecules.[2] In recent years, PPI inhibitors have thus been developed using large molecules such as other proteins,[3] and in particular antibodies,[4] or aptamers.[5] Synthetic foldamers,[6] because of their medium size (typically in the 0.5–5 kDa range) and well-defined structure in solution, appear as potent candidates to serve as scaffolds bearing proteinogenic side chains that would recognize protein surfaces.[7] In addition, foldamers generally show high resistance towards enzymatic degradation and represent attractive alternatives to peptidic and oligonucleotidic backbones. But the question remains: how to arrange individual proteinogenic side chains at the surface of a foldamer to target a given protein surface? Efforts towards this goal have largely concerned the development of α-helix mimetics,[8] combining structure-based design and the screening of small foldamer libraries.[9] But the targeting of large surfaces not complementary to a discrete protein secondary motif remains elusive. A major hurdle lies in the fact that detailed structural information about interactions between foldamers, or other medium-size molecules, and proteins is very scarce.[8i,k,10] In the absence of good lead compounds to form complexes that could be subjected to structural analysis and also in the absence of structural information to help designing good ligands, the discovery of foldamers to bind protein surfaces meets a sort of deadlock.

To solve this problem, we reasoned that it may be possible to obtain structural information about interactions at a foldamer–protein interface even in the absence of strong binding, provided some sort of attachment would link the two. For this purpose, helical aromatic oligoamide foldamers[11] based on 8-amino-2-quinolinecarboxylic acid (Q, see Scheme 1 b) appeared to be suitable. They feature highly stable conformations in protic media.[12,13] Monomers can be equipped with proteinogenic side chains and oligomers can be prepared using solid-phase synthesis (SPS).[14] Though they have not yet

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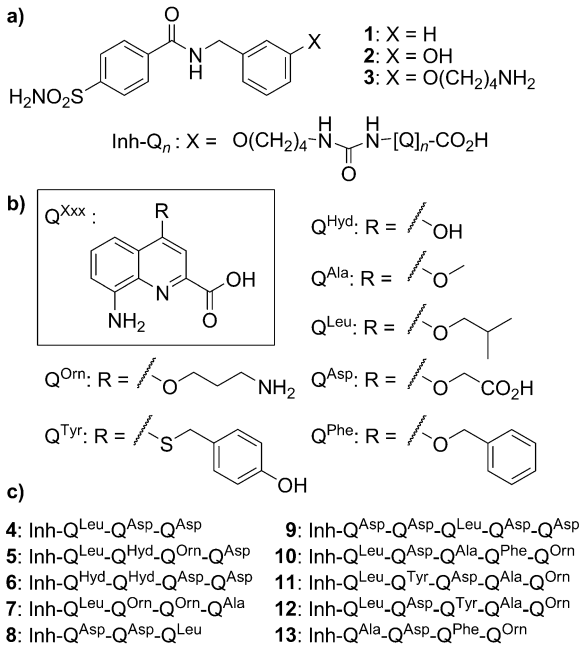

Scheme 1. a) Inhibitor constructs; b) Q^{Xxx} monomers; c) Inh-Q_n foldamers synthesized by SPS.

been shown to interact with proteins, tight binding to G-quadruplex DNA has been demonstrated.[15] In addition, helical aromatic oligoamides possess a remarkable ability to form single crystals even when their size exceeds 5 kDa,[16] thus allowing the hope of crystallizing protein–foldamer conjugates. We thus endeavored to prepare foldamers tethered to a protein ligand in order to crystallize a protein–ligand–foldamer complex and find a structural basis from which to tailor foldamer–protein interactions. As a relevant background to this study, previous examples described the exploration of protein binding properties by using recognition groups, including large peptides, attached to a protein ligand.[17] In the following, we report the design and structure elucidation at 2.1 Å resolution of a helical aromatic oligoamide foldamer–protein complex mediated by a protein ligand.

Human carbonic anhydrase II (HCA) was selected as a model target because it is commercial, relatively easy to crystallize, and because structurally simple benzene sulfonamide nanomolar inhibitors can be readily prepared.[17,18] Benzyl 4-aminosulfonylbenzamide (**1**; Scheme 1) has a K_D value of approximately 2 nM[17b,19] and has been cocrystallized in complex with the HCA active site, its sulfonyl group being coordinated to the Zn^{2+} ion of the enzyme.[19] Examination of this structure hinted at the preference for a functionalization of the benzyl group in the *meta* position to extend this inhibitor out of the binding pocket and covalently attach a foldamer. We prepared inhibitor **2** (see the Supporting Information) and confirmed its suitability by a crystal structure at 1 Å resolution of its complex with HCA, which crystallized in the classical $P2_1$ lattice and showed the phenol function pointing straight out of the binding site (protein database entry: 4MTY). We thus prepared inhibitor **3**, possessing an aliphatic amine at the end of an *n*-butyloxy linker, which was inserted to allow the foldamer some freedom to position itself at the protein surface. A method was then developed to attach the aliphatic amine of **3** to the N-terminal aromatic amine of a quinoline oligoamide chain on the solid phase. The terminal 8-aminoquinoline was activated on the Wang resin by using triphosgene, and amine **3** was then added to form a urea. In the following, we designate such inhibitor–urea–oligoamide constructs as Inh-Q_n (Scheme 1 a).

Proteinogenic side chains were attached by ether or thioether functions to the quinoline position 4; the side chains attached in this position point towards the outside of the helices. Monomers are designated Q^{Xxx} using the Xxx three-letter code of analogous α-amino acids when available. All Fmoc-Q^{Xxxx} monomers were prepared on a multigram scale in 4 or 5 steps and overall yields ranged from 20 to 60%, when starting from a common 8-nitro-4-(1*H*)-quinolinone precursor. We used the previously described Fmoc-Q^{Leu}, Fmoc-Q^{Asp}, and Fmoc-Q^{Orn} acid precursors, the latter two having their side chain protected as *tert*-butyl ester and *tert*-butyloxycarbonyl (Boc) amine, respectively.[14] We also introduced four new residues. To prepare Fmoc-Q^{Phe}, $CuSO_4$/$NaBH_4$ proved to be efficient to reduce the 8-nitro-quinoline precursor into the corresponding amine while preserving the benzyl ether function. Monomer Q^{Hyd} possesses a simple 4-hydroxy residue that was protected by a trifluoroacetic acid (TFA)-labile *para*-methoxybenzyl ether (PMB) during SPS. The PMB group resisted H_2/Pd–C reduction of the 8-nitro-quinoline in the presence of pyridine.[20] In Fmoc-Q^{Tyr}, an acid-stable thioether was used to attach the side chain to the quinoline; the thioether was formed by aromatic nucleophilic substitution of a 4-bromoquinoline precursor. The hydrogenation of the 8-nitro group was possible in this case too. NH_4HCO_2/Pd–C gave better results than H_2/Pd–C. Fmoc-Q^{Ala} caused no particular difficulty and indeed, the final compound could be afforded in excellent purity in a simple precipitation.

To screen different foldamers for their interactions with the surface of HCA, ten sequences were prepared by SPS,[14] all having an appended HCA inhibitor (Scheme 1 c). Crude oligomers are typically obtained in 60–90% purity. Side chain deprotection and cleavage from the resin was carried out in 95:2.5:2.5 TFA/iPr$_3$SiH/H_2O (vol/vol/vol). Sequences were purified by reversed-phase HPLC and obtained in 30–80% yield from initial Wang resin loadings. The choice of side chains of **4**–**13** was intended to be diverse. Most sequences carry a mix of charged and hydrophobic residues. A prevalence of Q^{Asp} was favored owing to the abundance of positively charged residues in the vicinity of the HCA active site.

Importantly, sequences **4**–**13** do not carry any stereogenic center and thus exist as a mixture of right- (*P*) and left- (*M*) handed enantiomeric helical conformers. Q_n sequences were kept short (3 to 5 residues) to allow helix handedness inversion to occur in solution. Indeed, helix stability rises so quickly upon increasing oligomer length[12b] that longer sequences do not undergo any equilibrium in protic media.[13] We then used circular dichroism (CD) as a preliminary screen. All compounds **4**–**13** were expected to have a high affinity for HCA imparted by their inhibitor moiety.

However, the desired interactions between the foldamer helix and the protein, if they existed, would be diastereoselective and thus induce a preferred handedness in the helical oligomer backbone.[15a,21]

The CD spectra of **4**–**13** were recorded in the presence of HCA and monitored over time to allow inversion of helix handedness to occur. For the CD signal to stabilize two days were necessary for sequences having 3 or 4 monomers and five days were necessary for sequences having 5 monomers. HCA does not absorb between 300–500 nm. Thus, CD bands in this region could be assigned to the quinoline chromophores. All CD spectra but one showed very weak bands indicating only marginal effects of the protein surface on foldamer handedness (Figure 1a). In contrast, an intense CD

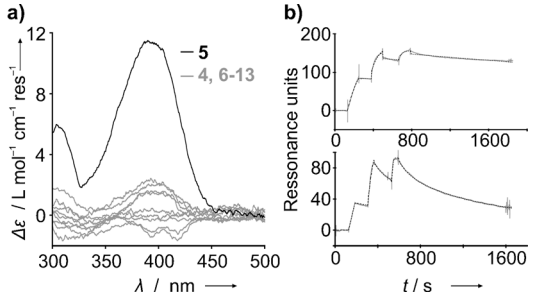

Figure 1. a) CD spectra of **4**–**13** (34.5 μM in 50 mM aqueous phosphate buffer, pH 7.4) in the presence of HCA (34.5 μM) at 20 °C, at equilibrium; b) SPR sensorgrams (gray line) in 2:98 DMSO/aqueous PBS pH 7.4 (vol/vol) at 25 °C. Increasingly concentrated solutions of **5** (250–500–1000 nM, top) and **3** (62.5–125–250 nM, bottom) were flowed over the HCA surface sequentially at 25 μL min^{-1} for 60 s. Curve fitting (dotted black line) assuming a Langmuir 1:1 model yielded $k_{on} = 2.8 \times 10^4$ M^{-1} s^{-1} and $k_{off} = 1.5 \times 10^{-4}$ s^{-1} for **5**; $k_{on} = 1.5 \times 10^6$ M^{-1} s^{-1}, $k_{off} = 7.7 \times 10^{-3}$ s^{-1} for **3**.

band emerged at 390 nm in the spectrum of **5**. Comparison of the $\Delta\varepsilon_{390}$ value (40 L mol^{-1} cm^{-1} for 4 residues) with those of related oligomers[22] suggested that an almost complete handedness bias of **5** towards a *P* helix had taken place upon confinement to the protein surface. Weak induced CD for **4** and **6**–**13** does not allow us to rule out foldamer–protein interactions, but reveals no *P*-versus-*M* helix selectivity. These compounds were thus not further investigated.

The binding of **5** to HCA was characterized by surface plasmon resonance (SPR).[23] Kinetic titration assays[24] were first performed with model compound **3** on HCA immobilized on the chip, and gave a K_D value of approximately 5 nM (Figure 1). Foldamer **5** showed an almost identical K_D value but its k_{on} and k_{off} rates were both 50 times smaller than for **3**. This slow binding and dissociation confirms that interactions take place between the foldamer and HCA, independent from the Inh part of **5**.

Cocrystals of the HCA–**5** complex were obtained by the hanging-drop technique and its structure was solved at 2.1 Å resolution (protein database entry: 4LP6). The complex crystallizes in space group $P2_1$ but the packing is unprecedented among over 400 reported HCA structures. The unit cell volume is twice that of the search model and of the HCA–**2** complex, so that two independent HCA–**5** subunits are present in the asymmetric unit (Figure 2a). The final model shows a continuous (2mFo-DFc) electron density map, contoured at 1.0 σ, all along both A and B HCA chains

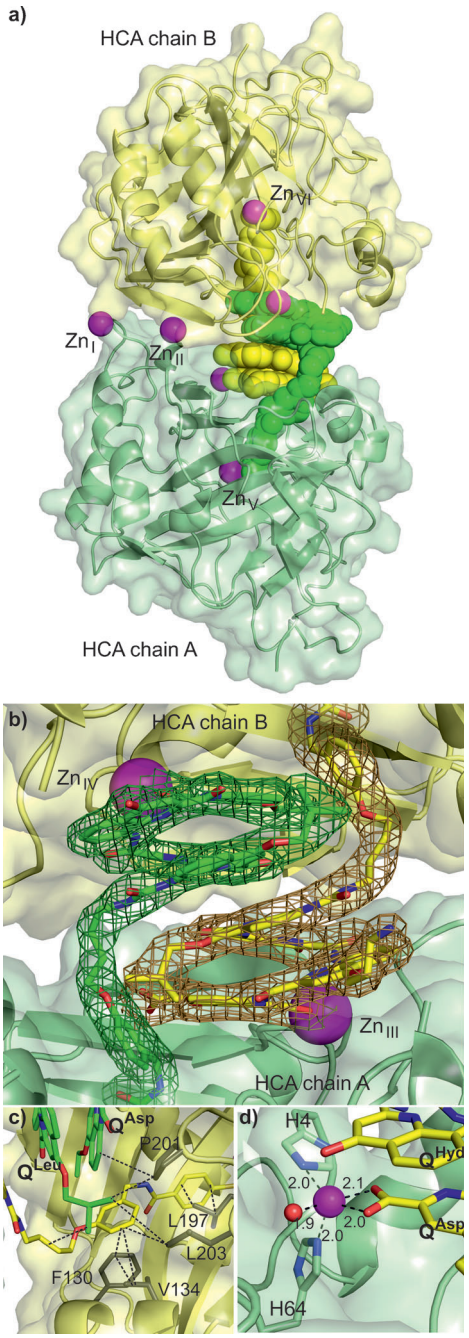

Figure 2. Crystal structure of the HCA–**5** complex. a) Asymmetric unit showing two HCA molecules in pale yellow (chain A) and green (chain B), two Inh-Q^{Leu}Q^{Hyd}Q^{Orn}Q^{Asp} molecules (**5**) in CPK representation and Zn^{2+} ions as purple spheres. Zn$_I$ and Zn$_{II}$ are involved in interactions between HCA chains A and B. Zn$_V$ and Zn$_{VI}$ belong to catalytic centers; b) foldamer backbones shown in sticks and contoured by 2 mFo-DFc density maps at 1σ level. Zn$_{III}$ and Zn$_{IV}$ take part in protein–foldamer interactions; c) contacts between the hydrophobic side chain of Q^{Leu} (green), the linker of the Inh of the other foldamer (yellow) and hydrophobic HCA residues (gray); d) Zn$_{III}$ (purple sphere) and its ligands. A neighbor Q^{Hyd} hydroxy group is found in a distal position. Distances are shown in Å.

(from residues 4 to 260) and the two well-defined Inh-Q_4 molecules (Figure 2b). As with other HCA structures no density is observed for the three first N-terminal residues of each chain.

The structure reveals the formation of a novel pseudo C_2-symmetrical HCA dimer (Figure 2a) involving new protein–protein contacts, foldamer–protein interactions, and foldamer–foldamer interactions. Within a dimer, protein–protein interactions occur through two water molecules hydrogen bonded to Ser172 residues of chains A and B and by two Zn^{2+} bridges (Figure S2). Coordination spheres involve Lys171 and Glu233 of one chain and Asp174 of the other chain. For one of these bridges, an additional ligand, Glu26 from a symmetry-related chain B, is also involved. Within the crystal lattice, contacts between dimers are in limited number, which may explain a high value (51.5 Å^2) for the average temperature factor.

As expected, the inhibitor moieties are bound to the HCA active sites in a conventional manner, with the sulfonamide group coordinated to the catalytic zinc, the benzenesulfonamide ring stacked over Leu197, and the benzyl ring in an edge-to-face arrangement with Phe130 (Figure 2c). The foldamers protrude from the protein surface and adopt right-handed conformations, in agreement with CD spectra. The foldamer bound to chain A is more disordered (higher temperature factor values, Q^{Orn} and Q^{Asp} side chains not observed in density map) than that bound to chain B. The active sites of HCA chains A and B face each other, thereby allowing a direct contact between the foldamers that contributes to the dimerization. The inhibitor-foldamer linkers are in an extended conformation. As a result, each foldamer extends beyond the HCA chain to which its inhibitor moiety is bound and establishes interactions with the other HCA chain. In addition, the two foldamers stack to clip into one another. Stacking interactions occur through the first quinoline units in each sequence (Q^{Leu}), which are found to be coplanar (average distance between rings: 3.46 Å) and rotated by almost 90°. The helix axes of the two foldamers deviate by only 7°.

At its C-terminal part, each helix interacts with the opposite HCA chain through a Zn^{2+} cation coordinated in a square-based pyramidal geometry (Figure 2d). The involved ligands are the main-chain C-terminal carboxylate moiety of the last quinoline, the His4 and His64 imidazole rings, and a water molecule. Cooperative metal binding by His4 and an HCA ligand had been hypothesized by others in earlier studies and is validated here.[17f] The hydroxy group of Q^{Hyd} is found at a distal position in the Zn^{2+} coordination sphere (mean $d_{Zn-O} = 3.8$ Å). The role of Zn^{2+} in foldamer–protein and protein–protein contacts seems to be essential to crystal packing. No crystal growth was observed in the absence of zinc acetate. When observed (foldamer bound to chain B), the polar Q^{Asp} and Q^{Orn} side chains are found to be hydrogen bonded to the opposite protein chain through bridging water molecules. The Q^{Leu} side chain is the only one that is clearly observed for both foldamers and involved in direct interaction with the protein, as it faces a hydrophobic environment involving Pro201, Leu203, the linker, and the phenyl ring of the inhibitor (Figure 2c).

These multiple foldamer–protein and protein–protein interactions do not alter the overall HCA structure. Indeed, the A and B chains are similar to each other (average r.m.s deviation on 257 Cα positions: 0.23 Å; largest r.m.s deviation: 0.93 Å), and similar to the native protein (average and largest Cα r.m.s deviations when superimposing HCA–**5** to the model are 0.31 Å and 0.95 Å for chain A, 0.33 Å and 1.14 Å for chain B, respectively). The reciprocal is also true: the main-chain helix of the foldamer is not altered by the multiple interactions its side chains, its inhibitor moiety, and its terminal carboxylate engage with the protein.

Altogether, the above results validate the strategy proposed in this study. Anchoring a foldamer to a modified inhibitor does not drastically change the K_D value, which remains in the nanomolar range. Accurate structural information quickly revealed intricate interactions between foldamers, proteins, and Zn^{2+} ions and an unexpected and unprecedented dimerization. Multiple suggestions for improvements and controls can be proposed from the structure of HCA–**5**. Can the Q^{Orn} and Q^{Asp} residues of **5** be modified to enhance foldamer–protein interactions? Can the Q^{Hyd} residue be modified to complete the coordination sphere of the foldamer–protein Zn^{2+} bridge? The crystals grew at a concentration of the HCA–**5** complex of 150 μM, which hints at a reasonable stability of the dimeric structure. Yet, this stability and its dependence on Zn^{2+} concentration should be assessed in solution. Investigations along these lines are in progress and will be reported in due course.

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