β-turns are secondary structure elements not only exposed on protein surfaces, but also frequently found to be buried in protein–protein interfaces. Protein engineering so far considered mainly the backbone-constraining properties of synthetic β-turn mimics as parts of surface-exposed loops. A β-turn mimic, Hot-Tap, that is available in gram amounts, provides two hydroxyl groups that enhance its turn-inducing properties besides being able to form side-chain-like interactions. NMR studies on cyclic hexapeptides harboring the Hot═Tap dipeptide proved its strong β-turn-inducing capability. Crystallographic analyses of the trimeric fibritin-foldon/α-hairpin – within a protein interface, as represented by the fibritin–foldon trimer, show how adaptation to complex local environments is achieved. Hot═Tap can mimic a β-turn buried within the protein fold by establishing polar side-chain-like interactions with neighboring structural elements.

**Results and Discussion**

**Synthesis of the β-turn Mimic Hot═Tap.** The synthesis of conformationally constrained β-turn mimics with native-like side chains is hampered by the expense of designing an oligocyclic scaffold that is adequately protected for solid-phase peptide synthesis (13). Polyhydroxylated dipeptides with diversified stereochemistries can be obtained from uronolactones in a straightforward way (14, 15). The oxidation of the primary hydroxy group of 2,3-isopropylidene-d-ribonolactone (1) yields 2,3-isopropylidene-L-riburonolactone, which reacts with L-cysteinemethylester to thiaindolizidinone 2 (Fig. 1B). Acidic isomerization of the acetonide liberates O6, which is subsequently activated to triflate 3. The crystalline azide (4, *SI Text*) forms under inversion of configuration. Hydrogenation, C-terminal saponification, and acylation with Fmoc-chloride yielded the Fmoc-protected Hot═Tap precursor 5 for solid-phase peptide synthesis as further described in *SI Text*. Evidence for the ring puckering is also given in *SI Text*.

**Evaluation of Hot═Tap in Hexapeptide Models.** The difference between Hot═Tap, an element that has a strong tendency to induce β-turns, and BTD, which passively fits to other backbone conformations as well, becomes obvious when comparing different β-hairpin mimics in cyclic hexapeptide models. Cyclic hexapeptides comprise two antiparallel β-hairpin turns and can assume various alternative conformations (17). The cyclic hexapeptides cyclo[Hot═Tap-Gly-], (6), cyclo[BTDT-Gly-], (7), and cyclo[Hot═Tap-Gly-BTD-Gly-] (8) differ in the number of hydroxyl groups on the β-valerolactam ring. Although the hydroxyl groups are oriented outward and do not directly interfere with the intramolecular hydrogen bonds of the two β-hairpin turns, the NMR data of peptide 7 differ from peptides 6 and 8, which contain Hot═Tap. The C2-symmetric hexapeptide 6 two-covalent ring connections (12), provides additional ring substituents, compared to BTD, that restrict alternative puckering states of the β-valerolactam ring. We show how polar hydroxyl substituents lock the ring puckering and provide secondary interactions within the protein environment. Atomic resolution structures of the Hot═Tap turn mimic within a protein–protein interface, as represented by the fibritin–foldon trimer, show how adaptation to complex local environments is achieved. Hot═Tap can mimic a β-turn buried within the protein fold by establishing polar side-chain-like interactions with neighboring structural elements.
shifts and temperature gradients caused by the conformational adaptability of BTD, which fits at least two different positions in the hexapeptide. The lower alternative conformation (yellow background).

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exhibits a large chemical shift dispersion of the amide protons and a reduced solvent accessibility of the glycine amide protons in the solvent DMSO. The Hot=Tap amide proton is oriented toward the solvent, rendering its chemical shift δ (ppm) significantly temperature-dependent with a gradient of ~5.36 ppm/K (SI Text and Fig. S1). The glycine NH, on the other hand, is shielded from the solvent, making its chemical shift less temperature-dependent with a gradient of only ~0.28 ppm/K. The NH chemical shifts and the temperature gradients of 6 show a reversed behavior compared to other bicyclic dipeptides investigated by us, where the bicyclic dipeptide was found outside the β-turn (9, 14, 18). A pyridinone dipeptide within a cyclic hexapeptide (9) is included in Fig. 2 as an example of a peptidomimetic that prefers extended conformations with a reversed hydrogen bonding pattern resulting in a reversed temperature dependency of the NH chemical shifts (18). The averaged temperature dependencies of the amide proton of BTD (~3.46 ppm/K) and of glycine (~2.42 ppm/K) in cyclic hexapeptide 7 are explained by the averaging between conformers wherein BTD occupies the short side of the hexapeptide (similar to 6) and conformers wherein BTD occupies the long side (similar to 9). The temperature gradients of 7 are close to the empirical limit of ±2 ppm/K for an amide that is incorporated in a hydrogen bond (17). The small chemical shift difference between the amide protons supports the notion of a flexible molecule with the peptide appearing dynamic and BTD alternating between the short side (i + 1 and i + 2 position of a β-turn) as well as in the long side (i and i + 1 position of a β-turn) of the cyclopeptide. The conformation of the cyclic hexapeptide 8 containing both BTD and Hot=Tap is again comparable with peptide 6. The glycine at the i + 3 position relative to the β-turn shows the lowest temperature gradient, but also the glycine next to BTD shows only low temperature dependency. In the mixed hexapeptide 8, Hot=Tap pushes BTD into the i + 1 and i + 2 position of the turn and a main conformation similar to 6 is observed. Cyclic hexapeptides show conclusively that Hot=Tap is different from BTD in strongly preferring the i + 1 and i + 2 position of a β-turn. NOEs further support this result, although only few NOEs are unambiguously assigned in the C2-symmetric cyclopeptides.

Overall, Hot=Tap surpasses the well-known BTD because of the restrained pucker of its six-membered δ-valerolactam ring (SI Text and Table S1). Although the bicyclic dipeptide BTD efficiently kinks the peptide backbone, neither this nor related turn mimics can completely restrict a given oligopeptide into a β-hairpin turn conformation. Hot=Tap not only kinks the peptide backbone as any other related ring structure would do but restricts a complete reverse turn.

Design of Hot=Tap-Foldon Hybrids. The foldon protein was first described as the C-terminal domain (G457-A486) of the T4 phage fibrin (19, 20), a trimeric protein that serves as fibrous "whiskers" during late stages of the assembly of T4 phage parti-

Fig. 1. (A) Chemical formula and systematic numbering of the β-turn mimic Hot=Tap, the structurally related dipeptide motif Thr-Pro and the bicyclic dipeptide BTD (5). Constrained oligocyclic dipeptides, which fit the backbone register of oligopeptides, are named by a six-letter code (Xaa=Yaa) (12). (B) Chemical synthesis of the protected dipeptide Hot=Tap derivative 5 for solid-phase peptide synthesis.
Fig. 3. (A) Schematic representation of the secondary structure of the FV-1. Amino acid exchanges are highlighted in orange. The bicyclic dipeptide is abbreviated with the six-letter code Hot=Tap. (B) Backbone of one subunit of the crystalline trimeric FV-1 with the side-chain and backbone modifications highlighted in orange. The chemical structure of the dipeptide mimic Hot=Tap is shown in the inlay (PPII: polyproline II helix).

Figures and data from Eckhardt et al., Proc Natl Acad Sci USA 2007; 104(43): 17155-17160.
tions only for the solvent-exposed side chains of Arg², Lys¹⁶, Glu¹⁹, and Phe²⁰. In contrast, the hydrophobic core of the fibrin–foldon—Ala⁶, Pro⁷, Tyr¹³, Trp²⁵, and Leu²⁷—is almost invariant; the rmsd values for its 26 Cα-positions range between 0.32 and 0.71 Å (Fig. 4B). Furthermore, the conformation of the FV-1 closely corresponds to chemically unmodified foldon units (27–30). For example, compared to the foldon unit within a genetically engineered fusion to a viral nucleocapsid protein (27), the rmsd of the Cα-positions is only 0.65 Å (Fig. S3).

Adaption of the Hot=Tap Turn Mimic into a Protein Interface. The β-turn mimic Hot=Tap occupies the anticipated i+1 and i+2 position within the β-turn loop Lys¹⁶-Glu¹⁹. The six-membered δ-valerolactam ring of the β-turn mimic Hot=Tap uniformly adopts an envelope conformation (E₈) thus directing the C8-hydroxyl group into an axial orientation (Fig. 5). In contrast, the five-membered thiazolidine ring of Hot=Tap occurs in at least two pucker conformations as shown in Fig. 5B. Five out of six monomers have an E₂ envelope conformation, but in the sixth monomer the thiazolidine ring in Hot=Tap adopts two alternative conformations. In the second conformer the sulfur atom of the thiazolidine flips out of the ring plane, thus adopting an E₁ conformation. Obviously, puckering of the thiazolidine allows adaptability of the Hot=Tap mimic to accommodate local structural variations of the FV-1. Due to the conformation of the bicyclic Hot=Tap mimic, its amino group projects equatorially from the six-membered ring, whereas its carboxyl group protrudes axially from the thiazolidine’s ring plane. In the FV-1 the β-hairpin loop conformation can thus be best described as belonging to the β₁₁ type, instead of the β₁ type that is found in unmodified foldon units. A second difference, compared with the wild-type foldon structure, is given by the alternative trace taken by the Hot=Tap moiety. Whereas the thiazolidine occupies almost exactly the same position as Glu¹⁸ in the wild-type fibrin–foldon (deviation between C3 atom and corresponding Cα atom: ~0.5 Å, Fig. S3), the δ-valerolactam ring is significantly shifted from the position occupied by Asp¹⁷ (deviation between C6 and Cα atom: ~1.6 Å). As the backbone cyclization that is implemented in Hot=Tap should restrict only rotary degrees of freedom along β-turn positions i + 1 and i + 2 without shifting the register of β-hairpins, the overall structural effects on the conformation of the Ala¹²-Leu²³ β-hairpin are minor. Accordingly, the hydrogen bond between positions i and i+3 (Lys¹⁶, Glu¹⁹) is almost unaffected (3.0 Å) due to the structure-inducing properties of the Hot=Tap mimic. The expected function of Hot=Tap as a generally strong β-turn inducer unable to adopt largely differing conformations is underlined by the fact that this building block is not tolerated in other positions of the foldon trimer (Table 1, FV-9 to FV-11).

In the wild-type fibrin–foldon the β-hairpin Lys¹⁶-Glu¹⁹ participates in trimer stabilization by forming several hydrogen bonds: first, between the side chain of Asp¹⁷ and the amide of Gly²⁰ of another monomer within the trimer, and secondly, by several water-bridged interactions between the amides deriving from Asp¹⁷ and Gly¹⁸ with the carbonyl group of Pro³ and the side chain of Glu³. Despite some differences for the conformation of the β-hairpin within the FV-1 as compared to the unmodified foldon, the functionalized Hot=Tap group fits to the surface of the neighboring monomer and is well nestled among side chains derived from residues Glu⁶, Arg⁸, and D-Ala¹⁹ (Fig. 4A). Here, Hot=Tap emulates the aforementioned interactions between the β-hairpin turn and the neighboring monomer. Instead of Asp¹⁷ in the unmodified foldon, the C7-hydroxyl group forms a hydrogen bond (3.0 Å) with the amide of the glycine surrogate D-Ala¹⁹ (Fig. 5A). One water molecule (wat1) corresponds to a water molecule found in the wild-type structure by bridging between the axial C8-hydroxyl and the Pro³ carbonyl; two additional water molecules are trapped in the trimer interface. One of them (wat2) forms a hydrogen bond between the C5-carbonyl of the Hot=Tap mimic and the amide of Ala⁶, whereas the other water molecule (wat3) invades the interface by bridging the amino group of Hot=Tap to the backbone carbonyl of Gin¹⁵.

Despite the different β-turn types adopted, the Hot=Tap turn mimic neither affects the intermolecular salt bridge between Glu⁶ and Arg¹⁵ (2.8 Å), which is crucial for trimer formation, (21) nor distorts the attached antiparallel β-sheet. The β-turns of three crystalline fibrin-foldon variants are shown in Fig. 6 together with the four hydrogen bonds formed by their constituting antiparallel β-sheets. Like the peptide backbones shown for the related FV-2 foldon variant that misses a turn mimic, the...
Conclusions and several noncovalent side-chain-like interactions allow the polar contacts to neighboring structural elements within a quaternary protein fold. Residual mobility of the bicyclic ring system and several noncovalent side-chain-like interactions allow the assembly of the intact protein oligomer and trapping of the turn mimic within a protein–protein interface.

\section*{Conclusion}

We replaced the two central amino acids of the $\beta$-turn in the mini-protein fibrinogen–foldon with the bicyclic dipeptide mimic Hot$\Rightarrow$Tap, which constrains the peptide backbone and forms polar contacts to neighboring structural elements within a quaternary protein fold. Residual mobility of the bicyclic ring system and several noncovalent side-chain-like interactions allow the assembly of the intact protein oligomer and trapping of the turn mimic within a protein–protein interface.

$\beta$-turn mimics are of general interest not only in the design of enzyme inhibitors but also as building blocks for synthetic proteins. Design of the latter may involve different strategies of simplification, e.g., the usage of reduced amino acid alphabets (31) or conformationally restricted building blocks like the synthetic turn mimic Hot$\Rightarrow$Tap. Accordingly, the engineering of diversity-minimized biopolymers may pave the way toward a “molecular bionics” of proteins and be in the long-term a new strategy to construct therapeutic proteins.

\section*{Materials and Methods}

\subsection*{Synthesis of the Hexapeptide Models.}

Fragment condensation of two adequately protected Hot$\Rightarrow$Tap-Gly tripeptides yielded the linear urethane protected Hot$\Rightarrow$Tap-Gly-Hot$\Rightarrow$Tap-Gly methyl ester. The linear peptides were deprotected under mild dilution conditions and cyclized to 5, 6, and 7, respectively (4).

\subsection*{Polypeptide Synthesis}

Compound 4 is a stable white powder convenient for the automated solid-phase peptide synthesis. Fibrin–foldon miniproteins were produced on the 0.1 mM scale using a modified standard procedure as given in SI Text.

\subsection*{Crystallization and Data Collection of Fibrin–Foldon Peptides.}

Puriﬁed protein was solved in low salt buffer (9 mM potassium phosphate, pH 7.0, 10% (v/v) D$_2$O) at a concentration of 2 mg/mL. After gentle heating the NMR tube was left in a warm water bath to cool to room temperature overnight, where spontaneous crystallization occurred. Crystals were shortly soaked in cryo buffer (9 mM potassium phosphate, pH 7.0, 10% (v/v) D$_2$O, 40% PEG4000) and flash frozen in liquid nitrogen. Data collection was performed at ID23-1 at the European Synchrotron Radiation Facility (ESRF) (Grenoble, France) yielding a low- and high-resolution dataset with 2.2 Å and 1.06 Å resolution in space group P1 for the FV-1 and a low- and high-resolution dataset at 2.25 Å and 0.98 Å in space group P3$_2$ for the FV-2 foldon.

\subsection*{Structure Solution of Fibrin–Foldon Peptides.}

Diffraction data were integrated and merged using mosflm (32) in space groups P1 and P3$_2$ for FV-1 and FV-2, respectively. Scaling of data was performed using SCALA 6.0 in the CCP4 package (33) and structure solution was carried out with the program Phaser (34). Residues 135–161 of the structure of a fibrin–foldon fusion protein (PDB code: 1TNV) (29) were used as search model assuming two trimers per asymmetric unit for the FV-1 and one trimer per asymmetric unit for the FV-2, respectively. The graphics program Coot (35) was used for manual model building and refinement was carried out with REFMAC5 from the CCP4i package (33, 36). Restraints used for refinement of the synthetic turn mimic were created using the MOE v2008.1 package (Chemical Computing Group Inc.) and MMFF94 as force field. Figures were made using the program PYMOL 0.99 (37).

\section*{Acknowledgments}

The authors thank the Deutsche Forschungsgemeinschaft (DFG), Uwe Linne for his assistance with HPLC-MS analyses, Tobias Craan for discussion, Sandor Brockhauser for support at ESRF beamline ID23-1, Grenoble, and Michael Marsch and Klaus Harms for the crystal structures of the synthetic intermediates discussed in SI Text. B.E. thanks the Evonik foundation for the Werner–Schwarze scholarship.