ACS chemical biology

Letters

pubs.acs.org/acschemicalbiology

Bicyclic Peptide Inhibitor Reveals Large Contact Interface with a Protease Target

Alessandro Angelini,[†,#] Laura Cendron,[‡,⊥] Shiyu Chen,[†] Jeremy Touati,[†] Greg Winter,[§] Giuseppe Zanotti,[‡,⊥] and Christian Heinis*[,†]

[†]Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

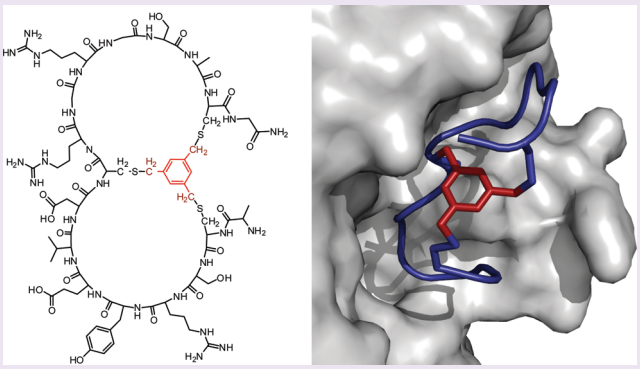
[‡]Department of Biological Chemistry, University of Padua, Viale G. Colombo 3, 35131 Padua, Italy

[⊥]Venetian Institute of Molecular Medicine (VIMM), Via Giuseppe Orus 2, 35129 Padua, Italy

[§]Laboratory of Molecular Biology, Medical Research Council, Hills Road, Cambridge CB2 0QH, U.K.

Supporting Information

ABSTRACT: From a large combinatorial library of chemically constrained bicyclic peptides we isolated a selective and potent (K_i = 53 nM) inhibitor of human urokinase-type plasminogen activator (uPA) and crystallized the complex. This revealed an extended structure of the peptide with both peptide loops engaging the target to form a large interaction surface of 701 Å^2 with multiple hydrogen bonds and complementary charge interactions, explaining the high affinity and specificity of the inhibitor. The interface resembles that between two proteins and suggests that these constrained peptides have the potential to act as small protein mimics.



Urokinase-type plasminogen activator (uPA), a trypsin-like serine protease that participates in the turnover of extracellular matrix (ECM) proteins, is implicated in tumor growth and invasion,[1] and inhibitors of human uPA are being developed for therapy.[2] However it has proved difficult to make small chemical inhibitors that are sufficiently selective or potent,[3] and inhibitors based on macromolecules such as monoclonal antibodies[4,5] or serpins[6] are expected to penetrate tumors poorly. Peptide-based drugs offer an alternative strategy, potentially with a large enough surface of interaction to obtain high potency and selectivity and yet small enough (<2 kDa) to diffuse into tissues. A chemical derivative of a tripeptide efficiently inhibiting human uPA has already been described (K_i = 20 nM).[7] However, although this inhibitor has a significantly better selectivity than previously developed tripeptides, it blocks some serine proteases at submicromolar concentrations that might be a limitation when applied at the high doses that showed therapeutic effects in mice (*e.g.*, K_i's for plasmin, plasma kallikrein, and trypsin are 750, 160, and 22 nM).[7] Cyclic peptide inhibitors have also been described.[8,9] They showed an excellent selectivity for human uPA, but their potency (upain-1: K_i = 6.7 μM at pH 6.0 and 29.9 μM at physiological pH and 37 °C)[9] is likely insufficient for a therapeutic effect.

To improve the potency of cyclic peptides we turned in this work to bicyclic peptides; rather than one large loop we used two smaller loops with the intention of providing a further constraint on the peptide backbone. We isolated the bicyclic peptide inhibitors from a combinatorial repertoire of peptides (library 1: 4.0 × 10^9 different peptides; Figure 1A) that had been cyclized by the trifunctional tris(bromomethyl)benzene (TBMB)[10] on the surface of filamentous bacteriophage,[11] panning the phage against human uPA. After two rounds of selection, we sequenced 30 phage clones, and identified lead inhibitors from the presence of common sequence elements (Figure 1A). A major class of sequences (UK1−UK17) shared residues common to mupain-1, a disulfide constrained monocyclic peptide previously isolated by phage display (CPAYSRYLDC; similar residues are underlined).[8] A minor class of sequences (UK18 and UK19) did not resemble any of the sequences of monocyclic peptides[8,9] previously described (Figure 1A), although it did include a GR motif as noted at the cleavage site of the human uPA substrate plasminogen (KKSPGR↓VVGGSV) and other preferred substrates, for example, the SGR↓S or YGR↓S[12,13] peptides.

We further characterized representative clones (UK3, UK5, UK11, UK16, and UK18). The peptides were expressed as fusions to the D1-D2 domains of phage protein 3 (g3p),[14] purified, and cyclized with TBMB (Supplementary Figure 1 and 2). The bicyclic UK18 fusion protein proved a much more potent inhibitor of human uPA activity (K_i = 71 ± 4 nM) than

Received: November 19, 2011
Accepted: February 3, 2012
Published: February 3, 2012

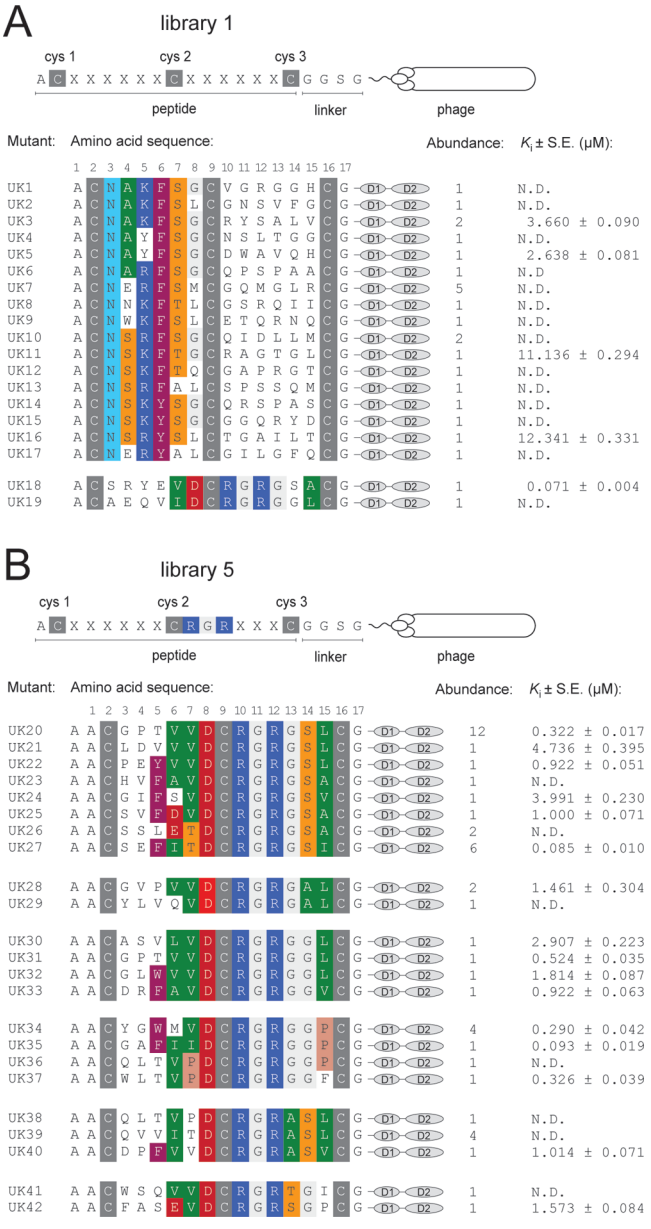


Figure 1. Phage selected bicyclic peptides against human uPA. (A) Amino acid sequences of clones isolated from library 1 having sequence similarities are shown. Identical or similar amino acids are highlighted in color (Rasmol color code). The inhibitory activities (K_i's) of five TBMB-cyclized peptides (as D1-D2 fusion peptides) are indicated. (B) In an attempt to obtain more potent inhibitors, we generated further variants while keeping Arg10 and Arg12 fixed (motif RGR in library 5 and motif DCRGRG in library 6, Supplementary Figures 4 and 5). However, we were unable to identify variants with improved potency (Supporting Information). The average values of at least three measurements are indicated. S.E., standard error; N.D., not determined.

the others (K_i's ranging from 2.6 to 12.3 μM) (Figure 1A). This is also consistent with the binding affinities to human uPA as measured by surface plasmon resonance (K_D = 157 ± 39 and 2078 ± 39 nM for bicyclic UK18 and UK5 fusion proteins, respectively) (Supplementary Figure 3 and Supplementary Table 1). We then chemically synthesized the UK18 peptide (H-ACSRYEVDCRGRGSACG-NH_2) and cyclized it with TBMB (Figure 2A); the bicyclic product inhibited human uPA with improved potency over the fusion protein (K_i = 53 ± 4 nM; Figure 2B and Supplementary Table 2).

We also synthesized a peptide similar to UK18 having serine residues instead of the three cysteines to disable oxidative cyclization (H-ASSRYEVDSRGRGSASG-NH_2) (Figure 2A); the linear peptide inhibited human uPA with low potency (K_i = 17.5 ± 0.7 μM) (Figure 2B and Supplementary Figure 6). A further variant was synthesized with a single serine residue and two cysteines (H-ACSRYEVDSRGRGSACG-NH_2) and cross-linked with bis(bromomethyl) benzene (DBMB) (Figure 2A); the monocyclic peptide inhibited human uPA with a potency (K_i = 383 ± 37 nM) intermediate between those of the linear and bicyclic peptide (Figure 2B and Supplementary Figure 6). Thus successive constraints on the conformational flexibility of the peptide lead to improved potency of inhibition.

The bicyclic UK18 peptide is a competitive inhibitor; the Michaelis–Menten constant (K_m^{app}) for the hydrolysis of a chromogenic peptide substrate by human uPA was found to increase linearly at higher inhibitor concentrations, while the maximal velocity (V_{max}) changed only slightly (Figure 2C). This indicates that the peptide binds at the active site of human uPA. The inhibition appears to be highly specific to human uPA, as the peptide only weakly inhibits a panel of related proteases (K_i's = 111–316 μM). The panel included mouse uPA, and human and mouse tissue-type plasminogen activators (tPA) which share the same primary physiological substrate and inhibitors as well as trypsin, thrombin, plasmin, plasma kallikrein and fXIa (Figure 2D, Supplementary Table 2). The high specificity is crucial for therapeutic use, since several of the proteases have important physiological functions. For example, human tPA is essential to prevent the formation of blood clots, and its inhibition could have adverse consequences in the clinic.

In order to investigate the binding mode of the bicyclic peptide to its target, we determined the X-ray structure of the uPA-UK18 complex at 1.9 Å resolution (Figure 3A and Supplementary Table 3). For residues in contact with the enzyme the electron density was very clear, allowing an unambiguous assignment of side chain orientations, while the density was weaker for residue Gly17, suggesting some flexibility of orientation inside the crystal (Supplementary Figure 7). The bicyclic peptide forms an extended structure with a total of eight intramolecular hydrogen bond interactions (Figure 3B, Supplementary Table 4 and Supplementary Figure 7). The first ring (residues 2–9) forms a large loop, and the second ring (residues 9–16) has a tight turn from residues Gly11 to Ser14 (see also Figure 4).

Both of the peptide rings make contacts with the enzyme, covering a total surface of 701 $Å^2$, with 14 hydrogen bonds, mainly mediated by the three residues Asp8, Arg10, and Arg12 (Figure 3B, Supplementary Table 5). These residues appear to be important as they are conserved between the sequences of UK18 and UK19, and in the X-ray structure Arg12 occupies the S1 substrate pocket of the protease, as expected for natural substrates of human uPA (Figure 3B and Supplementary Figure 7). However, the second ring does not have the required conformation for protease cleavage at this site, and the bicyclic peptide is not cleaved by human uPA.

Comparison of the structure with that of the monocyclic peptide inhibitor upain-1[15] reveals that both inhibitors bind to the same region of human uPA. It may also suggest why the binding affinity of UK18 for human uPA is 200-fold greater than that of upain-1. UK18 is a longer peptide (17 residues compared to 12) and has a more extended structure and a

dx.doi.org/10.1021/cb200478t | ACS Chem. Biol. 2012, 7, 817–821

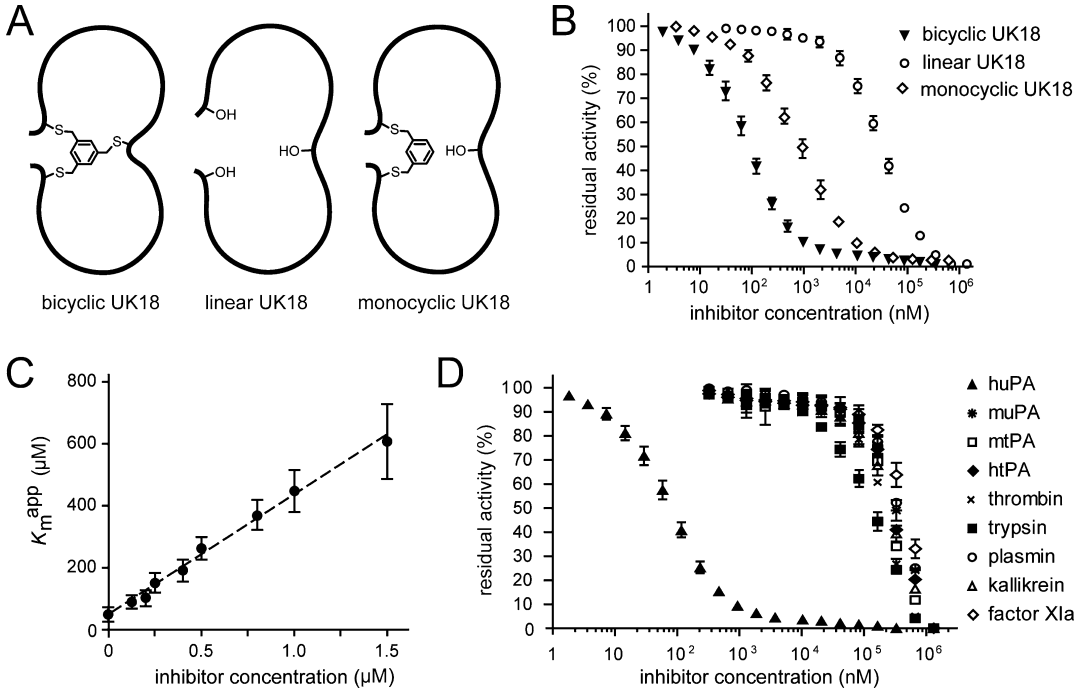


Figure 2. Inhibitory activity and specificity of UK18. (A) Schematic depiction of the bicyclic peptide UK18 (left), a linear peptide based on UK18 with the cysteine residues replaced by serines (middle) and a monocyclic derivative of UK18 having the middle cysteine replaced by serine and the flanking cysteines reacted with DBMB. (B) Residual activities of human uPA incubated with bicyclic UK18 or the linear or monocyclic derivative. The average values of at least three measurements are indicated. (C) Apparent K_m values (K_m^{app}) of human uPA in the presence of different UK18 concentrations were determined and plotted against the inhibitor concentration. The K_m^{app} increases linearly with the inhibitor concentration, suggesting that UK18 is a competitive inhibitor. The average values of at least three measurements are indicated. (D) The residual activities of human uPA and a range of homologous trypsin-like serine proteases were measured at different concentrations of bicyclic peptide UK18. The average values of at least three measurements are indicated (see also Supplementary Table 2).

larger surface of interaction (by more than 179 A^2; see caption of Supplementary Table 7). UK18 also makes more hydrogen bonds to human uPA (14 compared to 11), with three involving complementary charge interactions thought to be important for binding affinity (compared to two for upain-1).[16,17] These noncovalent interactions between peptide and protein appear to be facilitated by cyclization, as shown by the higher potency of bicyclic UK18 over its monocyclic and linear counterparts. Indeed the structures of human uPA in complex with the cyclic peptides resemble those of protein complexes, with constrained peptide backbones, large interfaces of interaction and (directional) hydrogen bonds.[17] More importantly the cyclic peptides appear to behave as proteins, with exquisite specificity and in the case of UK18 with high affinity of binding.

In summary, we report the development of a bicyclic peptide inhibitor of human uPA that is around 200-fold more potent than the best monocyclic peptide inhibitor previously isolated to this target. Such small highly constrained bicyclic peptides (<2 kD) appear to have properties typical of proteins, with a large interface of interaction with target, and multiple directional hydrogen and electrostatic bonds from both loops of the peptide, leading to good binding affinity and exquisite specificity. Such small protein mimics may thereby possess features of both small molecule and protein therapeutics.

METHODS

Phage Affinity Selection of Bicyclic Peptides. Phage were produced and peptides on phage were modified as described previously.[11] Human uPA (UPA-LMW, 33 kDa, Innovative Research) was biotinylated as described in the Supporting Information. Biotinylated protein (2 μg) was blocked in 0.5 mL of washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) containing 1% w/v BSA and 0.1% v/v Tween 20 for 30 min. At the same time, the chemically modified phage (about 10^{11} t.u. dissolved in 2 mL washing buffer) were blocked by addition of 1 mL of washing buffer containing 3% w/v BSA and 0.3% v/v Tween 20 for 30 min. Blocked antigen (0.5 mL) and phage (2 mL) were mixed together and incubated for 30 min on a rotating wheel at RT. Then 50 μL of magnetic streptavidin beads (Dynabeads M-280 from Invitrogen Dynal Biotech AS, Oslo, Norway), blocked in 0.5 mL of washing buffer containing 1% w/v BSA and 0.1% v/v Tween 20 for 30 min, were added to the phage/antigen mixture and incubated for 5 min at RT with rotation. The beads were washed eight times with washing buffer containing 0.1% v/v Tween 20 and twice with washing buffer. Phage were eluted by incubation with 100 μL of 50 mM glycine, pH 2.2 for 5 min. Eluted phage were transferred to 50 μL of 1 M Tris-Cl, pH 8.0 for neutralization and incubated with 50 mL of exponentially growing TG1 cells (OD_{600} = 0.4) for 90 min at 37 °C. The phage-infected cells were pelleted by centrifugation, resuspended in 2 mL of 2YT medium, and plated on a large 2YT/chloramphenicol (30 μg/mL chloramphenicol) plate. A second round of panning was performed following the same procedure but using neutravidin-coated magnetic beads in order to prevent the enrichment of streptavidin-specific peptides. Neutravidin beads were prepared by reacting 0.8 mg of neutravidin (Pierce) with 2×10^9 tosyl-activated magnetic beads (2×10^9 beads/mL; Dynabeads M-280, Invitrogen Dynal Biotech AS) according to the supplier's instructions. Affinity maturation selections with libraries 5 and 6 were performed essentially as the selection with the naïve library 1 but with less biotinylated human uPA (1 μg). The cloning of the phage libraries 5 and 6 is described in the Supporting Information.

Production of Bicyclic Peptides as D1-D2 Fusion Proteins. Phage selected peptides were expressed as fusion proteins of the domains D1 and D2 of phage protein 3 and purified by Ni-affinity

dx.doi.org/10.1021/cb200478t | ACS Chem. Biol. 2012, 7, 817–821

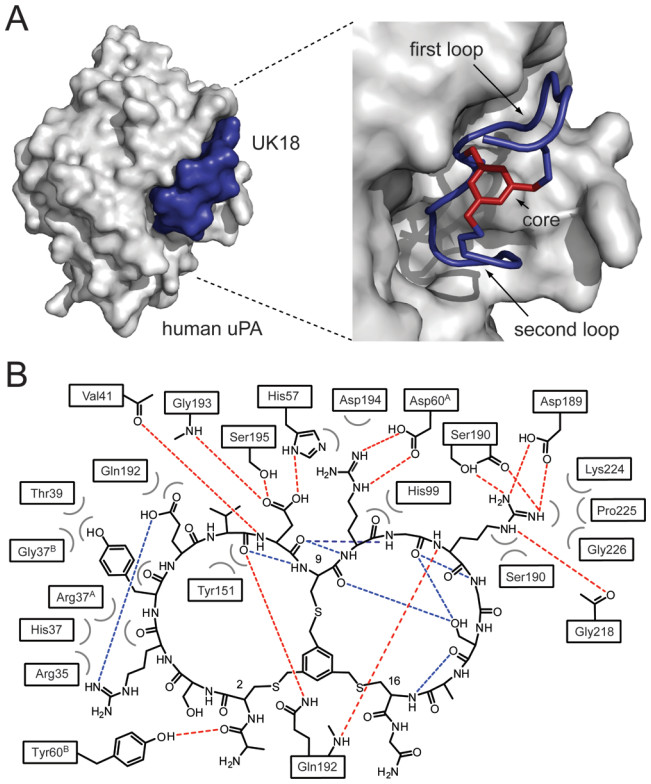


Figure 3. Crystal structure of human uPA in complex with the bicyclic peptide UK18. (A) Surface representation of human uPA (gray) in complex with UK18 (peptide ribbon in blue, mesitylene scaffold in red). A 701 Å^2 surface of human uPA is covered by UK18. (B) Schematic representation of molecular interactions between human uPA and UK18. Residues of human uPA are labeled according to the chymotrypsin numbering system. Potential intermolecular (red) and intramolecular (blue) hydrogen bonds are shown as dashed lines (Supplementary Table 4 and 5). Bent gray lines indicate residues of UK18 in close contact with human uPA (distances shorter than 4.0 Å that are not hydrogen bonds) (Supplementary Table 6).

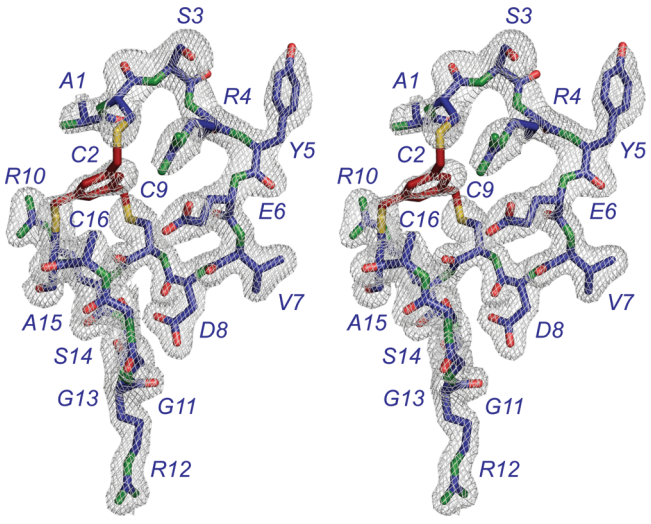


Figure 4. Stereoview of the bicyclic peptide UK18. Conformation and electron density map is calculated with coefficients ($2F_o - F_c$) and contoured at the 2σ level. The aromatic ring of the mesitylene core is shown in dark red. The side chains of the residues are shown as sticks. Carbon, oxygen, nitrogen and sulfur atoms are shown in blue, red, green and yellow, respectively.

chromatography and gel filtration as described in the Supporting Information. The peptide appendix was cyclized by mixing 0.5 mL of TBMB (500 μM) in acetonitrile with 4.5 mL of reduced peptide-D1-D2 (10 μM) in reaction buffer (20 mM NH_4HCO_3, 5 mM EDTA, pH 8.0, degassed) at 30 °C for 1 h. Nonreacted TBMB was removed by gel filtration using a PD-10 column (GE Healthcare, Uppsala, Sweden) and washing buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$) as solvent. The concentration was determined by measuring the optical absorption at 280 nm (GeneQuant 100, GE Healthcare). Cyclized peptide-D1-D2 fusion proteins were analyzed by SDS-PAGE and size exclusion chromatography. The molecular mass of peptide-D1-D2 fusion proteins, before and after modification with TBMB, was determined with electrospray ionization mass spectrometry (ESI−MS) as described in the Supporting Information.

Chemical Synthesis of Bicyclic Peptides. Linear peptides were synthesized by solid-phase peptide synthesis as described in the Supporting Information and cyclized with TBMB or DBMB as follows. The crude peptides UK18 and UK18^{CSC} (1 mM) in 5% v/v DMSO, 65% v/v 20 mM NH_4HCO_3, pH 8.0 and 30% v/v acetonitrile were reacted with TBMB and DBMB, respectively (1.5 mM) for 1 h at 30 °C. The reaction products (bicyclic UK18 and monocyclic UK18) and the linear UK18^{SSS} peptide were purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) using a Vydac C18 (218TP) column (22 mm × 250 mm) (Grace & Vydac, Hesperia, USA), a linear gradient with a mobile phase composed of eluant A (99.9% v/v H_2O, 0.1% v/v TFA) and eluant B (94.9% v/v acetonitrile, 5% v/v H_2O and 0.1% v/v TFA) and a flow rate of 20 mL/min. The purified peptides were freeze-dried and dissolved in H_2O for activity measurements. The purity of the modified and unmodified peptides was assessed by analytical RP-HPLC. The molecular mass was determined by MALDI-TOF mass spectrometry as described in the Supporting Information.

Determination of Inhibitory Activity and Specificity of Bicyclic Peptides. The inhibitory activity of bicyclic peptides was determined by incubating human uPA with different concentrations of inhibitor and quantification of the residual activity with a fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, Switzerland). Substrate, enzyme, and inhibitor concentrations as well as assay conditions are indicated in the Supporting Information. The inhibitory constant K_i was calculated using equations described in the Supporting Information. The specificity of UK18 was determined by measuring the inhibition of a panel of serine proteases using the same assay but different enzyme concentrations and substrates as described in the Supporting Information. The mode of inhibition was verified by quantifying V_{max}^{app} and K_m^{app} at different substrate and inhibitor concentrations using a chromogenic substrate (H-Glu-Gly-Arg-pNA, Bachem) as also described in the Supporting Information.

Crystallization and Structure Determination. The catalytic domain of human uPA with two mutations (C122A and N145Q) eliminating a surface-exposed free cysteine residue and a glycosylation site was expressed in mammalian cells and purified as described in the Supporting Information. The crystallization trials were carried out employing the method of hanging drop vapor diffusion. Crystals were obtained by mixing 1.2 μL of LMW huPA-C122A/N145Q protein (10 mg/mL, 340 μM) in 50 mM HEPES, 100 mM NaCl, pH 7.0, 0.6 μL of UK18 bicyclic peptide solution (6.5 mM), and 1.2 μL of reservoir solution and allowed to equilibrate against 500 μL of 50 mM sodium citrate at pH 4.3, 5% v/v PEG400, 2 M $(NH_4)_2SO_4$ and NaN_3. Crystallization occurred at 20 °C within 3−4 days. X-ray diffraction data of human uPA-UK18 complex were collected at beamline PXIII of the Swiss Light Source at the Paul Scherrer Institut (SLS, Villigen, Switzerland) and at the ID14-EH4 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Crystals belong to the *R*3 space group, with $a = b = 121.25$ Å, $c = 42.72$ Å (hexagonal setting). One monomer is present in the asymmetric unit. The structure was solved by molecular replacement as described in the Supporting Information. Intramolecular and intermolecular hydrogen bond interactions were defined with the program CONTACT (CCP4) and PROFUNC, respectively.

dx.doi.org/10.1021/cb200478t | *ACS Chem. Biol.* 2012, 7, 817−821

■ ASSOCIATED CONTENT

Supporting Information

Additional results on the affinity maturation of UK18, X-ray data collection and refinement statistics, additional views of the structure as well as a detailed discussion of the structure and the molecular interactions between the bicyclic peptide and the protease; complete experimental details as well as a characterization and validation of linear and cyclic peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: christian.heinis@epfl.ch.

Present Address

[#]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge MA, 02139, United States.

■ ACKNOWLEDGMENTS

The authors thank L. Menin for her help with mass spectrometry experiments and analysis as well as D. Hacker and S. Thurnheer for their help with the large scale mammalian cell culture protein expression. We acknowledge L. Grasso and L. Pollaro for help with SPR experiments. Furthermore, we are grateful to S. Tawffik, Y. Bouza, C. Varenne, J. Morales-Sanfrutos, and all of the group members for helpful discussions and technical assistance. We finally thank the staff of beamlines PXIII of SLS (Villingen, Switzerland) and ID14-EH4 of ESRF (Grenoble, France) for providing technical assistance during data collection. The financial contribution from the Swiss National Science Foundation (SNSF Professorship PP00P3_123524/1 to C.H.) is gratefully acknowledged.

■ REFERENCES

(1) Andreasen, P. A., Egelund, R., and Petersen, H. H. (2000) The plasminogen activation system in tumor growth, invasion, and metastasis. *Cell. Mol. Life Sci. 57*, 25−40.

(2) Mekkawy, A. H., Morris, D. L., and Pourgholami, M. H. (2009) Urokinase plasminogen activator system as a potential target for cancer therapy. *Future Oncol. 5*, 1487−1499.

(3) Schweinitz, A., Steinmetzer, T., Banke, I. J., Arlt, M. J., Sturzebecher, A., Schuster, O., Geissler, A., Giersiefen, H., Zeslawska, E., Jacob, U., Kruger, A., and Sturzebecher, J. (2004) Design of novel and selective inhibitors of urokinase-type plasminogen activator with improved pharmacokinetic properties for use as antimetastatic agents. *J. Biol. Chem. 279*, 33613−33622.

(4) Petersen, H. H., Hansen, M., Schousboe, S. L., and Andreasen, P. A. (2001) Localization of epitopes for monoclonal antibodies to urokinase-type plasminogen activator: relationship between epitope localization and effects of antibodies on molecular interactions of the enzyme. *Eur. J. Biochem. 268*, 4430−4439.

(5) Sgier, D., Zuberbuehler, K., Pfaffen, S., and Neri, D. (2010) Isolation and characterization of an inhibitory human monoclonal antibody specific to the urokinase-type plasminogen activator, uPA. *Protein Eng., Des. Sel. 23*, 261−269.

(6) Stoop, A. A., and Craik, C. S. (2003) Engineering of a macromolecular scaffold to develop specific protease inhibitors. *Nat. Biotechnol. 21*, 1063−1068.

(7) Henneke, I., Greschus, S., Savai, R., Korfei, M., Markart, P., Mahavadi, P., Schermuly, R. T., Wygrecka, M., Sturzebecher, J., Seeger, W., Gunther, A., and Ruppert, C. (2010) Inhibition of urokinase activity reduces primary tumor growth and metastasis formation in a murine lung carcinoma model. *Am. J. Respir. Crit. Care Med. 181*, 611−619.

(8) Andersen, L. M., Wind, T., Hansen, H. D., and Andreasen, P. A. (2008) A cyclic peptidylic inhibitor of murine urokinase-type plasminogen activator: changing species specificity by substitution of a single residue. *Biochem. J. 412*, 447−457.

(9) Hansen, M., Wind, T., Blouse, G. E., Christensen, A., Petersen, H. H., Kjelgaard, S., Mathiasen, L., Holtet, T. L., and Andreasen, P. A. (2005) A urokinase-type plasminogen activator-inhibiting cyclic peptide with an unusual P2 residue and an extended protease binding surface demonstrates new modalities for enzyme inhibition. *J. Biol. Chem. 280*, 38424−38437.

(10) Timmerman, P., Beld, J., Puijk, W. C., and Meloen, R. H. (2005) Rapid and quantitative cyclization of multiple peptide loops onto synthetic scaffolds for structural mimicry of protein surfaces. *ChemBioChem 6*, 821−824.

(11) Heinis, C., Rutherford, T., Freund, S., and Winter, G. (2009) Phage-encoded combinatorial chemical libraries based on bicyclic peptides. *Nat. Chem. Biol. 5*, 502−507.

(12) Ke, S. H., Coombs, G. S., Tachias, K., Corey, D. R., and Madison, E. L. (1997) Optimal subsite occupancy and design of a selective inhibitor of urokinase. *J. Biol. Chem. 272*, 20456−20462.

(13) Ke, S. H., Coombs, G. S., Tachias, K., Navre, M., Corey, D. R., and Madison, E. L. (1997) Distinguishing the specificities of closely related proteases. Role of P3 in substrate and inhibitor discrimination between tissue-type plasminogen activator and urokinase. *J. Biol. Chem. 272*, 16603−16609.

(14) Kather, I., Bippes, C. A., and Schmid, F. X. (2005) A stable disulfide-free gene-3-protein of phage fd generated by in vitro evolution. *J. Mol. Biol. 354*, 666−678.

(15) Zhao, G., Yuan, C., Wind, T., Huang, Z., Andreasen, P. A., and Huang, M. (2007) Structural basis of specificity of a peptidyl urokinase inhibitor, upain-1. *J. Struct. Biol. 160*, 1−10.

(16) Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M., and Winter, G. (1985) Hydrogen bonding and biological specificity analysed by protein engineering. *Nature 314*, 235−238.

(17) Jones, S., and Thornton, J. M. (1996) Principles of protein-protein interactions. *Proc. Natl. Acad. Sci. U.S.A. 93*, 13−20.