

Effects of Conformational Stability and Geometry of Guanidinium Display on Cell Entry by β -Peptides

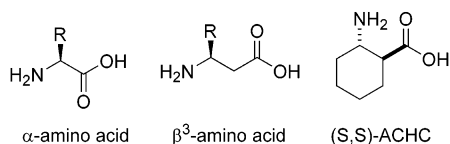
Terra B. Potocky,[§] Anant K. Menon,^{*,‡} and Samuel H. Gellman^{*,§}

Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received December 10, 2004; E-mail: gellman@chem.wisc.edu (S.H.G.); menon@biochem.wisc.edu (A.K.M.)

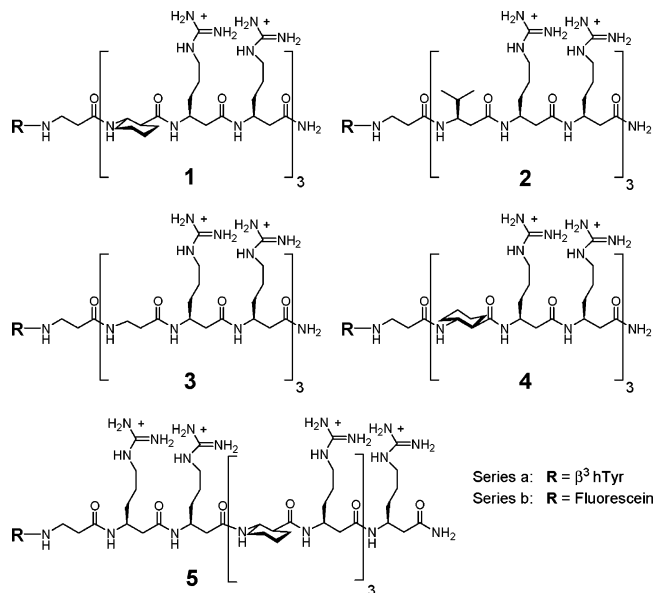
Peptides rich in Arg residues can enter the cytoplasm and ultimately the nucleus of a living cell from the external medium.^{1,2} Cell entry has also been demonstrated for unnatural molecules that display multiple guanidinium groups.^{2e,3} Engineered cell entry may be useful for drug delivery,⁴ but the mechanism is not yet clear and may vary as a function of entry agent, cell type, and/or other factors. As a step toward understanding how molecular structure influences cell entry activity, we have explored the effects of conformational stability and geometry of guanidinium display on this behavior.

α -Amino acid oligomers of ≤ 20 residues are very flexible, and it is difficult or impossible to generate sets of short α -peptides that manifest a wide range of conformational stabilities while being comparable in other characteristics. In contrast, conformational stability can be easily varied among short β -amino acid oligomers. β -Peptides containing exclusively β^3 -residues can adopt the 14-helix secondary structure (defined by 14-membered ring hydrogen bonds between backbone groups, C=O(i)-H-N(i-2)).⁵ For most β^3 -sequences, however, 14-helicity is observed only in structure-promoting solvents, such as methanol, and not in aqueous solution.^{5c} The preorganized *trans*-2-aminocyclohexanecarboxylic acid (ACHC) residue has a much stronger 14-helical propensity than do β^3 -residues.⁶ ACHC and β^3 -homovaline (β^3 hVal) have comparable net hydrophobicities,^{6c} so comparison of β -peptides in which these two residues are swapped allows one to examine the impact of 14-helix stability on other molecular properties of interest.



β -Peptides **1–4** contain repeating triads (X- β^3 hArg- β^3 hArg), where the choice of X is intended to influence 14-helix stability. β -Peptide **1** is designed to form a very stable 14-helix in aqueous solution (X = (S,S)-ACHC), with the six β^3 hArg residues clustered along one side (Figure 1). β -Peptide **2** (X = β^3 Val) is expected to have diminished 14-helical propensity relative to that of **1**.^{6c} In **3**, the X residues are β hGly, which is even more flexible than β^3 hVal; 14-helical folding is therefore unlikely for **3**. The configurational switch of the ACHC residues in **4** (X = (R,R)-ACHC) relative to those of diastereomer **1** (X = (S,S)-ACHC) should prevent 14-helix formation for **4**. Overall, the likelihood of 14-helix formation should decrease dramatically from **1** to **4**.

β -Peptide **5** should form a 14-helix in which the β^3 hArg residues are distributed around the periphery rather than segregated along one side, as in sequence isomer **1** (Figure 1). For both **1** and **5**, the 14-helix conformation is expected to be highly populated in aqueous



solution. Thus, comparing **1** and **5** should indicate whether cell entry activity is affected by the spatial arrangement of guanidinium groups. Circular dichroism (CD) data indicate that **1a–5a** display the expected folding behavior.⁷

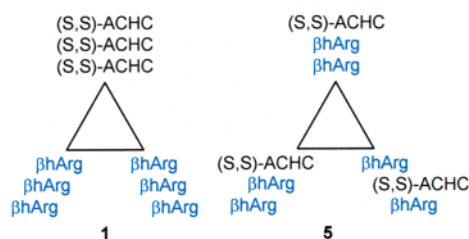


Figure 1. A 14-helical wheel diagram of **1** and **5**, showing the differential display of guanidinium residues about the helical axis.

β -Peptides **1b–5b** bear an N-terminal 6-carboxyfluorescein unit to allow evaluation of cell entry behavior by fluorescence microscopy. β -Peptide **1b** entered cells to a greater extent than did **2b–5b** (Figure 2A). Cell entry by **1b** seemed to peak within 60 min, with $\sim 70\%$ of the HeLa cells showing nuclear staining. Entry by **1b** was completely blocked in the presence of NaN_3 , which implies an energy-dependent uptake process.⁸ Incubation of cells with **1b** in the presence of NH_4Cl led to only endosomal uptake (no green fluorescence in the nucleus; see Supporting Information). β -Peptide **2b** displayed modest cell entry, but only after 60 min, whereas the less structured **3b** did not result in significant nuclear staining until 120 min. β -Peptides **4b–5b** appeared to enter ca. 12–18% of the cells, although the behavior varied between 15 and 120 min. Because of this erratic variation, we regard $\leq 15\%$ uptake as a nonspecific background effect (dashed line in Figure 2A).

[§] Department of Chemistry.
[‡] Department of Biochemistry.

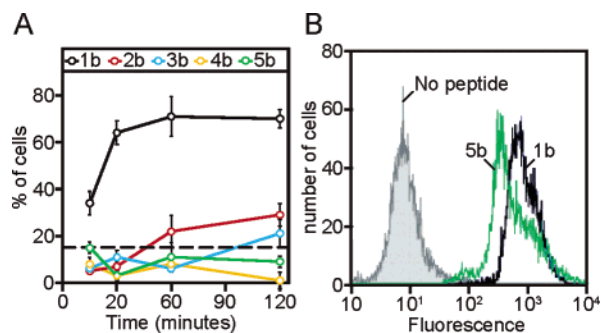


Figure 2. (A) Internalization of **1b–5b** over time; $8 \mu\text{M}$ β -peptide was incubated with cells for 15, 30, 60, or 120 min. The cells with nuclear staining were counted. Each data point is an average of at least four separate experiments; 70–100 cells were counted per experiment. The error bars denote standard error.⁹ Data below the dashed line (15%) are considered insignificant as variations between 0 and 15% occur over time. (B) Surface binding comparison of β -peptides **1b** and **5b** by flow cytometry. Cells treated with NaN_3 were incubated with $8 \mu\text{M}$ peptide for 10 min at 37°C , washed, and analyzed by flow cytometry. β -Peptides **2b–4b** show similar histograms to that of **5b** (see Supporting Information).

Previous studies have indicated that binding to the cell surface is a prerequisite for entry by Arg-rich α -peptides¹⁰ and their cargo-conjugates.^{4c,11} We used flow cytometry to probe for differences in binding to the surface of HeLa cells among **1b–5b**. All five hexacationic β -peptides bind to the cell surface, with ca. 2-fold higher binding for **1b** relative to that of **2b–5b** (Figure 2B and Supporting Information). Because this difference is small, we conclude that the observed variations in cell entry are *not* primarily caused by differences in cell surface binding.

The role of endocytosis^{2e,f,10b,11,12} in cell entry by Arg-rich peptides and their cargo-conjugates is a topic of ongoing debate. We monitored uptake of **1b–5b** via microscopy, looking for the punctate pattern of internal fluorescence that indicates endosomal distribution. β -Peptide **1b** showed extensive endocytic uptake after 15 min, while **2b** did not display significant endocytic uptake until 30 min. The other three β -peptides showed endocytic uptake only after 60 min. These differences parallel the variations in extent of cell entry observed across the series **1b–5b**, which is consistent with the hypothesis that endocytic uptake is necessary for access of these β -peptides to the nucleus. These observations do not rule out the direct entry pathway. In addition, these observations suggest that endocytic uptake does not guarantee access to the cytoplasm or nucleus.

The results reported here show that both the spatial arrangement of guanidinium groups (**1b** vs **5b**) and the rigidity of the molecular scaffold that displays the guanidinium groups (**1b** vs **2b–4b**) affect the entry of an oligocation into live cells. Our ability to examine the influence of these structural features on cell entry depends on the unique control of helix stability offered by β -peptides. The molecular designs we have introduced should be useful for exploring the mechanism(s) of cell entry by guanidinium-rich compounds, which ultimately could allow us to design cationic oligomers with improved cargo delivery ability.

Acknowledgment. This paper is dedicated to Professor Peter B. Dervan on the occasion of his 60th birthday. This work was supported by NIH Grants GM56414 to S.H.G. and GM55427 to A.K.M. Confocal images were obtained at the W.M. Keck

Laboratory for Biological Imaging at the University of Wisconsin. Flow cytometry was performed at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Facility. We thank Anita Pottekat and Saulius Vainauskas for HeLa cell cultures, Adam Steinberg for preparation of figures, and Erik Puffer for helpful discussions.

Supporting Information Available: Fmoc- β -amino acid and β -peptide synthesis, circular dichroism, and biological assay procedures. Figures of circular dichroism, ammonium chloride treatment, and flow cytometry data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) For reviews on cationic peptides, see: (a) Futaki, S. *Int. J. Pharm.* **2002**, *245*, 1. (b) Prochiantz, A. *Curr. Opin. Cell Biol.* **2000**, *12*, 400. (c) Lundberg, P.; Langel, U. *J. Mol. Recognit.* **2003**, *16*, 227. (d) For a review on amphipathic peptide delivery, see: (e) Fernandez-Carneado, J.; Kogan, M. J.; Pujals, S.; Giralt, E. *Biopolymers* **2004**, *76*, 196. For a discussion of fixation artifacts common in experiments with cationic peptides, see: (f) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. *J. Biol. Chem.* **2003**, *278*, 585. (g) Vives, E. *J. Mol. Recognit.* **2003**, *16*, 265. Because of the problems stemming from cell fixation and uncalibrated quantification via flow cytometry initially brought to light by Richard et al., the articles cited below are limited to those describing experiments utilizing live cells and flow cytometry with correction for surface binding of peptides.
- (2) (a) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. *J. Peptide Res.* **2000**, *56*, 318. (b) Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. *J. Med. Chem.* **2002**, *45*, 3612. (c) Thorén, P. E. G.; Persson, D.; Isakson, P.; Goksör, M.; Önfelt, A.; Nordén, B. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 100. (d) Terrone, D.; Sang, S. L. W.; Roudaia, L.; Silviu, J. R. *Biochemistry* **2003**, *42*, 13787. (e) Potocky, T. B.; Menon, A. K.; Gellman, S. H. *J. Biol. Chem.* **2003**, *278*, 50188. (f) Fischer, R.; Kohler, K.; Fotin-Mlecsek, M.; Brock, R. *J. Biol. Chem.* **2004**, *279*, 12625. (g) For an interesting model study, see: Sakai, N.; Takeuchi, T.; Futaki, S.; Matile, S. *Chem. Biochem.* **2004**, *5*, 1.
- (3) (a) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003. (b) García-Echeverría, C.; Ruetz, S. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 247. (c) Seebach, D.; Namoto, K.; Mahajan, Y. R.; Bindschädl, P.; Sustmann, R.; Kirsch, M.; Ryder, N. S.; Weiss, M.; Sauer, M.; Roth, C.; Werner, S.; Beer, H.-D.; Munding, C.; Walde, P.; Voser, M. *Chem. Biodiversity* **2004**, *1*, 65.
- (4) (a) Fawell, S.; Seery, J.; Daikh, Y.; Moore, C.; Chen, L. L.; Pepinsky, B.; Barsoum, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 664. (b) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. *Science* **1999**, *285*, 1569. (c) Wadia, J. S.; Stan, R. V.; Dowdy, S. F. *Nat. Med.* **2004**, *10*, 310. (d) Saalik, P.; Elmquist, A.; Hansen, M.; Padari, K.; Saar, K.; Viht, K.; Langel, U.; Pooga, M. *Bioconjugate Chem.* **2004**, *15*, 1246. (e) Caron, N. J.; Quenneville, S. P.; Tremblay, J. P. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 12. For a review on cargo delivery, see: (f) Trehin, R.; Merkle, H. P. *Eur. J. Pharm. Biopharm.* **2004**, *58*, 209.
- (5) (a) Seebach, D.; Matthews, J. L. *Chem. Commun.* **1997**, *21*, 1015. (b) Gellman, S. H. *Acc. Chem. Res.* **1998**, *31*, 173. (c) Cheng, R. P.; Gellman, S. H.; Degrado, W. F. *Chem. Rev.* **2001**, *101*, 3219.
- (6) (a) Appella, D. H.; Barchi, J. J.; Durell, S. R.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 2309. (b) Raguse, T. L.; Lai, J. R.; Gellman, S. H. *J. Am. Chem. Soc.* **2003**, *125*, 5592. (c) Raguse, T. L.; Porter, E. A.; Weisblum, B.; Gellman, S. H. *J. Am. Chem. Soc.* **2002**, *124*, 12774.
- (7) See data in Supporting Information.
- (8) Steinman, R. M.; Silver, J. M.; Cohn, Z. A. *J. Cell Biol.* **1974**, *63*, 949.
- (9) Standard error = (standard deviation)/ \sqrt{n} , where n equals the number of experiments.
- (10) (a) Tyagi, M.; Rusnati, M.; Presta, M.; Giacca, M. *J. Biol. Chem.* **2001**, *276*, 3254. (b) Fuchs, S. M.; Raines, R. T. *Biochemistry* **2004**, *43*, 2438 and references therein.
- (11) (a) Lundberg, M.; Wikström, S.; Johansson, M. *Mol. Ther.* **2003**, *8*, 143. (b) Drin, G.; Cottin, S.; Blanc, E.; Rees, A. R.; Tamsamani, J. *J. Biol. Chem.* **2003**, *278*, 31192. (c) Nakase, I.; Niwa, M.; Takeuchi, T.; Sonomura, K.; Kawabata, N.; Koike, Y.; Takehashi, M.; Tanaka, S.; Ueda, K.; Simpson, J. C.; Jones, A. T.; Sugiura, Y.; Futaki, S. *Mol. Ther.* **2004**, *10*, 1011. (d) Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A. *J. Am. Chem. Soc.* **2004**, *126*, 9506.
- (12) We use the term endocytosis to describe any capture of cargo from the plasma membrane, including macropinocytosis. For a review, see: Connor, S. D.; Schmid, S. *Nature* **2003**, *422*, 37.

JA042566J