

CH₂), 2.42 (t, 2H, CH₂), 3.48 (s, 9H, NCH₃), 3.72 (2H, NCH₂); FAB-MS: 276 ([M – Br]⁺ calcd 276). **1c**: ¹H NMR (CDCl₃): δ = 0.90 (t, 3H, CH₃), 1.37 (14H, CH₂), 1.77 (2H, CH₂), 2.26 (4H, CH₂), 3.48 (s, 9H, NCH₃), 3.60 (2H, NCH₂); FAB-MS: 276 [M⁺ – Br] (calcd 276), 634 [2M⁺ – Br] (calcd 633).

Thermally induced polymerization of **1b**, **2a**, and **2b** was carried out by heating a N₂-purged glass tube containing a powder sample in a Kugelrohr apparatus at 170 °C for 2 h. For extraction of the included polymer from **3b**, the composite powder was suspended in aqueous hydrofluoric acid (55 wt %) at room temperature for 1 h, and N₂ was bubbled into the resulting clear solution to flush out volatile fractions.

Absorption and fluorescence spectra were recorded in reflection mode on JASCO U-best V-560 and FL-777W spectrophotometers, respectively, with attachments for powder samples. Infrared spectra of KBr pellet samples were recorded on a JASCO FTIR-610 spectrophotometer. X-ray diffraction patterns were recorded on a Rigaku RINT 2400 diffractometer with CuK_α radiation (20 kV, 50 mA) and a step size of 0.004° at a scan speed of 1° min^{–1}. Optical and fluorescence micrographs were taken by an Olympus VANOX AH2-FL microscope, and excitation (395–415 nm) and emission filters (>455 nm) were used for fluorescence microscopy. Thermogravimetric analysis (TGA) was carried out on a Shimadzu TGA-50 at a heating rate of 10 °C min^{–1}. Transmission electron microscopy (TEM) was performed on a Hitachi H-9000UHR electron microscope operated at 300 kV.

Received: April 13, 2001

Revised: August 1, 2001 [Z16942]

- [1] a) M. Fujitsuka, R. Nakahara, T. Iyoda, T. Shimidzu, H. Tsuchiya, *J. Appl. Phys.* **1993**, 74, 1283–1286; b) H. Hong, M. Terabia, H. Chayet, D. Davidov, E. Z. Faraggi, Y. Avny, R. Neumann, S. Kerstein, *J. Appl. Phys.* **1998**, 79, 3082–3088; c) A. V. Yakimov, V. N. Savvate'ev, H. Hong, D. Davidov, *Synth. Met.* **1999**, 102, 955–956.
- [2] K. Tajima, T. Aida, *Chem. Commun.* **2000**, 2399–2412.
- [3] A. Sarkar, S. Okada, H. Matsuzawa, H. Matsuda, H. Nakanishi, *J. Mater. Chem.* **2000**, 10, 819–828.
- [4] a) J. S. Beck, J. C. Vartuli, W. J. Roth, M. E. Leonowicz, C. T. Kresge, K. D. Schmitt, C. T.-W. Chu, D. H. Olson, E. W. Sheppard, S. B. McCullen, J. B. Higgins, J. L. Schlenker, *J. Am. Chem. Soc.* **1992**, 114, 10834–10843; b) A. Corma, *Chem. Rev.* **1997**, 97, 2373–2419; c) J. Y. Ying, C. P. Mehnert, M. S. Wong, *Angew. Chem.* **1999**, 111, 58–82; *Angew. Chem. Int. Ed.* **1999**, 38, 56–77.
- [5] C.-G. Wu, T. Bein, *Science* **1994**, 266, 1013–1014.
- [6] D. J. Cardin, S. P. Constantine, A. Gilbert, A. K. Lay, M. Alvaro, M. S. Galletero, H. Garcia, F. Marquez, *J. Am. Chem. Soc.* **2001**, 123, 3141–3142.
- [7] T. Nguyen, J. Wu, V. Doan, B. J. Schwartz, S. H. Tolbert, *Science* **2000**, 288, 652–656.
- [8] K. Kageyama, S. Ogino, T. Aida, T. Tatsumi, *Macromolecules* **1998**, 31, 4069–4073.
- [9] K. Kageyama, J. Tamazawa, T. Aida, *Science* **1999**, 285, 2113–2115.
- [10] K. Tajima, G. Ogawa, T. Aida, *J. Polym. Sci. Part A* **2000**, 38S, 4821–4825.
- [11] *Advances in Polymer Science*, Vol. 63 (Ed.: H.-J. Cantow), Springer, Berlin, **1984**, and references therein.
- [12] Q. Huo, D. Zhao, J. Feng, K. Weston, S. K. Buratto, G. D. Stucky, S. Schacht, F. Schüth, *Adv. Mater.* **1997**, 9, 974–978.
- [13] See Supporting Information.
- [14] The lowest possible temperature for the polymerization^[13] was chosen to avoid undesirable side reactions.
- [15] G. Wenz, M. A. Müller, M. Schmidt, G. Wegner, *Macromolecules* **1984**, 17, 837–850.
- [16] J. Olmsted III, M. Strand, *J. Phys. Chem.* **1983**, 87, 4790–4792.
- [17] H. R. Bhattacharjee, A. F. Preziosi, G. N. Patel, *J. Chem. Phys.* **1980**, 73, 1478–1480.
- [18] F. Marlow, M. D. McGehee, D. Zhao, B. F. Chmelka, G. D. Stucky, *Adv. Mater.* **1999**, 11, 632–636.

Design and Evolution of a Miniature Bcl-2 Binding Protein**

Jason W. Chin and Alanna Schepartz*

The design of molecules that bind protein surfaces with nanomolar affinity and high specificity presents a major challenge for chemical biologists.^[1] Molecules that possess these properties have potential as therapeutic leads for protein targets^[2] and new tools for proteome research.^[3] We have described a general strategy for the design of miniature α -helical proteins^[4] that bind DNA with high affinity and selectivity.^[5] This protein grafting strategy involves identifying the α -helical residues used for DNA recognition by a natural DNA-binding protein and substituting them on the small, stable protein scaffold avian pancreatic polypeptide (aPP).^[6] Grafting of the DNA contact residues of the bZIP protein GCN4^[7] followed by functional selection generated a folded miniature protein that bound DNA with exceptional affinity and specificity.^[8] Here we demonstrate the versatility of protein grafting for the design of selective miniature protein-binding proteins (Figure 1) and report highly potent and specific ligands for human Bcl-2 and Bcl-X_L.^[9]

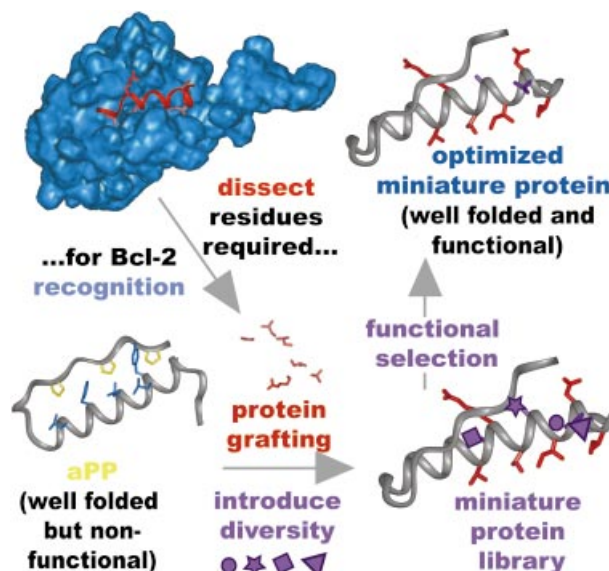


Figure 1. Protein grafting and evolution of high affinity miniature protein ligands for Bcl-2 and Bcl-X_L.

[*] Prof. A. Schepartz, J. W. Chin
Department of Chemistry
Yale University
PO Box 208107, New Haven, CT 06520 (USA)
Fax: (+1) 203-432-3486
E-mail: alanna.schepartz@yale.edu

[**] We thank the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory (Yale University School of Medicine, New Haven, CT) for oligonucleotide and peptide synthesis and amino acid analysis and Professor Jennifer Doudna (Yale University) for use of a Perseptive Voyager-DE (MALDI-TOF) mass spectrometer. We are grateful also to Dr. Junying Yuan and Dr. Alexi Degterev (Harvard Medical School) for a generous gift of Bcl-X_L-His₆ and Stacey E. Rutledge for helpful comments. This work was supported by the National Institutes of Health.

Supporting information for this article is available on the WWW under <http://www.angewandte.com> or from the author.

Bcl-2 and Bcl-X_L are central antagonists of programmed cell death (apoptosis).^[10] Bak,^[11] and other members of the Bcl-2 protein family, are death agonists. The pro-apoptotic activity of Bak can be localized to a 16-residue sequence known as a BH3 domain (Bcl-2 homology domain 3).^[12] The BH3 domain of Bak binds Bcl-2^[13] and Bcl-X_L,^[14] and these interactions can induce apoptosis in both cell-free *Xenopus* extracts^[15] and HeLa cells.^[12] High Bcl-2 levels are found in 80% of B-cell lymphomas as well as certain other cancers,^[16] and can protect malignant cells from apoptosis induced by gamma irradiation and clinically used anticancer drugs.^[17] Inhibition of the anti-apoptotic function of Bcl-2 in tumors is an attractive strategy for restoring apoptosis in cancer cells and increasing their susceptibility to chemo- or radiotherapy.^[18]

Our design of a miniature protein that binds Bcl-2 and Bcl-X_L began with the structure of the Bak_{72–87}·Bcl-X_L complex.^[14] This structure shows Bak_{72–87} bound as a three-turn α -helix to an irregularly shaped hydrophobic cleft on Bcl-X_L. Several observations suggest that the complexes of Bak_{72–87} with Bcl-X_L and Bcl-2 are structurally homologous. First, the backbones of free Bcl-2 and Bcl-X_L superimpose with a root mean square deviation (RMSD) of 1.91 Å.^[13] Second, nine of ten residues within Bcl-X_L that contact Bak are also found in Bcl-2. The lone exception is L112 in Bcl-X_L, which is mutated conservatively to M115 in Bcl-2. Two other differences are notable in the primary sequence that forms the hydrophobic groove of Bcl-2. A108 of Bcl-X_L is mutated to D111 in Bcl-2 and S126 of Bcl-X_L is mutated to R129 in Bcl-2. These mutations alter the charge distribution in the binding pocket on Bcl-2 with respect to the binding pocket on Bcl-X_L.^[13] Finally, Bak binds both Bcl-2 and Bcl-X_L, although with somewhat different affinities.^[13]

We considered all possible alignments of the primary sequences of Bak_{72–87} and the aPP α -helix and assessed their relative merits in three ways. First, each alignment was scored for conflicts, positions where a residue important for maintaining aPP structure was aligned with a residue important for binding Bcl-X_L. Alignments with multiple conflicts were

disfavored as we believed they would produce miniature proteins that were either well folded or had high affinity for Bcl-X_L, but not *both* these properties. Second, each alignment was scored on the basis of unfavorable steric clashes in a model of each miniature protein·Bcl-X_L complex created by using INSIGHT II (Figure 2). Alignments were ranked on the basis of overlap between the van der Waals surfaces of Bcl-X_L and the miniature protein. Finally, we considered the backbone RMSD of each alignment.

Our scoring procedure favored alignment III, in which residues 21–34 of aPP aligned with residues 74–87 of Bak (Figure 2) and a library of 1×10^5 miniature proteins (20^4) was designed (Figure 3a).^[19] All library members contained six

a)

Bak (72–87)	GQVGRQLAIIIGDDINR
aPP	G SQ T Y GDDAPVEDLIRFYNDLQQYLVNVTTRHY
PPBH3LIB	G SQ T Y GDDAPVEDLIRFVXRLLXYTXDXINR

b)

	#	K_d (nM)
Bak (72–87) GQVGRQLAIIIGDDINR	–	4933 ± 450
20 25 30 34		
PPBH3-2.....FVGRLLRYFGDEINR	6	80 ± 12
PPBH3-3.....FVGRLLAYFGDEINR	2	120 ± 20
PPBH3-1.....FVGRLLAYFGDEINR	3	52 ± 5
PPBH3-4.....FVSRL-RYIADLINR	2	N.D.
.....FVRRLLGYIDDIINR	1	
.....FVLRLLWYIPDGINR	1	
.....FVRRLLVYIWDIINR	1	

Figure 3. a) Alignment of Bak_{72–87}, aPP and the miniature protein library. Color coding is described in the legend for Figure 2. Positions varied in PPBH3LIB are in purple. b) Selected miniature proteins. The number of times (#) each sequence was represented among 16 clones is shown. PPBH3-1–4 were synthesized to carry a C-terminal cysteine and labeled with acetamidofluorescein^[22] to facilitate fluorescence polarization analysis. The K_d shown^[24] represents the stability of each PPBH3-Flu·Bcl-2 complex $\pm$ the standard error of at least three trials. PPBH3-4 underwent a time-dependent polarization change in the absence of Bcl-2 and its affinity for Bcl-2 could not be measured (no other peptide tested exhibited this property).

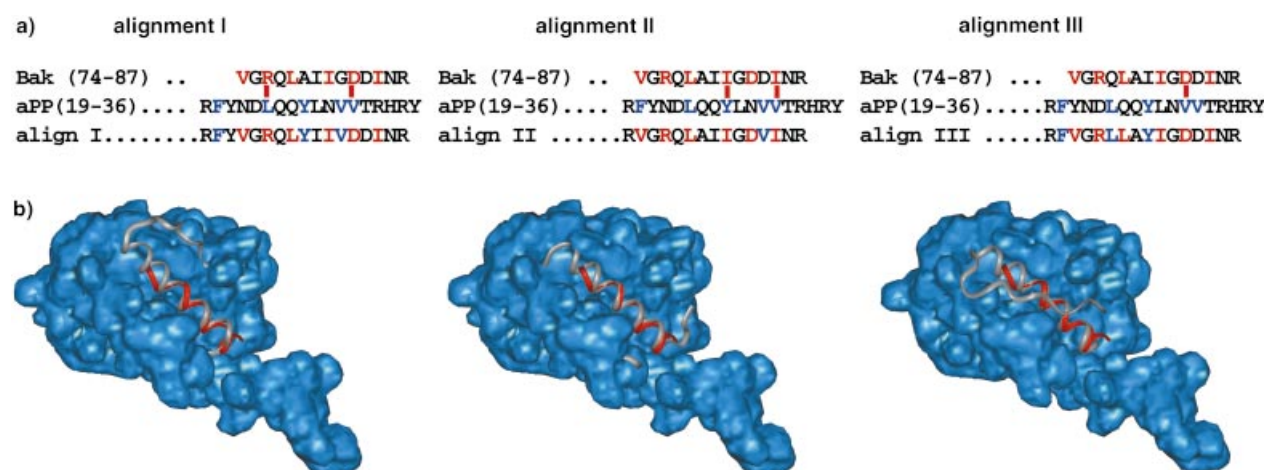


Figure 2. Alignment of aPP and Bak used to guide protein design. a) The three highest scoring alignments of aPP with residues 72–87 of Bak (Bak_{72–87}). Residues in red contribute to Bcl-X_L binding by Bak; residues in blue contribute to folding of aPP. Conflicts between the two are indicated with a red bar. b) Models of miniature proteins resulting from each alignment (gray) superimposed on the Bak_{72–87} backbone (red) when in complex with Bcl-X_L (blue). The conflict in alignment III (between D83 of Bak and V30 of aPP) was expected to be insignificant as residues 30 and 31 are used interchangeably by PP-fold proteins for hydrophobic core formation.^[6, 19]

Bak₇₂₋₈₇ residues (V74, R76, L78, I81, D83, and I85) that contribute significantly to Bcl-X_L affinity^[14] and four aPP residues (V14, F20, L24, Y27) that comprise the aPP hydrophobic core.^[6] The four remaining residues between V21 and I34 were varied across all 20 amino acids. The library of M13 phage produced was sufficient to examine greater than 97 % of all potential DNA sequences.^[20]

Phage were sorted for five rounds on the basis of their affinity for GST-Bcl-2. Seven distinct sequences were represented in the sixteen clones sequenced after round 5 (Figure 3b). Analysis of the four sequences represented multiple times indicated a strong preference for amino acids similar to those in Bak and other BH3 domains at all four randomized positions.^[21] Surprisingly, PPBH3-1, PPBH3-2 and PPBH3-3 each contained phenylalanine at position 28, although this position was not randomized and was encoded as isoleucine. We believe this mutation is functionally significant for two reasons. First, isoleucine was present at position 28 in all 10 independent clones sequenced from the library prior to selection and in all 15 independent clones sequenced after two selection rounds. Second, a clone containing isoleucine at position 28 in addition to G₂₂, A₂₆, G₂₉, and E₃₁ was identified in round 2. This clone differs from Bak₇₂₋₈₇ at only one selected position, but was out-competed in later rounds by sequences containing F₂₈.

The four sequences represented multiple times in round 5, as well as Bak₇₂₋₈₇, were labeled with iodoacetamidofluorescein^[22] and their affinities for Bcl-2 determined by fluorescence polarization analysis.^[23, 24] Under the conditions of our assay the Bak₇₂₋₈₇·Bcl-2 complex was characterized by an equilibrium dissociation constant (K_d) of $4.9 \pm 0.5 \mu\text{M}$. Miniature proteins PPBH3-2 and PPBH3-3 bound Bcl-2 with 40- and 60-fold higher affinity than Bak₇₂₋₈₇, respectively (Figure 3b). PPBH3-1, however, bound Bcl-2 with 100-fold higher affinity to form a complex with a K_d of $52 \pm 5 \text{ nM}$. Comparison of the Bcl-2 affinities of Bak₇₂₋₈₇ and PPBH3-1 suggests that the polyproline helix in PPBH3-1 contributes as much as $\Delta\Delta G = 2.5 \text{ kcal mol}^{-1}$ to PPBH3-1·Bcl-2 complex stability (Figure 3b).

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of PPBH3-1 and Bak₇₂₋₈₇ and explore the structural basis for the enhanced affinity of PPBH3-1 for Bcl-2. The CD spectrum of free PPBH3-1 at 4 °C was concentration independent between 1 and 7 μM and exhibited minima at 208 and 222 nm, as expected for a protein containing one or more α -helices. The mean residue ellipticity (Θ_{mre}) at 222 nm of $-20000 \text{ deg cm}^2 \text{ dmol}^{-1}$ suggested that approximately 60 % of PPBH3-1 possessed an α -helical conformation.^[25] The stability of the fold was examined by monitoring the temperature-dependence of Θ_{mre} at 222 nm. PPBH3-1 underwent a cooperative melting transition with a T_m of 65 °C. These data suggest that PPBH3-1 adopts a stable, well-folded, monomeric, aPP-like structure containing an extended, contiguous α -helix. By contrast, the CD spectrum of Bak₇₂₋₈₇ showed little α -helix ($\Theta_{\text{mre}} = -2000 \text{ deg cm}^2 \text{ dmol}^{-1}$). We conclude that the increased affinity of PPBH3-1 for Bcl-2 relative to Bak₇₂₋₈₇ ($\Delta\Delta G = 2.5 \text{ kcal mol}^{-1}$) reflects preorganization of the otherwise unstructured Bak₇₂₋₈₇ functional epitope.

One advantage of molecules that bind proteins in the nanomolar concentration range may be their ability to discriminate effectively among numerous protein surfaces in a biological milieu. To evaluate the specificity of the interaction between PPBH3-1 and Bcl-2, we monitored the affinity of PPBH3-1 for several proteins that themselves bind hydrophobic peptides or small molecules. PPBH3-1 bound calmodulin, a protein notorious for its ability to bind many α -helical peptides and proteins,^[26] with $K_d > 100 \mu\text{M}$, and bound carbonic anhydrase II^[27] and protein kinase A^[28] with K_d values between 30 and 40 μM (Figure 4b). The large differ-

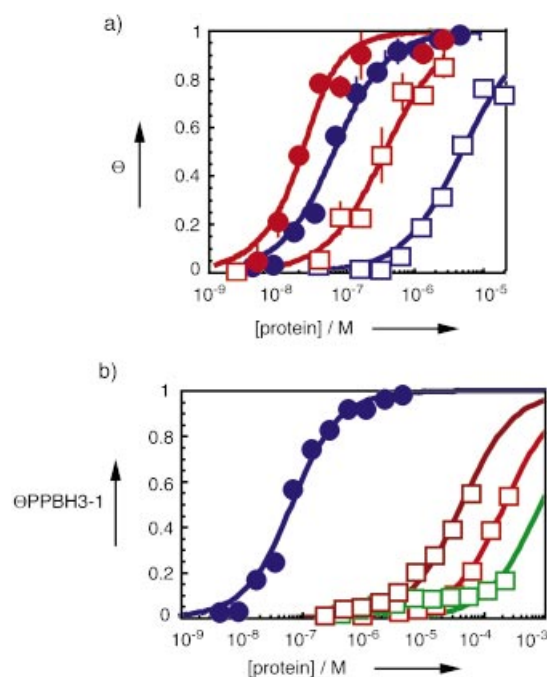


Figure 4. Fluorescence polarization analysis of affinity and specificity. a) Binding isotherms illustrating the affinity of PPBH3-1 (●) and Bak₇₂₋₈₇ (□) for Bcl-2 (blue) and Bcl-X_L (red) at 4 °C in PBS. Θ represents the fraction bound. Data was fit as previously described.^[24] Error bars represent the standard error of at least three trials. b) Binding isotherms illustrating the affinity of the PPBH3-1 for Bcl-2 (●), calmodulin (green), PKA (brown), and carbonic anhydrase II (pink). $\Theta_{\text{PPBH3-1}}$ represents the fraction of PPBH3-1 bound.

ences between the stabilities of these complexes and PPBH3-1·Bcl-2 suggest that the latter is stabilized by a highly stereospecific set of van der Waals contacts. By comparison, Bak₇₂₋₈₇ bound carbonic anhydrase II and calmodulin with K_d values greater than 100 μM and protein kinase A with a K_d value greater than 10 μM .

A critical test of how well protein grafting reproduces the Bak₇₂₋₈₇ epitope is how well PPBH3-1 discriminates between related Bcl-2 proteins. Bak₇₂₋₈₇ itself prefers Bcl-X_L; the Bak₇₂₋₈₇·Bcl-X_L complex ($K_d = 0.35 \pm 0.04 \mu\text{M}$) was 14-fold more stable than the Bak₇₂₋₈₇·Bcl-2 complex ($K_d = 4.9 \pm 0.5 \mu\text{M}$; $\Delta\Delta G = 1.4 \pm 0.1 \text{ kcal mol}^{-1}$) (Figure 4a). Indeed, PPBH3-1 displays a similar preference ($\Delta\Delta G = 1.1 \pm 0.2 \text{ kcal mol}^{-1}$). The PPBH3-1·Bcl-X_L complex ($K_d = 7 \pm 2 \text{ nM}$) was 7-fold more stable than the PPBH3-1·Bcl-2 complex ($K_d = 52 \pm 5 \text{ nM}$). Thus the prefolded recognition

helix in PPBH3-1 differentiates the molecular surfaces of Bcl-2 and Bcl-X_L as well as Bak_{72–87} although it does so in the nM concentration range. It remains to be seen whether PPBH3-1 can evolve further to discriminate more effectively the subtle differences in the Bcl-2 and Bcl-X_L binding pockets.

In summary, here we extend the utility of protein grafting and evolution^[5, 8] to produce miniature protein ligands for protein surfaces. The combined features of high affinity, high specificity, and a compact fold imply that miniature proteins could be used to dissect, modulate, or analyze a single protein function, irrespective of the other functions the protein may regulate within the proteome. In this regard, miniature proteins may possess significant advantages over molecules that down-regulate transcription or those that possess micro-molar affinities and poor or uncharacterized specificities.

Experimental Section

Phage display selection:^[29] The phage display library, PPBH3LIB, was created by cloning an insert encoding the α -helix of library residues 18–35 into the vector pJC20 between unique BglII and NotI restriction sites.^[8] M13 Phage were produced, propagated, and purified as previously described,^[8] except that XL1-blue cells were used in place of TG-1 cells in rounds 3–5. To select for Bcl-2 binding proteins, approximately 10¹⁰ phage were incubated for 2 h in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% (v/v) Tween-20) with human GST-Bcl-2 (Sc4096, Santa Cruz Biotechnology) which had been immobilized on glutathione-coated 96-well microtiter plates (Pierce #15140). Rounds 1 and 2 were performed at 4 °C and rounds 3–5 at 25 °C. Wells were then washed 10 times with TBST for 1 min at 1 min intervals in round 1 and for 5 min at 5 min intervals in rounds 2 through 5. Bound phage were eluted with 0.1N HCl–glycine (pH 2.2) and neutralized with 2M Tris (pH 9.5) before infecting log-phase *E. coli*.

Peptide purification and analysis: All peptides were synthesized by solid-phase methods,^[30] purified to homogeneity by HPLC, labeled with acetamidofluorescein^[22] if necessary, and characterized by MALDI-TOF mass spectrometry and amino acid analysis. CD spectra were recorded on an Aviv 202 CD spectrometer at 4 °C in phosphate-buffered saline (PBS) and were background corrected but not smoothed. PPBH3–1 and Bak_{72–87} were used at 7 and 24 μ M respectively. Binding analyses were performed by using a Photon Technologies 814 fluorimeter at 4 °C in PBS buffer^[22] except for experiments with calmodulin which were measured in buffer containing 20 mM HEPES (pH 7.2), 130 mM KCl, 1 mM CaCl₂.

Received: April 27, 2001 [Z17005]

- [1] A. G. Cochran, *Chem. Biol.* **2000**, 7, R85; M. W. Pecuh, A. D. Hamilton, *Chem. Rev.* **2000**, 100, 2479.
- [2] A. Schepartz, P. S. Kim, *Curr. Opin. Chem. Biol.* **1998**, 2, 9.
- [3] T. Kodadek, *Chem. Biol.* **2001**, 8, 105; R. M. T. deWilt, C. R. Mundy, B. D. Gorick, I. M. Tomlinson, *Nat. Biotechnol.* **2000**, 18, 989; G. MacBeath, S. L. Schreiber, *Science* **2000**, 289, 1760.
- [4] For previous examples of miniaturized or minimized proteins, see: A. C. Braisted, J. A. Wells, *Proc. Natl. Acad. Sci. USA* **1996**, 93, 5688; M. D. Struthers, R. P. Cheng, B. Imperiali, *Science* **1996**, 271, 342; R. B. Hill, W. F. DeGrado, *J. Am. Chem. Soc.* **1998**, 120, 1138; H. Domingues, D. Cregut, W. Sebal, H. Oschkinat, L. Serrano, *Nat. Struct. Biol.* **1999**, 6, 652; C. Vita, E. Drakopoulou, J. Vizzavona, S. Rochette, A. Menez, C. Roumestand, Y. S. Yang, L. Ylistigui, A. Benjouad, J. C. Gluckman, *Proc. Natl. Acad. Sci. USA* **1999**, 96, 13091; L. Martin, P. Barthe, O. Combes, C. Roumestand, C. Vita, *Tetrahedron* **2000**, 56, 9451.
- [5] N. J. Zondlo, A. Schepartz, *J. Am. Chem. Soc.* **1999**, 121, 6938.
- [6] T. L. Blundell, J. E. Pitts, I. C. Tickle, S. P. Wood, C.-W. Wu, *Proc. Natl. Acad. Sci. USA* **1981**, 78, 4175; I. Glover, I. Haneef, J. Pitts, S. Wood, D. Moss, I. Tickle, T. Blundell, *Biopolymers* **1983**, 22, 293.
- [7] T. E. Ellenberger, C. J. Brandl, K. Struhl, S. C. Harrison, *Cell* **1992**, 71, 1223; W. Keller, P. König, T. J. Richmond, *J. Mol. Biol.* **1995**, 254, 657; P. König, T. Richmond, *J. Mol. Biol.* **1993**, 233, 139.
- [8] J. W. Chin, A. Schepartz, *J. Am. Chem. Soc.* **2001**, 123, 2929; J. W. Chin, R. M. Grotzfeld, M. A. Fabian, A. Schepartz, *Bio. Med. Chem. Lett.* **2001**, 11, 1501.
- [9] For examples of other molecules that bind Bcl-2 or Bcl-X_L, see: Degterev, A., A. Lugovskoy, M. Cardone, B. Mulley, G. Wagner, T. Mitchison, J. Y. Yuan, *Nat. Cell Biol.* **2001**, 3, 173; S. Tzung, K. M. Kim, G. Basanez, C. D. Giedt, J. Simon, J. Zimmerberg, K. Y. J. Zhang, D. M. Hockenbery, *Nat. Cell Biol.* **2001**, 3, 183; J. Wang, L. Dongxiang, Z. Zhang, S. Shan, X. Han, S. M. Srinvasa, C. M. Croce, E. S. Alnemri, Z. Huang, *Proc. Natl. Acad. Sci. USA* **2000**, 97, 7124.
- [10] M. G. Vander Heiden, C. B. Thompson, *Nat. Cell Biol.* **1999**, 1, E209.
- [11] S. N. Farrow, J. H. M. White, I. Martinou, T. Raven, K. T. Pun, C. J. Grinham, J. C. Martinou, R. Brown, *Nature* **1995**, 374, 731; T. Chittenden, E. A. Harrington, R. O'Connor, C. Flemington, R. J. Lutz, G. I. Evan, B. C. Guild, *Nature* **1995**, 374, 733; M. C. Kiefer, M. J. Brauer, V. C. Powers, J. J. Wu, S. R. Umansky, L. D. Tomei, P. J. Barr, *Nature* **1995**, 374, 736.
- [12] E. P. Holinger, T. Chittenden, R. J. Lutz, *J. Biol. Chem.* **1999**, 274, 13298.
- [13] A. M. Pettros, A. Medek, D. G. Nettesheim, D. H. Kim, H. S. Yoon, K. Swift, E. D. Matayoshi, T. Oltersdorf, S. W. Fesik, *Proc. Natl. Acad. Sci. USA* **2001**, 98, 3012.
- [14] M. Sattler, H. Liang, D. Nettesheim, R. P. Meadows, J. E. Harlan, M. Eberstadt, H. S. Yoon, S. B. Shuker, B. S. Chang, A. J. Minn, C. B. Thompson, S. W. Fesik, *Science* **1997**, 275, 983.
- [15] S. C. Cousulich, V. Worrall, P. J. Hedge, S. Green, P. R. Clarke, *Curr. Biol.* **1997**, 7, 913.
- [16] A. M. Berghella, P. Pellegrini, I. Contasta, T. Del Beato, D. Adorno, *Cancer Biother. Radiopharm.* **1998**, 13, 225; J. K. Buolamwini, *Curr. Opin. Chem. Biol.* **1999**, 3, 500.
- [17] J. Y. Yang, X. Liu, K. Bhalla, C. N. Kim, A. M. Ibrado, J. Cai, T. Peng, D. P. Jones, X. Wang, *Science* **1997**, 275, 1129; J. C. Reed, *Curr. Opin. Oncol.* **1999**, 1, 68.
- [18] D. W. Nicholson, *Nature* **2000**, 407, 810; A. Piche, J. Grim, C. Rancourt, J. Gomez-Navarro, J. C. Reed, D. T. Curiel, *Cancer Res.* **1998**, 58, 2134.
- [19] X. Li, M. J. Sutcliffe, T. W. Schwartz, C. M. Dobson, *Biochemistry* **1992**, 31, 1245.
- [20] R. C. Ladner in *Phage Display of Peptides and Proteins* (Eds: B. K. Kay, J. Winter, J. McCafferty), Academic Press, San Diego, **1995**, p. 156.
- [21] A. Kelekar, C. B. Thompson, *Trends Cell Biol.* **1998**, 8, 324.
- [22] J. J. Kohler, A. Schepartz, *Biochemistry* **2001**, 40, 130.
- [23] T. Heyduk, Y. Ma, H. Tang, R. H. Ebright, *Methods Enzymol.* **1996**, 274, 492.
- [24] T. Kenakin, *Pharmacologic Analysis of Drug/Receptor Interactions*, 2nd ed., Lippencott Raven, Philadelphia, **1993**.
- [25] P. C. Lyu, J. C. Sherman, A. Chen, N. R. Kallenbach, *Proc. Natl. Acad. Sci. USA* **1991**, 88, 5317.
- [26] W. E. Meador, A. R. Means, F. A. Quirocho, *Science* **1991**, 257, 1251.
- [27] A. Liljas, K. K. Kannan, P.-C. Bergsten, I. Waara, K. Fridborg, B. Strandberg, U. Carlbom, L. Jarup, S. Lovgren, M. Petef, *Nature (London) New Biol.* **1972**, 235, 131.
- [28] D. R. Knighton, J. H. Zheng, L. F. Teneyck, N. H. Xuong, S. S. Taylor, J. M. Sowadski, *Science* **1991**, 253, 414.
- [29] S. S. Sidhu, *Curr. Opin. Biotechnol.* **2000**, 6, 610.
- [30] S. Aimoto, *Curr. Org. Chem.* **2001**, 5, 45.