NMR Evidence for the Reassembly of an α/β Domain after Cleavage of an α-Helix: Implications for Protein Design

X.-M. Yang,[†] W.-F. Yu,[†] J.-H. Li,[†] J. Fuchs,[‡] J. Rizo,[§] and M. L. Tasayco*[,†]

Chemistry Department, The City College of the CUNY New York, New York 10031
Department of Biochemistry, University of Minnesota Saint Paul, Minnesota 55108
Department of Biochemistry and Pharmacology Molecular Genetics and Howard Hughes Medical Institute University of Texas Southwestern Medical Center, Dallas, Texas 75235

Received January 6, 1998

Advances in structural biology have begun to reveal the existence of partially[1] and completely[2] disordered proteins which become more structured upon binding other molecules under nativelike conditions. However, the ability to predict these complementary partners is still in its infancy and we believe that detailed studies of association/folding processes might shed light on this issue. In this regard, it would be interesting to revisit the pioneering work on fragment complementation of Taniuchi,[3] as well as the more recent work by others.[4] In these experiments, the same principles that govern protein folding are apparently involved in the binding of two or more chains.[5] Nussinov and co-workers[6] have indeed found hydrophobic folding units, commonly found in monomeric proteins, at protein interfaces. These elegant results reflect that nature has found ways of assembling the same units using pieces of various shapes. Since most of the sequential information needed to acquire folding is available, although not on a single chain, one can speculate that many cleavages will provide fragments capable of reassembling the native structure. However, most of the successful reassemblies have been limited to loops. We have chosen oxidized *E. coli* thioredoxin (Trx), a small α/β -protein,[7] as a model hydrophobic folding unit to study the effect of the site and number of cleavages on the reassembly. Here we report the first NMR evidence of successful reassembly (1−37/38−108) by fragment complementation after cleavage[8] of an α-helix.

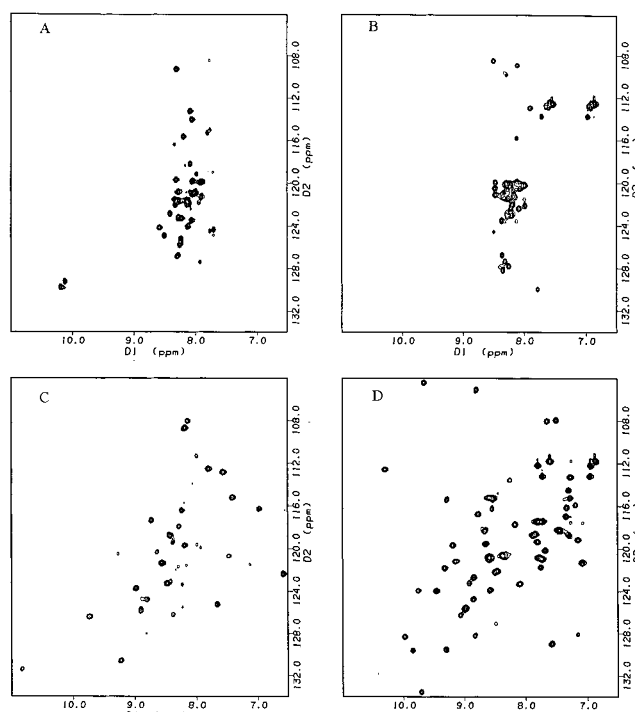

Figure 1. ^{1}H−^{15}N HSQC spectra of ^{15}N−N fragment (A), ^{15}N−C fragment (B), a 2.5:1 stoichiometric mixture of unlabeled C fragment and ^{15}N−N fragment (C), and a 1:2.5 stoichiometric mixture of ^{15}N−C fragment and unlabeled N fragment (D). All of the samples were prepared in 10 mM KP$_i$, pH 6.5, 90% H_2O/10% D_2O at 20°.

Because of the high sensitivity of ^{1}H and ^{15}N chemical shifts to structural changes, ^{1}H−^{15}N HSQC spectra (see Figure 1) provide a powerful tool to probe the conformation of a given polypeptide sequence. The ^{1}H−^{15}N HSQC spectra of the N- and C-terminal fragments exhibit a narrow dispersion of backbone ^{1}H chemical shifts (0.80 and 0.95 ppm, respectively), which is characteristic of disordered polypeptides.[9] In contrast, the ^{1}H−^{15}N HSQC spectra of the ^{15}N-labeled N- and C-terminal fragments in the presence of an excess of the unlabeled complementary fragment show a much broader dispersion of backbone ^{1}H chemical shifts (3.15 and 3.11 ppm, respectively), which is consistent with the formation of a noncovalent complex (1−37/38−108) with well-defined structure.[10]

The HSQC spectra of the 1:1 stoichiometric mixture of 1−37 and 38−108 (data not shown) show the resonance cross-peaks of the individual fragments[11] in equilibrium with their noncovalent complex. This complex is apparently less stable than a previously

* To whom correspondence should be addressed.
† The City College of the CUNY.
‡ University of Minnesota.
§ University of Texas Southwestern Medical Center.

(1) (a) Muchmore, S. W.; Sattler, M.; Liang, R. P.; Meadows, H.; Harlan, J. E.; Yoon, H. S.; Nettesheim, D.; Chang, B. S.; Thompson, C. B.; Wong, S.-L.; Ng, S.-C.; Fesik, S. *Nature* **1996**, *381*, 335−311. (b) Kriwacki, R. W.; Hengst, L.; Tennant, L.; Reed, S. L.; Wright, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11504−11509. (c) Chang, B. S.; Minn, A.; Muchmore, S.; Fesik, S. W.; Thompson, C. *EMBO J.* **1997**, *16*, 968−977. (d) Eberstadt, M.; Huang, B.; Olejniczak, E. T.; Fesik, S. W. *Nat. Struct. Biol.* **1997**, *4*, 983−985. (e) Riek, R.; Hornemann, S.; Wider, G.; Glockshuber, R.; Wüthrich, K. *FEBS Lett.* **1997**, *413*, 282−288.

(2) (a) Daughdrill, G. W.; Chadsey, M. S.; Karlinsey, J. E.; Hughes, K. T.; Dahlquist, F. W. *Nat. Struct. Biol.* **1997**, *4*, 285−291. (b) Daughdrill, G. W.; Hanely, L. J.; Dahlquist, F. W. *Biochemistry* **1998**, *37*, 1076−1082.

(3) Taniuchi, H.; Parr, G. R.; Jullierat, M. A. *Methods Enzymol.* **1986**, *131*, 185−217.

(4) (a) Sancho, J.; Fersht, A. R. *J. Mol. Biol.* **1992**, *224*, 741−747. (b) Tasayco, M. L.; Carey, J. *Science* **1992**, *255*, 594−597. (c) de Prat Gay, G.; Fersht, A. *Biochemistry* **1994**, *33*, 7957−7963. (d) Tasayco, M. L.; Chao, K. *Proteins: Struct., Funct. Genet.* **1995**, *22*, 41−44. (e) Kobayashi, N.; Honda, S.; Yoshi, H.; Uedaira, H.; Munekata, E. *FEBS Lett.* **1995**, *366*, 99−103. (f) Neira, J. L.; Davis, B.; Ladurner, A. G.; Buckle, A. M.; de Prat Gay, G.; Fersht, A. R. *Folding Des.* **1996**, *1*, 189−208. (g) Ladurner, A. G.; Itzhaki, L. S.; de Prat Gay, G.; Fersht, A. R. *J. Mol. Biol.* **1997**, *273*, 317−329. (h) Neira, J. L.; Fersht, A. R. (personal communication on their recent work with barnase fragments).

(5) The same type of forces are at play in protein folding and binding (Xu, D.; Lin, S.-L.; Nussinov, R. *J. Mol. Biol.* **1997**, *265*, 68−84). However, the hydrophobic effect appears to drive folding (Dill, K. A. *Biochemistry* **1990**, *29*, 7133−7155) and the electrostatic interactions seem to predominate in binding.

(6) Tsai, C.-J.; Nussinov, R. *Protein Sci.* **1997**, *6*, 1426−1437.

(7) Dyson, H. J.; Holmgren, A.; Wright, P. E. *Biochemistry* **1989**, *28*, 7074−7087.

(8) The fragments were generated using CNBr (Slabý, I.; Holmgren, A. *J. Biol. Chem.* **1975**, *250*, 1340−1347) and purified under denaturing conditions by molecular sieve chromatography and reverse phase FPLC. The purity was established by SDS-PAGE, isoelectric enfocusing, and mass spectroscopy. Each individual fragment or their mixture was concentrated to at least 1 mM in 10 mM potassium phosphate (KP$_i$) at pH 6.5. All NMR spectra were recorded in a Varian-500 at Hunter College and analyzed with FELIX 1.1.

(9) Alexandrescu, A. T.; Abeygunawardana, C.; Shortle, D. *Biochemistry* **1994**, *33*, 1063−1072.

(10) Upfield ^{1}H resonances indicate nativelike side chain packing.

(11) The 1D-NMR spectra of the freshly isolated fragments show no concentration dependence indicative of self-association. Molecular sieve chromatography of the 1:1 stoichiometric mixture indicates the presence of at least 10% of the individual fragments.

(12) Analysis of 1:1 stoichiometric mixtures of 1−73 and 74−108 indicates a noncovalent complex in equilibrium with negligible amounts of monomeric fragments: Chaffotte, A.; Li, J.-H.; Georgescu, R.; Goldberg, M.; Tasayco, M. L. *Biochemistry* **1997**, *36*, 16040−16048.

S0002-7863(98)00065-1 CCC: $15.00 © 1998 American Chemical Society
Published on Web 07/24/1998

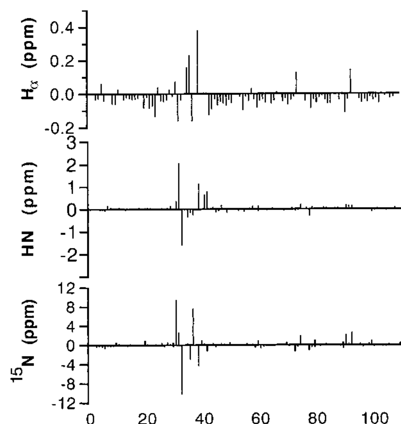

Figure 2. Chemical shift differences between the H_α, HN, and ^{15}N of the complex (1−37/38−108) and uncleaved Trx.

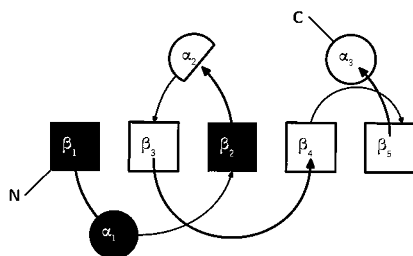

Figure 3. Scheme of the topology of the complex (1−37/38−108). The regions corresponding to the N and the C fragment are depicted with solid and empty symbols, respectively.

reported complex (1−73/74−108)[4d,12] derived from Trx, but more active in vitro (27% of Trx's activity[13]) than the other one (1%). Assignment of the ^1H and ^{15}N resonances of each ^{15}N-labeled protein fragment in the complex were obtained using 3D $^1H-^{15}N$ TOCSY-HSQC and NOESY-HSQC experiments.[14] The small differences in the majority of HN, ^{15}N, and H_α chemical shifts between the fragments and uncleaved Trx (Figure 2) reflect the nativelike features of the reassembly with perturbations near the cleavage site (W31−A39). A comprehensive analysis of NOE patterns confirms the presence of a β-sheet and three α-helices.[15] The observed differences are not unexpected since (i) residues 37 and 38 of uncleaved Trx become the new N- and C-termini without the constraints of a peptide bond and (ii) Met37 has been modified to a homoserine which is in equilibrium with its lactone.[16] In conclusion, our NMR analysis demonstrates the reassembly of the backbone topology (Figure 3) and side chain packing.

We have shown that cleavage of an α-helix of Trx does not prevent reassembly. To our knowledge, this is the first NMR evidence that disruption of an element of secondary structure does not prevent reassembly. Analogous results have been obtained, however, by random circular permutation of aspartate transcarbamoylase,[17] which illustrates that the appearance of new N- and C-termini within α-helices has no effect on its activity. In conclusion, studies of fragment complementation and circularly permuted proteins[18] indicate that the folded state is tolerant to such perturbations. However, the number of successful fragment complementations is smaller than the number of known protein dissections.[4e,g,19] This observation suggests that the site and number of cleavages have a profound effect on the energetics but not on the structure of the folded state. Indeed, the relationship between structure and stability of the complexes (1−37/38−108; 1−73/74−108) derived from Trx is not simple and might be due to (i) entropic differences between the cleavage of a loop and a helix, (ii) the electrostatic effect of the new termini on the helix macrodipole,[20] and (iii) the competition between intra-[21] and intermolecular[19e,22,23] processes involving the individual fragments. Thus, more work is still needed to predict which cleavage sites lead to stable reassemblies.

The de novo design of proteins with desirable properties demands a profound understanding of protein folding. During the past decade, the classification of thousands of protein sequences according to a much smaller number of structural motifs[24] has opened the way to the design and prediction[25] of protein structure. Modern algorithms based on the idea of "threading" [26] have been partially successful in the prediction of structure and are continuously being improved.[27] Progress has also been made in the design of small-sized α-helical,[28] β-sheet,[29] and α/β protein domains.[30] The next step might be the rational design of binding proteins of pharmaceutical interest. Our results might have implications in this area; for instance, one could imagine inserting one fragment in the sequence of a hydrophobic unit while its complementary fragment is inserted into another.

Acknowledgment. We thank Francisco Figueirido and Jose Luis Neira for insightful comments. M.L.T. is a recipient of financial support from NIGMS (5R29GM53808-02) and the RCMI grant to CCNY.

Supporting Information Available: HSQC spectra of the 1:1 stoichiometric mixtures, summary of NOE connectivities, upfield region of the 1D-NMR spectra, and line widths of resonances from the isolated fragments at various concentrations (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

JA980065Q

(13) The initial velocity of protein-catalyzed reduction of DTNB in the presence of *E. coli* thioredoxin reductase, NADPH, 10 mM KP_i at pH 5.7 was measured by visible spectroscopy according to the following: Luthman, M.; Holmgren, A. *Biochemistry* **1982,** *21*, 6628−6633. The activity of the complex was determined using an excess of C-terminal fragment. Controls were obtained for each individual fragment.

(14) Zhang, O.; Kay, L.; Olivier, J.; Forman-Kay, J. *J. Biomol. NMR* **1994**, *4*, 845−858.

(15) The second α-helix of this complex is shorter than the one in uncleaved Trx.

(16) Gross, E.; Witkop, B. *J. Biol. Chem.* **1962**, *237*, 1856−1860.

(17) Graf, R.; Schachman, H. K. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11591−11596.

(18) Lindqvist, Y.; Schneider, G. *Curr. Opin. Struct. Biol.* **1997**, *7*, 422−427.

(19) (a) Dyson, H. J.; Merutka, G.; Waltho, J. P.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1992**, *226*, 795−817. (b) Dyson, H. J.; Sayre, J. R.; Merutka, G.; Shin, H. C.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1992**, *226*, 819−835. (c) Kemmink, J.; Creighton, T. E. *J. Mol. Biol.* **1993**, *234*, 861−878. (d) Bruix, M.; Muñoz, V.; Campos-Olivas, R.; Del Bosque, J. R.; Serrano, L.; Rico, M. *Eur. J. Biochem.* **1997**, *243*, 384−392. (e) Reymond, M. T.; Merutka, G.; Dyson, H. J.; Wright, P. E. *Protein Sci.* **1997**, *6*, 706−716. (f) Blanco, F. J.; Serrano, L. *Eur. J. Biochem.* **1995**, *230*, 634−649.

(20) Muñoz, V.; Serrano, L. *J. Mol. Biol.* **1995**, *245*, 275−296 and references therein.

(21) The accumulated NMR evidence indicates that protein fragments might acquire marginally stable native β-turns (Blanco, F. J.; Jiménez, M. A.; Rico, M.; Santoro, J.; Herranz, J.; Nieto, J. L. *Eur. J. Biochem.* **1991**, *200*, 345−351), β-hairpins (Blanco, F. J.; Ramírez-Alvarado, M.; Serrano, L. *Curr. Opin. Struct. Biol.* **1998**, *8*, 107−111), and α-helices ((a) Brown, J. E.; Klee, W. A. *Biochemistry* **1971**, *10*, 470−476. (b) Jiménez, M. A.; Bruix, M.; González, C.; Blanco, F. J.; Nieto, J. L.; Herranz, J.; Rico, M. *Eur. J. Biochem.* **1993**, *211*, 569−581 and ref 19a) or non-native (Blanco, F.; Ortiz, A. R.; Serrano, L. *Folding Des.* **1997**, *2*, 123−133) conformations (Dyson, H. J.; Wright, P. E. *Annu. Rev. Biophys. Biophys. Chem.* **1991**, *20*, 519−538), which might affect the recognition between disordered fragments.

(22) (a) Dyson, H. J.; Wright, P. E. *Curr. Opin. Struct. Biol.* **1993**, *3*, 60−65. (b) Ramírez-Alvarado, M.; Serrano, L.; Blanco, F. J. *Protein Sci.* **1997**, *6*, 162−174.

(23) This might involve a competition between self-association and non-self-association.

(24) Chothia, C.; Hubbard, T.; Brenner, S.; Barns, H.; Murzin, A. *Annu. Rev. Biophys. Biomol. Struct.* **1997**, *26*, 597−627 and references therein.

(25) Jones, D. T. *Curr. Opin. Struct. Biol.* **1997**, *7*, 377−387.

(26) Madej, T.; Gibrat, J. F.; Bryant S. H. *Proteins* **1995**, *23*, 356−369.

(27) Rost, B.; Schneider, R.; Sander, C. *Proteins* **1997**, *270*, 471−480.

(28) (a) Beasley, J. R.; Hecht, M. H. *J. Biol. Chem.* **1997**, *272*, 2031−2034. (b) Bryson, J. W.; Betz, S. F.; Lu, H. S.; Suich, D. J.; Zhou, H. X.; O'Neil, K. T.; DeGrado, W. F. *Science* **1995**, *270*, 935−941.

(29) Ilyina, E.; Roogta, V.; Mayo, K. H. *Biochemistry* **1997**, *36*, 5245−5250 and references therein.

(30) (a) Struthers, M. D.; Ottesen, J. J.; Imperiali, B. *Folding Des.* **1998**, *3*, 95−103. (b) Dahiyat, B. I.; Mayo, S. L. *Science* **1997**, *278*, 82−87. (c) Struthers, M. D.; Cheng, R. P.; Imperiali, B. *Science* **1996**, *271*, 342−345.