J|A|C|S
COMMUNICATIONS

Published on Web 04/09/2003

Protein Assembly by Orthogonal Chemical Ligation Methods

Bradley L. Nilsson,[†] Robert J. Hondal,[‡,§] Matthew B. Soellner,[†] and Ronald T. Raines*[,†,‡]

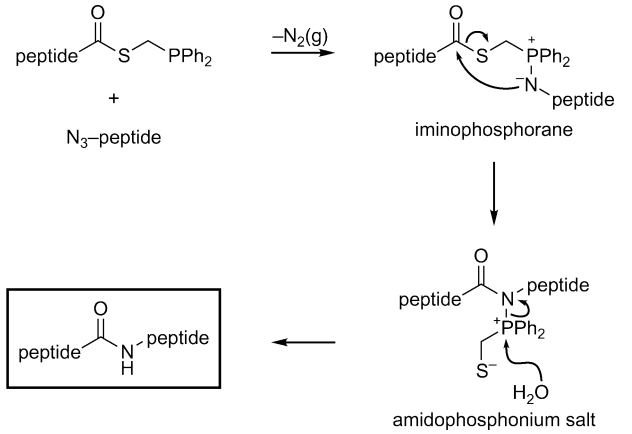
Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received December 14, 2002; E-mail: raines@biochem.wisc.edu

Chemical synthesis harbors the potential to provide ready access to natural proteins as well as to create nonnatural ones. Indeed, numerous proteins have already been assembled from synthetic peptides.[1,2] "Native chemical ligation"—the coupling of a peptide[3] (or protein[4]) containing a C-terminal thioester with another peptide containing an N-terminal cysteine residue—has been especially efficacious.[1] Emerging strategies for protein assembly avoid the need for a cysteine residue at the ligation junction. The Staudinger ligation is one such strategy.[5]

In our version of the Staudinger ligation, a peptide containing a C-terminal phosphinothioester reacts with a peptide containing an N-terminal azide to give an amide with no residual atoms, as in Scheme 1.[5b,c] The initial intermediate is an iminophosphorane, which rearranges to an amidophosphonium salt. Hydrolysis yields the amide and a phosphine oxide. Previously, we showed that this reaction can be used to form dipeptides in high isolated yield (>90%) and with the retention of α-carbon stereochemistry.[5b,c] Herein, we describe the first use of the Staudinger ligation to couple peptides on a solid support. We use the fragment thus produced to assemble a protein via native chemical ligation.[3,4] The synthesis of a protein by this route expands the versatility of chemical approaches to protein production.

Scheme 1



As a model protein, we chose ribonuclease A (RNase A;[6] 124 amino acid residues), which was the first protein to succumb to total synthesis.[7] Herein, RNase A was assembled from three fragments, which comprised residues 1−109, 110−111, and 112−124. The route is shown in Scheme 2.

RNase A(110−111) was synthesized as a C-terminal phosphinothioester using a sulfonamide-linker ("safety-catch") resin.[8] The fully loaded resin was activated with iodoacetonitrile.[8b] Treatment with an excess of diphenylphosphinomethanethiol[5b,c] in DMF for 18 h liberated FmocCys(Trt)Glu(O*t*Bu)SCH$_2$PPh$_2$, which was isolated in 64% yield.

RNase A(112−124) was synthesized as an N-terminal azide. The $n-1$ peptide was synthesized by using standard Fmoc-protection and HATU activation on a hydroxyethylpolystyrene (PEGA) resin, which has diverse solvent compatibility. α-Azido glycine[5a,b] (residue 112) was used to cap the $n-1$ peptide by its activation with PyBOP, HOBt, and DIPEA in DMF. The identity of $N_3CH_2C(O)$-Asn(Trt)ProTyr(*t*Bu)ValProValHis(Trt)Phe Asp(O*t*Bu)AlaSer(*t*Bu)-Val was confirmed by cleaving a small amount of the α-azido peptide from the resin with TFA and analyzing by MALDI mass spectrometry.

RNase A(110−111) and RNase A(112−124) were coupled by Staudinger ligation directly on the PEGA resin. Four equivalents of RNase A(110−111) in DMF/H_2O (10:1) was incubated with the resin over 12 h. After cleavage from the resin, side-chain deprotection, and HPLC purification, RNase A(110−124) was isolated in 61% yield.

RNase A(110−124) was synthesized again by this route, now incorporating an NMR probe of protein structure. Specifically, [^{13}C′,^{13}C$^{\alpha}$,^{15}N]proline[9] was inserted at position 114. The Asn113−Pro114 peptide bond resides in the cis (*E*) conformation in the properly folded protein, but in a mixture of cis and trans conformations in peptide fragments.[10]

RNase A(1−109) was produced by biosynthesis as a C-terminal thioester with *N*-methylmercaptoacetamide, as described previously.[4c,11] Both unlabeled and labeled RNase A(110−124) contained an N-terminal cysteine residue. Ligation of RNase A(1−109) and RNase A(110−124) in aqueous buffer, folding, and purification yielded intact RNase A. Its molecular mass was verified by MALDI mass spectrometry.

The ability to incorporate labeled amino acids at specific sites is a distinct advantage of producing proteins by chemical synthesis.[1,2] The route in Scheme 2 was used to incorporate [^{13}C′,^{13}C$^{\alpha}$,^{15}N]-Pro114 into RNase A, and a 1D HSQC NMR experiment was used to probe for proper folding of the resulting protein. In Fmoc-[^{13}C′,^{13}C$^{\alpha}$,^{15}N]ProOH, the carbamyl C−N bond was a mixture of cis and trans isomers (Figure 1). In contrast, the Asn113−[^{13}C′,^{13}C$^{\alpha}$,^{15}N]Pro114 peptide bond in labeled RNase A was a single species, consistent with this C−N bond being only in the cis conformation. Moreover, the chemical shift of the α-proton of [^{13}C′,^{13}C$^{\alpha}$,^{15}N]Pro114 in the synthetic RNase A was identical to that of unlabeled Pro114 in natural RNase A.[12]

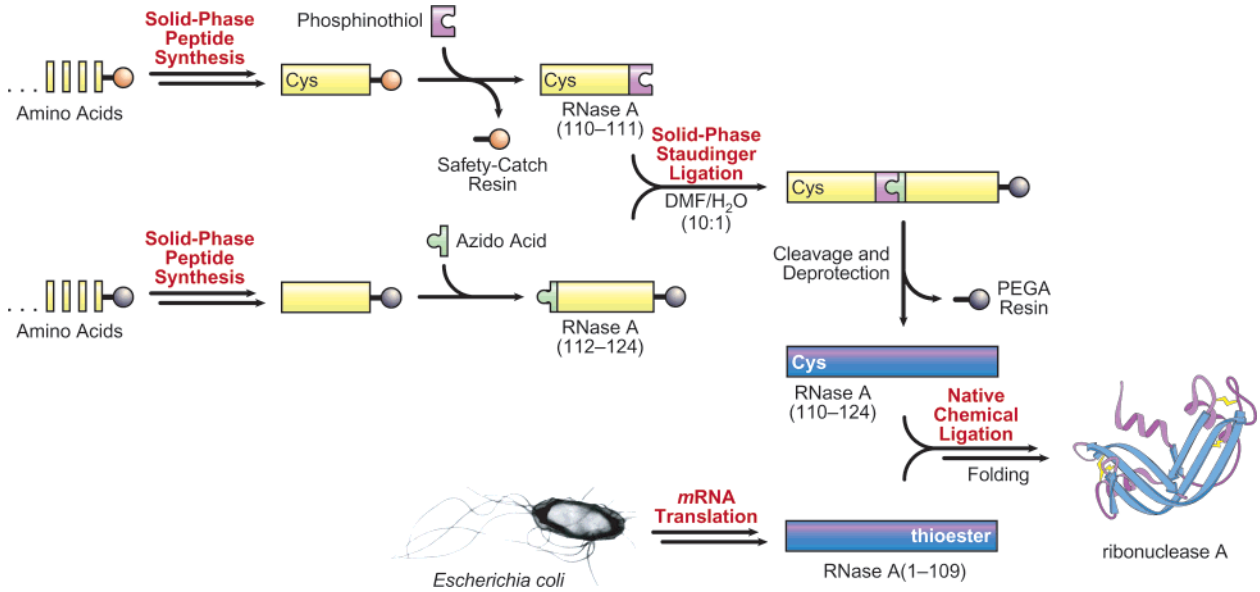
Enzymatic activity provides an extremely sensitive measure of protein structure.[13] The enzymatic activity of the RNase A synthesized as in Scheme 2 ($k_{cat}/K_M = 0.94 \times 10^7$ M^{-1} s^{-1}) was nearly equal to that of the wild-type enzyme produced by recombinant DNA technology ($k_{cat}/K_M = 1.1 \times 10^7$ M^{-1} s^{-1}).[11,14]

Thus, the solid-phase assembly of peptides with the Staudinger ligation has not only been realized, but used to assemble a functional enzyme. This method for amide bond formation is orthogonal and complementary to other ligation methods.[5] The enzyme created

[†] Department of Chemistry.
[‡] Department of Biochemistry.
[§] Current address: Department of Biochemistry, College of Medicine, University of Vermont, Burlington, VT 05405.

10.1021/ja029752e CCC: $25.00

Scheme 2



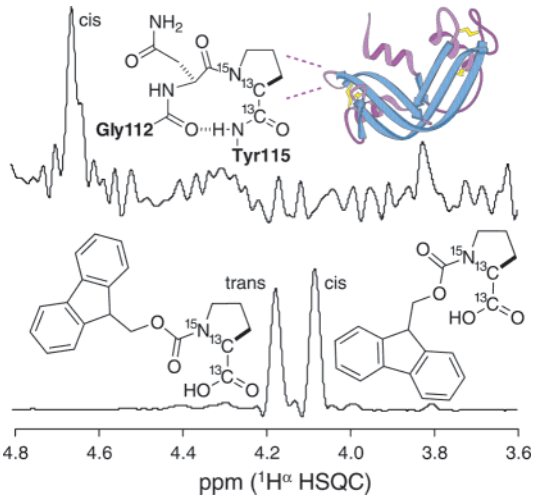


Figure 1. Observation of the α-proton of [^{13}C′,^{13}Cα,^{15}N]proline by 1D ^{15}N-CT-HSQC NMR spectroscopy. Top: [^{13}C′,^{13}Cα,^{15}N]Pro114 RNase A in D_2O (δ 4.66 ppm). Bottom: Fmoc[^{13}C′,^{13}Cα,^{15}N]ProOH in D_2O/CD_3-OD (1:1), assigned by 2D HMBC NMR spectroscopy.

herein is remarkable in that its peptide bonds were synthesized in four distinct processes: *m*RNA translation by a ribosome, solid-phase peptide synthesis, native chemical ligation, and solid-phase Staudinger ligation (Scheme 2). We anticipate that the two solid-phase processes alone could be the basis for an automated means to assemble proteins, making enzymes and other proteins more accessible targets for synthetic chemistry.

Acknowledgment. We thank W. M. Westler for help and advice with NMR experiments. This work was supported by grant GM44783 (NIH). B.L.N. was supported by the Abbott Laboratories Fellowship in Synthetic Organic Chemistry (University of Wisconsin-Madison). R.J.H. was supported by Postdoctoral Fellowship GM20190 (NIH). HSQC and HMBC NMR spectroscopy was performed at the National Magnetic Resonance Facility at Madison, which was supported by grant P41 RR02301 (NIH). The SIR[9] was supported by grant RR02231 (NIH).

Supporting Information Available: Procedures and additional data for syntheses and analysis reported herein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960.
(2) (a) Tam, J. P.; Yu, Q.; Miao, Z. *Biopolymers* **1999**, *51*, 311−332. (b) Borgia, J. A.; Fields, G. B. *Trends Biotechnol.* **2000**, *15*, 243−251. (c) Miranda, L. P.; Alewood, P. F. *Biopolymers* **2000**, *55*, 217−226. (d) Tam, J. P.; Xu, J.; Eom, K. D. *Biopolymers* **2001**, *60*, 194−205.
(3) (a) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Liebigs Ann. Chem.* **1953**, *583*, 129−149. (b) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.
(4) (a) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6705−6710. (b) Severinov, K.; Muir, T. W. *J. Biol. Chem.* **1998**, *273*, 16205−16209. (c) Evans, T. C., Jr.; Benner, J.; Xu, M. Q. *Protein Sci.* **1998**, *7*, 2256−2264.
(5) For use of the Staudinger ligation to form peptide bonds, see: (a) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939−1941. (b) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2001**, *3*, 9−12. (c) Soellner, M. B.; Nilsson, B. L.; Raines, R. T. *J. Org. Chem.* **2002**, *67*, 4993−4996. For other important precedents, see: (d) Staudinger, H.; Meyer, J. *Helv. Chim. Acta* **1919**, *2*, 635−646. (e) Bosch, I.; Romea, P.; Urpí, F.; Vilarrasa, J. *Tetrahedron Lett.* **1993**, *34*, 4671−4674. (f) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010. (g) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143.
(6) Raines, R. T. *Chem. Rev.* **1998**, *98*, 1045−1066.
(7) (a) Gutte, B.; Merrifield, R. B. *J. Am. Chem. Soc.* **1969**, *91*, 501−502. (b) Hirschmann, R.; Nutt, R. F.; Veber, D. F.; Vitali, R. A.; Varga, S. L.; Jacob, T. A.; Holly, F. W.; Denkewalter, R. G. *J. Am. Chem. Soc.* **1969**, *91*, 506−507. (c) Merrifield, R. B. *Science* **1984**, *232*, 341−347.
(8) (a) Kenner, G. W.; McDermott, J. R.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1971**, 636−637. (b) Backes, B. J.; Ellman, J. A. *J. Org. Chem.* **1999**, *64*, 2322−2330.
(9) [^{13}C′,^{13}Cα,^{15}N]Proline was a gift from the National Stable Isotope Resource at Los Alamos (SIR).
(10) An, S. S. A.; Lester, C. C.; Peng, J.-L.; Li, Y.-J.; Rothwarf, D. M.; Welker, E.; Thannhauser, T. W.; Zhang, L. S.; Tam, J. P.; Scheraga, H. A. *J. Am. Chem. Soc.* **1999**, *121*, 11558−11566.
(11) (a) Hondal, R. J.; Nilsson, B. L.; Raines R. T. *J. Am. Chem. Soc.* **2001**, *123*, 5140−5141. Also see: (b) Arnold, U.; Hinderaker, M. P.; Nilsson, B. L.; Huck, B. R.; Gellman, S. H.; Raines, R. T. *J. Am. Chem. Soc.* **2002**, *124*, 8522−8523.
(12) Rico, M.; Bruix, M.; Santoro, J.; Gonzalez, C.; Neira, J. L.; Nieto, J. L.; Herranz, J. *Eur. J. Biochem.* **1989**, *183*, 623−638.
(13) Knowles, J. R. *Science* **1987**, *236*, 1252−1258.
(14) Assays for ribonucleolytic activity used 6-carboxyfluorescein-dArU(dA)$_2$−6-carboxytetramethylrhodamine as the substrate (Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. *Nucleic Acids Res.* **1999**, *27*, 3696−3701).

JA029752E