

data. Another method much in vogue then was the use of the analytical ultracentrifuge, by which, on the same time photograms used for measuring the sedimentation coefficient (by monitoring the movement of a boundary through Schlieren optics), one could also assess the diffusion coefficient (18).

More recent methods for measuring diffusion coefficients of macromolecules are based on dynamic laser light scattering (19). Clearly, all these methods require dedicated equipment and are not easily available to scientists in nonspecialized laboratories. The present method is based on commonly available equipment (an instrument for capillary zone electrophoresis or just a capillary, pump, and UV detector), now standard in most biochemical laboratories, and allows an easy and reproducible determination of  $D$  values for both small analytes and macromolecules. However, a relatively large difference between  $D_{\text{exp}}$  and  $D_{\text{tab}}$  for macromolecules indicates that slower flows should be used in such cases.

The present method allows for a quick and precise estimation of the molecular diffusion coefficient and, thus, of the radius of a molecule in a wide range of molecular mass values and might be useful in a larger number of chemical and biochemical laboratories than in the past. An additional advantage of this application of Taylor's approach is that only small volumes of solution (fractions of nanoliters) are required.

## REFERENCES AND NOTES

- G. Taylor, *Proc. R. Soc. London Ser. A* **219**, 186 (1953).
- G. K. Batchelor, *An Introduction to Fluid Dynamics* (Cambridge Univ. Press, Cambridge, 1970).
- G. Taylor, *Proc. R. Soc. London Ser. A* **225**, 473 (1954).
- R. Aris, *ibid.* **235**, 67 (1956). In his original paper (1), Taylor neglected axial molecular diffusion and obtained only the second, although the most important, term in Eq. 2. That limited application of the theory, as specified in (3), to the case  $RU/D \gg 6.9$ . This limitation is not restrictive; however, Eq. 2 given by Aris is valid even for very slow flows in narrow-bore tubes where axial molecular diffusion may be comparable to Taylor's dispersion.
- The characteristic diffusion time for a solute in a tube is, by order of magnitude, the time necessary for a solute particle to diffuse over the capillary cross section given by  $R^2/D$ . In (1) it was defined as the time necessary for a radial nonuniformity to reduce its amplitude  $e$  times and was found to be given by  $R^2/3.8^2 D$ .
- A. Bourria, J. Coull, G. Houghton, *Proc. R. Soc. London Ser. A* **261**, 227 (1961).
- H. R. Bailey and W. B. Gogarty, *ibid.* **269**, 352 (1962).
- E. V. Evans and C. N. Kenney, *ibid.* **284**, 540 (1965).
- W. N. Gill, *ibid.* **298**, 335 (1967).
- \_\_\_\_\_ and R. Sankarasubramanian, *ibid.* **316**, 341 (1970).
- It is worth remembering that the theory and experimental methodology for capillary zone electrophoresis had been developed by the mid-1970s [S. Hjertén, *Chromatogr. Rev.* **9**, 122 (1967); R. Virtanen, *Acta Polytech. Scand.* **123**, 1 (1974)], al-

- though, extensive applications and further development of the method did not begin until a decade later [J. W. Jorgenson and K. D. Lukacs, *Science* **222**, 266 (1983)].
- Equation 4 is obtained by setting  $k = 0$  in equations 13 and 14 of E. Grushka, in *Methods of Protein Separation*, N. Catsimpoolas, Ed. (Plenum, New York, 1975), vol. 1, p. 161.
  - This allowed for recalculation of the found coefficients to another temperature, provided the dependence of the viscosity on temperature is known. As diluted water solutions or distilled water was used, the viscosity dependence on temperature was assumed to be that of water in all the cases [CRC Handbook of Chemistry and Physics (CRC Press, Boca Raton, FL, 1987), p. F-37].
  - American Institute of Physics Handbook (McGraw-Hill, New York, 1957), pp. 2-193.

- The capillary surface was initially coated with linear polyacrylamide, according to the method of S. Hjertén, *J. Chromatogr.* **347**, 191 (1985). Then, a dextran layer was grafted to the polyacrylamide as reported by K. Ganzler *et al.*, *Anal. Chem.* **64**, 2665 (1992).
- H. R. Mahler and E. H. Cordes, *Biological Chemistry* (Harper and Row, New York, ed. 2, 1971).
- R. H. Stokes, *J. Am. Chem. Soc.* **72**, 763 (1950).
- H. Neurath, *Chem. Rev.* **30**, 357 (1942).
- B. J. Berne and R. Pecora, *Dynamic Light Scattering* (Wiley, New York, 1976).
- Supported in part by a grant from Consiglio Nazionale delle Ricerche, Comitato di Chimica e Medicina e Biologia, and Radius in Biotechnology (European Space Agency, Paris) to P.G.R.

27 May 1994; accepted 15 September 1994

## Synthesis of Proteins by Native Chemical Ligation

Philip E. Dawson, Tom W. Muir, Ian Clark-Lewis, Stephen B. H. Kent\*

A simple technique has been devised that allows the direct synthesis of native backbone proteins of moderate size. Chemoselective reaction of two unprotected peptide segments gives an initial thioester-linked species. Spontaneous rearrangement of this transient intermediate yields a full-length product with a native peptide bond at the ligation site. The utility of native chemical ligation was demonstrated by the one-step preparation of a cytokine containing multiple disulfides. The polypeptide ligation product was folded and oxidized to form the native disulfide-containing protein molecule. Native chemical ligation is an important step toward the general application of chemistry to proteins.

Proteins owe their diverse properties to the precisely folded three-dimensional structures of their polypeptide chains. This is the defining feature of a protein, rather than size or molecular mass per se. Merely describing the three-dimensional structure of a protein is insufficient to fully explain its biological properties. A better understanding of how structure dictates the biological properties of a protein would be achieved by systematically varying the covalent structure of the molecule and correlating the effects with the folded structure and biological function.

In this report, we describe an important extension of the chemical ligation method (1) to allow the preparation of proteins with native backbone structures. The principle of "native chemical ligation" is shown in Fig. 1. The first step is the chemoselective reaction of an unprotected synthetic peptide- $\alpha$ -thioester (2, 3) with another unprotected peptide segment containing an amino-terminal Cys residue to give a thioester-linked intermediate as the initial co-

valent product. Without change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site. The target full-length polypeptide product is obtained in the desired final form without further manipulation. We believe that general synthetic access of this type will allow almost unlimited variation of the covalent structure of the protein molecule.

Model studies were undertaken with small peptides to investigate the native chemical ligation approach (4). These studies were consistent with the mechanism shown in Fig. 1, in which the initial thioester ligation product was not observed as a discrete intermediate because of the rapid rearrangement to form a stable peptide bond. Facile intramolecular reaction results from the favorable geometric arrangement of the  $\alpha$ -NH<sub>2</sub> moiety with respect to the thioester formed in the initial chemoselective ligation reaction. The use of such "entropy activation" for peptide bond formation is based on principles enunciated by Brenner (5) and more recently adopted by others (6).

Study of a variety of model peptides established that native chemical ligation was generally applicable to peptides containing the full range of functional groups

P. E. Dawson, T. W. Muir, S. B. H. Kent, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA.

I. Clark-Lewis, Biomedical Research Center, Department of Biochemistry, 2222 Health Sciences Mall, University of British Columbia, Vancouver, BC V6T 1Z3 Canada.

\*To whom correspondence should be addressed.

normally found in proteins (7). As described in this report, native chemical ligation is limited to reaction at an amino-terminal Cys residue. It was important to prevent the side chain thiol of this Cys from oxidizing to form a disulfide-linked dimer, because this was unreactive in the ligation. An excess of thiol corresponding to the thioester leaving group was used to keep the Cys residues in reduced form without inter-

fering with the ligation reaction. The amino-terminal peptide segment must be prepared by chemical synthesis to equip it with the necessary  $\alpha$ -COSR functionality (where R is an alkyl group) (2). Furthermore, for optimal ligation, this component should have an unhindered (that is, non  $\beta$ -branched) carboxyl-terminal amino acid. Solubilizing agents such as urea or guanidine hydrochloride did not interfere with

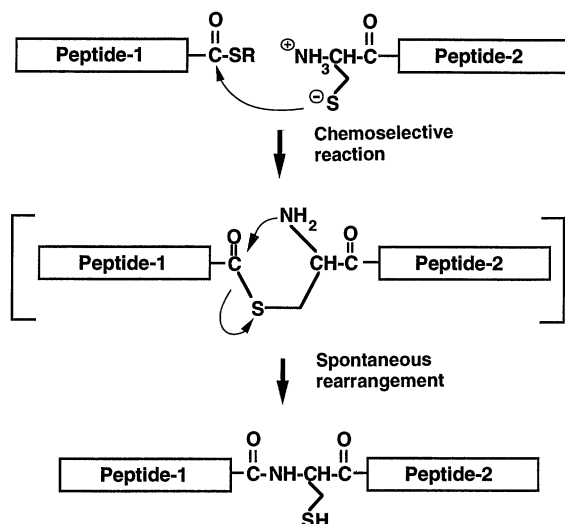
the ligation and could be used to enhance the concentration of peptide segments, and thus increase the reaction rate.

Further model reactions showed that the use of better thioester leaving groups resulted in faster ligation reactions. We applied this observation to the native chemical ligation of peptides from the extracellular domain of a human cytokine receptor (8) (Fig. 2). Use of the 5-thio-2-nitrobenzoic acid (-SNB) leaving group, corresponding to the reduced form of Elman's reagent, gave rapid reaction in high yield. As described in the legend to Fig. 2, the reaction between the peptide segments was observed to have gone essentially to completion in less than 5 min, giving the 50-residue product with a native peptide bond at the site of ligation. Thus, rapid native chemical ligation can be achieved by use of a thioester leaving group with suitably tuned properties.

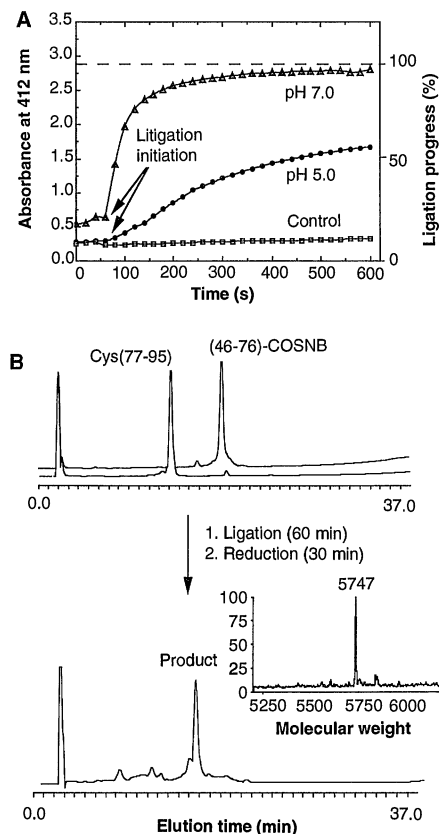
Application of the native chemical ligation method to the total synthesis of a protein molecule was illustrated by the preparation of human interleukin 8 (IL-8) (9). The 72-amino acid polypeptide chain contains four Cys residues, which form two functionally critical disulfide bridges in the native protein molecule (9). The total synthesis of IL-8 is shown in Fig. 3. The two unprotected synthetic peptide segments reacted cleanly to give the full-length polypeptide chain in reduced form without further chemical manipulation (10). This successful ligation was particularly significant because the 33- and 39-residue IL-8 segments each contained two Cys residues and together encompassed 18 of the 20 genetically encoded amino acids found in proteins. The purified product was folded and oxidized as previously described (9) to give IL-8 with a mass precisely 4 daltons less than that of the original ligation product, indicating the formation of two disulfide bonds. The properties of this folded product were identical to those of authentic IL-8 samples (11). This result unambiguously confirmed the formation of a peptide bond at the ligation site, because the thioester-to-amide rearrangement must have taken place to give the free Cys<sup>34</sup> side chain that formed the native disulfide bond (see Fig. 3A).

What is likely to be the impact of native chemical ligation on the study of proteins? Proteins are usually studied by expression in genetically engineered microorganisms with the methods of recombinant DNA-based molecular biology. Methods such as site-directed mutagenesis (12) have had a revolutionary impact on the ability to prepare large numbers of modified proteins in useful amounts for systematic study (13). Innovative approaches have increased the range of amino acids that can be incorporated in

**Fig. 1.** The principle of native chemical ligation. The synthetic segment, peptide 1, which contains a thioester at the  $\alpha$ -carboxyl group, undergoes nucleophilic attack by the side chain of the Cys residue at the amino terminal of peptide 2 (R is an alkyl group). The initial thioester ligation product undergoes rapid intramolecular reaction because of the favorable geometric arrangement [involving a five-membered ring] of the  $\alpha$ -amino group of peptide 2, to yield a product with a native peptide bond at the ligation site. Both reacting peptide segments are in completely unprotected form, and the target peptide is obtained in final form without further manipulation.



**Fig. 2.** Rapid native chemical ligation reaction, illustrated by the synthesis of a peptide segment corresponding to residues 46 to 95 from the external domain of the human IL-3 receptor  $\beta$ -subunit (8). **(A)** Monitoring by ultraviolet (UV) absorbance. Ligation was initiated by adding [Cys<sup>77</sup>](77-95) to purified Msc(46-76) $\alpha$ COSNB (27) at the stated pH and the reaction was monitored by UV [the substituted aryl thiolate leaving group has a characteristic UV absorption at 412 nm ( $\epsilon_{\text{TNB}, 412 \text{ nm}} = 13,700 \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$ )]. At pH 7.0, the reaction was essentially complete within 5 min. No reaction was observed when Msc(46-76) $\alpha$ COSNB was exposed to a 10-fold molar excess of Leu-enkephalin (amino-terminal residue, Tyr) at pH 5.0. This control experiment confirms the absolute requirement for an amino-terminal Cys residue at the site of ligation. **(B)** Monitoring by HPLC. Purified [Cys<sup>77</sup>](77-95) (0.98 mM) and (46-76) $\alpha$ COSNB (0.9 mM) were reacted in 8 M urea, pH 5.0, 50 mM ammonium acetate buffer at 23°C. Analytical HPLC ( $\text{C}_{18}$  reversed phase; 22.5 to 45% acetonitrile at 0.7% per minute; monitored at 214 nm) of the individual components is shown (upper trace). After 1 hour, the ligation solution was exposed to the reducing agent tris(2-carboxyethyl)phosphine at pH 9.0 and subsequently raised to pH 13 to remove the N $\alpha$ -Msc moiety. Analytical HPLC, under the same conditions, of the crude product is shown (lower trace). The 50-residue product had the expected molecular mass by electrospray mass spectrometry [observed, 5747.0 daltons; calculated (average isotope composition), 5747.4 daltons]. The ligation product was shown to be stable at high pH and to reducing conditions, and formed an intramolecular disulfide. These observations are consistent with the presence of a native peptide bond at the site of ligation.



expression systems (14) and promise to significantly extend the utility of biosynthetic modification of the covalent structure of proteins. However, there appear to be limitations inherent to the nature of ribosomal protein synthesis (14).

In favorable cases, chemical synthesis has already made important contributions to the exploration of the relationship of protein structure to function. Stepwise solid phase synthesis has permitted the de novo preparation of small proteins (15), and there have been several notable examples of the use of this method of total protein synthesis to explore the molecular basis of biological function (16). Another method that has in special instances allowed chemistry to be applied to the study of proteins is semisynthesis through the conformationally assisted religation of peptide fragments (17). An important extension of the semisynthesis approach is the use of enzymatic ligation of cloned or synthetic peptide segments (18). Although these methods currently have severe limitations, there continues to be serious interest in the wider application of the tools of organic chemistry to the study of proteins (15).

Recently, we introduced the chemical ligation of unprotected peptide segments as an improved route to the total synthesis of proteins (1). The key aspect of this approach

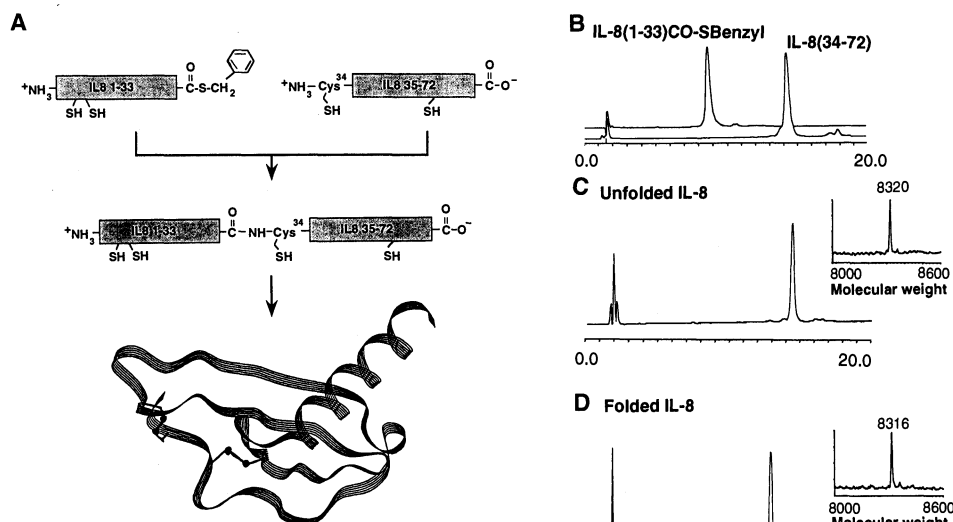
was the use of chemoselective reaction to specifically and unambiguously join peptides by formation of an unnatural (that is, non-peptide) backbone structure at the ligation site. It has permitted the facile preparation of a wide range of backbone-modified proteins, including analogs of protein domains (19) and of the human immunodeficiency virus-1 (HIV-1) proteolytic enzyme (1, 20). Chemical ligation has also proven to be useful for the routine, reproducible synthesis of large amounts of proteins in high purity with full biological activity (21), as well as for the straightforward production of protein-like molecules of unusual topology (22). However, the range of proteins accessible by this technique is limited by the size of the synthetic peptide segments (23). A useful extension would occur if we had direct synthetic access to native backbone polypeptide chains up to the size of typical protein domains (24). Chemical ligation would then allow us to string these domains together to explore the world of proteins in a general fashion.

Native chemical ligation provides precisely that capability. It combines the formation of a native peptide bond at the ligation site with the advantages of chemoselective reaction of unprotected peptides (1). This second-generation ligation chemistry dramatically increases the size of native back-

bone polypeptides directly accessible by total chemical synthesis (25). It can be usefully applied to a wide range of synthetic targets, including proteins of moderate size, and it allows direct access to protein functional domains (24). Native chemical ligation is a foundation stone of a general modular approach to the total chemical synthesis of proteins (26).

## REFERENCES AND NOTES

1. Chemical ligation [M. Schnölzer and S. B. H. Kent, *Science* **256**, 221 (1992)] involved the chemoselective reaction of unprotected peptides to give a product with an unnatural backbone structure at the ligation site. Use of unprotected peptides circumvented the difficulties inherent to classical chemical synthesis, namely, complex combinations of protecting groups that lead to limited solubility of many synthetic intermediates [for example, K. Akaji *et al.*, *Chem. Pharm. Bull. (Tokyo)* **33**, 184 (1985)]. In contrast, the chemical ligation technique has allowed us to make good use of the ability to routinely make, purify, and characterize unprotected peptides 50 or more residues in length (23).
2. Peptide 1 in Fig. 1. The key feature is the  $\alpha$ COSR moiety, which is readily generated from the peptide- $\alpha$ COSH prepared by highly optimized stepwise solid-phase peptide synthesis on a thioester resin. The thioester resin was prepared by a generalized version (L. E. Canne and S. B. H. Kent, manuscript in preparation; details can be obtained from the authors) of the Blake-Yamashiro procedure (3). Peptide products were cleaved, purified, and characterized by standard methods (23).
3. J. Blake, *Int. J. Pept. Protein Res.* **17**, 273 (1981); D. Yamashiro and C. H. Li, *ibid.* **31**, 322 (1988).
4. To help explore the mechanism of the reaction, the peptide Leu-Tyr-Arg-Ala-Gly- $\alpha$ COSBzl (Bzl, benzyl) was reacted with Ac-Cys (Ac, acetyl). The exact mass of the resulting ligation product was determined by electrospray mass spectrometry and was consistent with a thioester-linked peptide as the ligation product generated by nucleophilic attack of the Ac-Cys side chain on the  $\alpha$ -thioester moiety of the peptide. Reaction of Leu-Tyr-Arg-Ala-Gly- $\alpha$ COSBzl with H-Cys-Arg-Ala-Glu-Tyr-Ser (containing an unblocked  $\alpha$ -NH<sub>2</sub> functional group) proceeded rapidly at pH 6.8 (below pH 6, the reaction proceeded very slowly, suggesting the involvement of the ionized thiolate form of the Cys side chain) and gave a single product of the expected mass. This product lacked susceptibility to nucleophiles and had the ability to form disulfide-linked dimeric peptides, indicating unambiguously the formation of a native amide bond at the ligation site.
5. M. Brenner, in *Peptides. Proceedings of the Eighth European Peptide Symposium*, H. C. Beyerman, Ed. (North-Holland, Amsterdam, 1967), pp. 1-7.
6. D. S. Kemp and R. I. Carey, *J. Org. Chem.* **58**, 2216 (1993); C.-F. Liu and J. P. Tam, *J. Am. Chem. Soc.* **116**, 4149 (1994).
7. Even free internal Cys residues may be present in either of the reacting segments. Internal Cys residues can undergo ester exchange with the peptide- $\alpha$ -thioester component; however, this reaction is unproductive because no rearrangement to the amide bond can occur; the thioester formed is readily reversible and remains a productive part of the reacting system.
8. R. D'Andrea *et al.*, *Blood* **83**, 2802 (1994).
9. M. Baggiolini and I. Clark-Lewis, *FEBS Lett.* **307**, 97 (1989); I. Clark-Lewis, B. Dewald, M. Loetscher, B. Moser, M. Baggiolini, *J. Biol. Chem.* **269**, 16075 (1994); I. Clark-Lewis, *Biochemistry* **30**, 3128 (1991); K. Rajarathnam, I. Clark-Lewis, B. D. Sykes, *ibid.* **29**, 1689 (1994).
10. Analogous methods have required removal of protecting groups (3, 6) or conversion of intermediates to the final form (6), or both steps. No previous method has allowed the chemical reaction of unprotected peptide segments to directly yield a native backbone final product.
11. Titration in an assay for neutrophil elastase release



**Fig. 3.** Synthesis of IL-8 by native chemical ligation. (A) Synthetic scheme leading to folded [Ala<sup>33</sup>]IL-8 (28). (B) Analytical HPLC (C<sub>18</sub> reversed phase; 25 to 45% acetonitrile at 1% per minute; monitored at 214 nm) of the synthetic peptide segments (29), IL-8(1-33) $\alpha$ COSBzl and IL-8(34-72), each shown before reaction was initiated (30). (C) Analytical HPLC under the same conditions of the purified ligation product, IL-8(1-72)(SH)<sub>4</sub>, in fully reduced form. (Inset) Electrospray mass spectrum (raw data displayed as a single charge state): observed molecular mass 8319.8 daltons; calculated molecular mass (average isotope composition), 8319.8 daltons. (D) Air oxidation of the purified 1-72 ligation product to form the folded [Ala<sup>33</sup>]IL-8 molecule, shown after HPLC purification. The earlier elution of the folded, disulfide cross-linked native protein compared with the reduced polypeptide is typical (9). Folding and oxidation conditions: polypeptide at 0.2 mg/ml, 1 M guanidine-HCl, pH 8.5 Tris buffer, and vigorous stirring in air at ambient temperature. (Inset) Electrospray mass spectrometry of the oxidized and folded synthetic IL-8 (raw data displayed as a single charge state). Observed molecular mass, 8315.6 daltons; calculated molecular mass (average isotope composition), 8315.8 daltons.

- (9) demonstrated that the potencies [median effective dose ( $ED_{50}$ ) = 0.3 nM] and maximal responses of the folded, ligated [Ala<sup>33</sup>]IL-8 and the corresponding molecule obtained by conventional synthesis (9) were indistinguishable and identical to native sequence IL-8.
12. M. Smith, *Angew. Chem. Int. Ed. Engl.* **33**, 1214 (1994).
  13. C. Eigenbrot and A. Kossiakoff, *Curr. Opin. Biotechnol.* **3**, 333 (1992).
  14. C. J. Noren, S. J. Anthony-Cahill, M. C. Griffith, P. G. Schultz, *Science* **244**, 182 (1989); J. A. Ellman, D. Mendel, P. G. Schultz, *ibid.* **255**, 197 (1992); V. W. Cornish et al., *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2910 (1994).
  15. T. W. Muir and S. B. H. Kent, *Curr. Opin. Biotechnol.* **4**, 420 (1993).
  16. M. Miller et al., *Science* **246**, 1149 (1989); A. Wlodawer et al., *ibid.* **245**, 616 (1989); L. H. Huang, H. Cheng, A. Pardi, J. P. Tam, W. V. Sweeney, *Biochemistry* **30**, 7402 (1991); K. Rajarathnam et al., *Science* **264**, 90 (1994).
  17. R. E. Offord, in *Protein Design and the Development of New Therapeutics and Vaccines*, J. B. Hook and G. Poste, Eds. (Plenum, New York, 1990), pp. 253-282; C. J. A. Wallace and I. Clark-Lewis, *J. Biol. Chem.* **267**, 3852 (1992).
  18. L. Abrahmsen et al., *Biochemistry* **30**, 4151 (1991); T. K. Chang, D. Y. Jackson, J. P. Burnier, J. A. Wells, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
  19. Ligated 10F3, the integrin-binding module of fibronectin: 95 residues [M. Williams, T. Muir, M. Ginsberg, S. B. H. Kent, *J. Am. Chem. Soc.*, in press].
  20. Catalytic contribution of flap-substrate hydrogen bonds in HIV-1 protease explored by chemical synthesis: homodimer of 99-residue subunits [M. Baca and S. B. H. Kent, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11638 (1993)].
  21. R. C. deLisle Milton, S. C. F. Milton, M. Schnölzer, S. B. H. Kent, in *Techniques in Protein Chemistry IV* (Academic Press, New York, 1992), pp. 257-267.
  22. Four-helix bundle template-assembled synthetic protein: molecular mass 6647 daltons [P. E. Dawson and S. B. H. Kent, *J. Am. Chem. Soc.* **115**, 7263 (1993)]; homogeneous multivalent artificial protein: molecular mass 19,916 daltons [K. Rose, *ibid.* **116**, 30 (1994)]; artificial neoprotein mimic of the cytoplasmic domains of a multichain integrin receptor: molecular mass 14,194 daltons [T. W. Muir, M. J. Williams, M. H. Ginsberg, S. B. H. Kent, *Biochemistry* **33**, 7701 (1994)]; peptide dendrimer: molecular mass 24,205 daltons [C. Rao and J. P. Tam, *J. Am. Chem. Soc.* **116**, 6975 (1994)].
  23. By using optimized stepwise solid-phase methods [M. Schnölzer, P. Alewood, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res.* **40**, 180 (1992)], the preparation in good yield and high purity of peptides up to 60 residues is routine; in favorable cases, peptides with >80 residues can be prepared.
  24. A. L. Berman, E. Kolker, E. N. Trifonov, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4044 (1994).
  25. The carboxyl-terminal peptide segment or protein module could also be expressed by standard recombinant DNA methods; provided the product contained an amino-terminal Cys residue, it could be reacted with the synthetic amino-terminal peptide- $\alpha$ COSR by using the native chemical ligation described here to give a product in which part of the protein had been derived from chemical synthesis and part from ribosomal synthesis.
  26. A modular strategy for the total synthesis of proteins has been developed based on the convergent chemical ligation of unprotected peptides [L. E. Canne, S. K. Burley, S. B. H. Kent, paper presented at the Annual Meeting of the Protein Society, San Diego, July 1994]. Protein domains (modules) were prepared by chemical ligation of 50 to 70 residue segments; these domains were then stitched together to give the target protein. Mutually compatible ligation chemistries are required: Intradomain ligation should optimally yield a stable, peptide-like bond, whereas interdomain ligation will tolerate a wider variation of properties of the structure formed at the ligation site.
  27. Crude synthetic Msc(46-76) $\alpha$ COSH [Msc, 2(methylsulfonyl)ethyloxycarbonyl] was converted to the 5-thio-2-nitrobenzoic acid ester (-COSNB) by treatment with 5,5'-dithio-bis(2-nitrobenzoic acid) [10 equivalents (eq)] in 8 M urea, pH 4.0 50 mM ammonium acetate buffer. This thioester-containing material was found to be completely stable below pH 6.0, and was readily purified by reversed-phase high-performance liquid chromatography (HPLC).
  28. [Ala<sup>33</sup>]IL-8 was chosen as the synthetic target for convenience; previous work had shown that this mutant IL-8 had full biological activity (9), and a supply of the Boc-Ala (Boc, butyloxycarbonyl) thioester resin was on hand for other applications. The folded structure shown is based on the x-ray structure of the IL-8 monomer [E. T. Baldwin et al., *Proc. Natl. Acad. Sci. U.S.A.* **88**, 502 (1991)].
  29. The IL-8 peptide segments were prepared by optimized stepwise solid-phase synthesis (23) and were purified by reversed-phase HPLC and characterized by standard methods. Crude synthetic segment IL-8(1-33) $\alpha$ COSH was converted to the thiobenzyl ester by reaction with benzyl bromide (15 eq) in 6 M guanidine-HCl, pH 4.6, sodium acetate buffer, prior to purification under standard reversed-phase HPLC conditions.
  30. The segments (1-33) $\alpha$ COSBzl (5.0 mg, 1.3  $\mu$ mol) and 34-72 (4.8 mg, 1.1  $\mu$ mol) were reacted in 0.5 ml 6 M guanidine-HCl, pH 7.6, phosphate buffer at 23°C in the presence of benzyl mercaptan (5  $\mu$ l). After suitable reaction time (48 to 72 hours), a ligation yield of ~60% was obtained. The product was purified by reversed-phase HPLC and characterized by electrospray mass spectrometry.
  31. We gratefully acknowledge the assistance of T. Walters and M. Baca in the early stages of this work, of L. Canne in providing the thioester resin, of B. Dewald for some of the elastase release assays, and of R. Simon and S. Clark for critical comments on the manuscript. Supported by funding from NIH [GM48897-01 and GM48870-03 (S.B.K.); GM 50969-01 (I.C.L.)].

16 August 1994; accepted 21 September 1994

## A Theropod Dinosaur Embryo and the Affinities of the Flaming Cliffs Dinosaur Eggs

Mark A. Norell,\* James M. Clark,† Dashzeveg Demberelyin, Barsbold Rhinchen, Luis M. Chiappe, Amy R. Davidson, Malcolm C. McKenna, Perle Altangerel, Michael J. Novacek

An embryonic skeleton of a nonavian theropod dinosaur was found preserved in an egg from Upper Cretaceous rocks in the Gobi Desert of Mongolia. Cranial features identify the embryo as a member of Oviraptoridae. Two embryo-sized skulls of dromaeosaurids, similar to that of *Velociraptor*, were also recovered in the nest. The eggshell microstructure is similar to that of ratite birds and is of a type common in the Djadokhta Formation at the Flaming Cliffs (Bayn Dzak). Discovery of a nest of such eggs at the Flaming Cliffs in 1923, beneath the *Oviraptor philoceratops* holotype, suggests that this dinosaur may have been a brooding adult.

Dinosaur eggs are abundant in Upper Cretaceous rocks of the Gobi Desert (1, 2), but embryonic skeletons from these deposits are scarce. Definitive remains include numerous bird embryos (3) and a single fragmentary specimen of an ornithischian hind limb (4). Because the definitive taxonomic identity of eggs requires the presence of identifiable embryonic remains within them, the identity of most egg types present in Upper Cretaceous beds in Mongolia has been unclear (1, 2).

In 1993, a rich Upper Cretaceous fossil locality in the Gobi Desert was discovered (5). The site, Ukhaa Tolgod, is in the north-eastern Nemegt Basin, Omnogov Aimak, near the salt extraction settlement of Daus.

In addition to over 300 mammal and lizard skulls, 20 theropod skeletons (including several adult and juvenile oviraptorids), and many protoceratopsian and ankylosaurid dinosaurs discovered at this locality, at least five types of eggs were found. Many of these were arranged in nests. One egg, from a heavily weathered nest, contains the nearly complete skeleton of an embryonic oviraptorid dinosaur (Fig. 1). Also among the broken eggshell fragments in this nest were two tiny skulls (~5 cm long) of a dromaeosaurid theropod, one preserved with eggshell adhering to it (Fig. 2).

The red sandstones of Ukhaa Tolgod probably belong to either the Djadokhta Formation or the Barun Goyot Formation and lie 35 km east of the Barun Goyot type section (6). Limited studies indicate the presence of taxa typical of either or both the Barun Goyot and the Djadokhta formations (that is, *Velociraptor*, *Mononykus*, and the mammals *Zalambdalestes*, *Bulganbaatar*, *Nemegtbaatar*, and *Catopsalis*). These faunas are considered correlative with the Judithian North American land mammal age and the Campanian marine stage (7), although this correlation is poorly constrained (8).

M. A. Norell, J. M. Clark, L. M. Chiappe, A. R. Davidson, M. C. McKenna, M. J. Novacek, Department of Vertebrate Paleontology, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA.

Dashzeveg D. and Barsbold R., Geological Institute, Mongolian Academy of Sciences, Ulaan Baatar 11, Mongolia.

Perle A., Mongolian Museum of Natural History, Ulaan Baatar 46, Zaluuchudin Gudama-1, Mongolia.

\*To whom correspondence should be addressed.

†Present address: Department of Biological Sciences, George Washington University, Washington, DC 20052, USA.