curred in these lysate mixtures. Further, the first centriole to appear during astral formation in our cell-free system must have assembled de novo within 4 min after HSA-extracts and U-lysates were mixed. This conclusion is based on the fact that neither centrosomes (*13–15*) nor centrioles (*16, 17*) have been found in unactivated *Spisula* oocytes or in U-lysates (*18*). In addition, asters in lysates of *Spisula* oocytes prepared 2.5 min after activation did not contain centrioles, whereas those prepared 4.5 min after activation contained a single centriole (*18*). Finally, we did not find asters or centrioles in either HSA-extracts or U-lysates, even when we incubated them for 15 min at room temperature.

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20. Oocytes were obtained and parthenogenetically activated with KCl as previously described (*13, 14, 19*). Four minutes after activation, lysates were prepared and asters isolated (*19, 21*). For analysis of spindle-associated asters, oocytes were activated and incubated at 23°C with gentle stirring. Fifteen minutes after activation oocytes were centrifuged and washed with glycerol buffers (*19*), and spindles were isolated by resuspension in MT stabilizing buffer (MSB) [20 mM Mes, 10 mM EGTA, 5 mM $MgSO_4$, and 20% glycerol (pH 6.3 to 6.5)] containing 1% Triton X-100 or NP-40. Samples were centrifuged at 1000*g* for 10 min. The pellets were resuspended in MSB, centrifuged again, fixed for 15 to 30 min in MSB containing 1% glutaraldehyde, washed in distilled H_2O, postfixed in 1% OsO_4, and embedded in Epon. Serial 0.25-μm sections were prepared with a diamond knife, stained with uranyl acetate followed by lead citrate, and examined with the Wadsworth Center's AEI-EM7 high-voltage electron microscope operated at 800 kV (*7, 22*).
Lysates were prepared, and aster content was analyzed as described (*19*). To remove asters and centrosomes, we centrifuged lysates prepared 4 min after activation (A/4-lysates) at 29,000*g* at 4°C for 15 min. The clarified middle cytosolic layer (HSA-extract) was collected and analyzed for aster content with hexylene glycol (*19*). This procedure was repeated until the HSA-extract was unable to form asters. Aliquots of lysates and HSA-extracts were removed, and proteins analyzed by one-dimensional gel electrophoresis (*23*). Proteins were separated on 7.5% acrylamide gels, and lanes were loaded on the basis of equal volume. We prepared U-lysates by washing oocytes in 1 M glycerol solution (*14, 19*), suspending them in 1 M glycerol containing 10 mM NaH_2PO_4 (pH 8.0) for 1 min, and washing them in aster buffer [20 mM Pipes, 100 mM NaCl, and 5 mM $MgSO_4$ (pH 7.2)]. After centrifugation and aspiration, we lysed oocytes by gentle vortexing and kept them on ice until use. For IMF, 10-μl aliquots of the experimental mixture were diluted into 100 μl of MSB-containing detergent and fixed by the addition of 3 ml of phosphate-buffered saline (pH 7.2) containing 0.5% glutaraldehyde and 0.4% Triton X-100, centrifuged onto polylysine-coated cover slips, and processed as described (*24*) with monoclonal antibody to tubulin (TU27) and rhodamine secondary antibodies (Calbiochem). Samples were photographed with an Axiophot microscope (Zeiss). For HVEM, aliquots of mixtures of HSA-extract and U-lysate were diluted 1:10 to 1:20 in MSB-containing detergent and centrifuged at 1000*g* for 10 min. The pellets were fixed, embedded, sectioned, and analyzed as described (*20, 22*).
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Constructing Proteins by Dovetailing Unprotected Synthetic Peptides: Backbone-Engineered HIV Protease

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Backbone-engineered HIV-1 protease was prepared by a total chemical synthesis approach that combines the act of joining two peptides with the generation of an analog structure. Unprotected synthetic peptide segments corresponding to the two halves of the HIV-1 protease monomer polypeptide chain were joined cleanly and in high yield through unique mutually reactive functional groups, one on each segment. Ligation was performed in 6 molar guanidine hydrochloride, thus circumventing limited solubility of protected peptide segments, the principal problem of the classical approach to the chemical synthesis of proteins. The resulting fully active HIV-1 protease analog contained a thioester replacement for the natural peptide bond between Gly^{51}-Gly^{52} in each of the two active site flaps, a region known to be highly sensitive to mutational changes of amino acid side chains.

Protein engineering has been mainly carried out by site-directed mutagenesis (*1*) or other techniques of genetic manipulation (*2*) and, with limited exceptions (*3*), has been restricted to substitutions based on the genetically coded amino acids. The chemical synthesis approach (*4*) to the systematic variation of protein structure is in principle a much more general one and offers great flexibility in the incorporation of non-coded moieties ranging from unnatural amino acids (*5*) to fixed elements of three-dimensional (3-D) structure (*6*).

An ideal approach to protein engineering would involve the chemical ligation of readily available, large unprotected synthetic peptide segments to give the modified polypeptide chain corresponding to the target protein or functional domain. Here we describe a method for the preparation of protein analogs in which the act of joining two peptides generates an analog structure within the protein molecule. Such an approach allows the use of highly selective chemistries for the ligation reaction in a form of chemical dovetailing in which the reacting moieties on the segments to be joined have reactivities tailored to one another. This approach does away with the need for protecting groups for other functionalities present in the protein molecule (*7, 8*) and provides a flexible, general route to the total chemical synthesis of a wide range of protein analogs, including a new class of protein analog, namely, "backbone-engineered" proteins, in which the peptide bond has been replaced for structure-activity studies. Application of this approach is exemplified by the preparation of a fully functional backbone-engineered enzyme.

Human immunodeficiency virus–1 protease (HIV-1 PR) is a virally encoded en-

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zyme that cuts polypeptide chains with high specificity and is essential for virus replication (9). The 22.5-kD HIV-1 PR molecule is made up of two identical 99–amino acid (aa) polypeptide chains. Comparison of the crystal structures of the empty (5) and inhibitor-bound (*10*) enzyme revealed that on binding a substrate-derived inhibitor the HIV-1 PR molecule undergoes significant conformational changes that are particularly pronounced in two exterior, functionally important "flap" regions (Fig. 1, A and B). From these crystallography studies it appears that peptide bonds in the flap regions of the HIV-1 PR polypeptide chain backbone are involved in the formation of β-sheet and β-turn structure, in the interaction that occurs between the two subunits of the active dimer at the tip of each flap in the enzyme-inhibitor (substrate) complex, and in hydrogen-bonding interactions with bound peptide inhibitors (and, presumably, substrates) (Fig. 1, A and B). Mutagenesis studies show that the flap region is highly sensitive to changes in the amino acid sequence (*11*).

These observations make the flap region especially interesting as a target for protein backbone modifications to investigate the role of peptide bond interactions in HIV-1 protease activity. We used a chemical synthesis approach to introduce a pseudo-peptide molecule bond (Fig. 1, C and D) into the HIV-1 PR between Gly^{51}-Gly^{52} in the flap region (Fig. 1, A and B) to produce a backbone-engineered analog. We took advantage of two facts. First, Gly is the only achiral amino acid and therefore there is no concern about loss of optical purity. Second, the Gly^{51}-Gly^{52} peptide bond is located near the middle of the 99-aa monomer chain, which means that the two peptide segments that were ligated were each ~50 residues in length. The synthesis of high-purity ~50-aa peptides is straightforward with current highly optimized solid-phase methods (*4*, *12*).

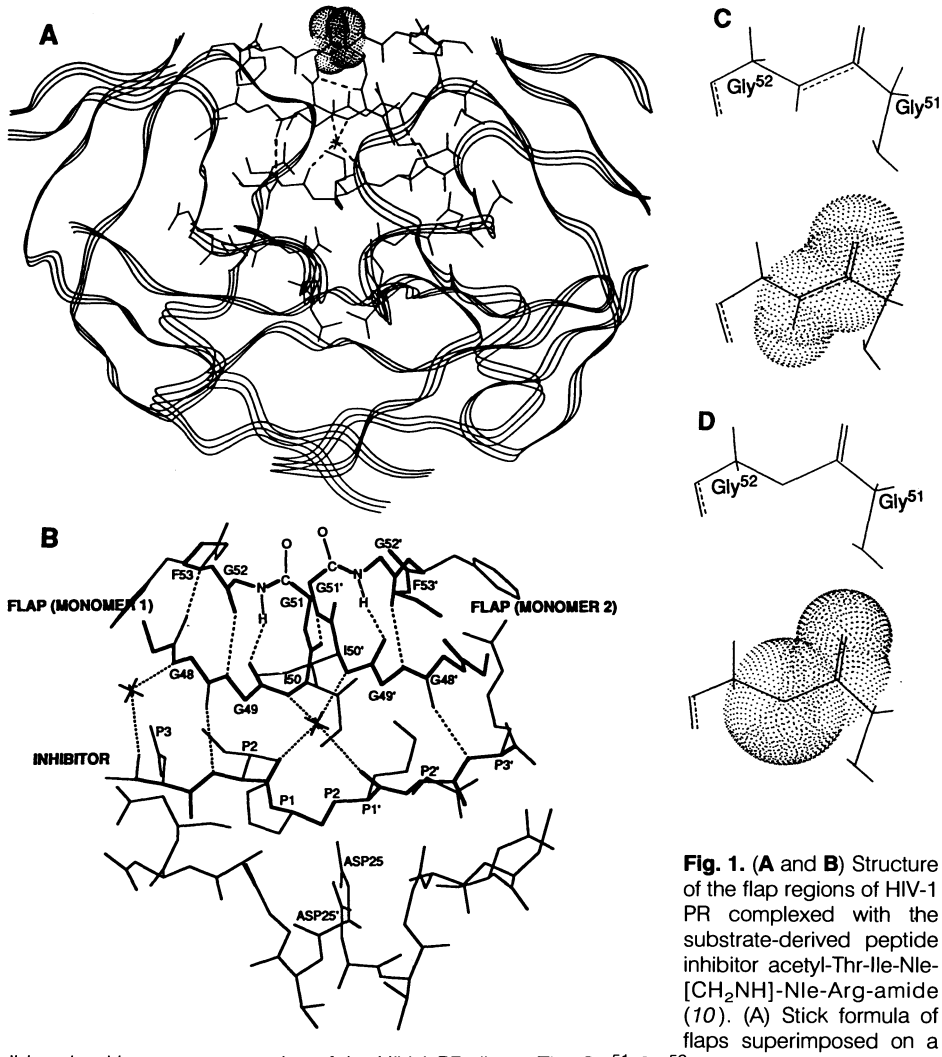

Fig. 1. (**A** and **B**) Structure of the flap regions of HIV-1 PR complexed with the substrate-derived peptide inhibitor acetyl-Thr-Ile-Nle-[CH_2NH]-Nle-Arg-amide (*10*). (A) Stick formula of flaps superimposed on a ribbon backbone representation of the HIV-1 PR dimer. The Gly^{51}-Gly^{52} peptide bond replaced in this work is highlighted in each flap. Backbone movements of up to 7 Å were observed when the antiparallel β-sheet flap structures closed down over the inhibitor (substrate) peptide chain, excluding bulk water and allowing the interactions that are responsible for catalyzing the hydrolysis of specific peptide bonds. (B) Expanded scale diagram of region including the flaps, the inhibitor, and the active site. Hydrogen-bonding interactions shown include those between the peptide bonds at the tips of the flaps ($Gly^{51'}$ N–H to Ile^{50} >CO), and several between flaps and inhibitor (substrate) either directly (Gly^{48} > CO and $Gly^{48'}$ > CO) or (in case of Ile^{50} NH and $Ile^{50'}$ NH) through a unique water molecule. (**C** and **D**) Chemical structure of the peptide bond mimetic that was used to replace the Gly^{51}-Gly^{52} peptide bond in the native HIV-1 PR sequence. (C) Natural peptide bond between the two Gly residues. The peptide chain runs from right to left (compare with B). (D) Analog with a thioester bond between the two Gly residues. The dotted surfaces shown are van der Waals contact radii of atoms in the peptide bond and in the isosteric replacement.

Our strategy was to react the unprotected amino-terminal half of HIV-1 PR with the corresponding unprotected carboxyl-terminal half to give the full-length molecule with the newly formed pseudo-peptide bond at the ligation site (Fig. 2). For this approach the two halves of the target polypeptide chain must have unique mutually reactive functional groups at their COOH- and NH_2-terminals, respectively, so that the ligation reaction occurs in an unambiguous way. In this case, we used a nucleophilic substitution reaction where a sulfur nucleophile at the terminus of one of the peptide segments was used to attack an alkyl bromide at the terminus of the other segment, thereby covalently linking the two segments together through a thioester analog of the peptide bond (Fig. 2). The high chemical selectivity of this S_N2 type reaction under the conditions used allowed us to carry out the ligation of the two peptide segments with all other functionalities unprotected.

The peptide segments were chemically synthesized by stepwise solid-phase methods (*13*), cleaved and deprotected with anhydrous hydrogen fluoride, purified by high-performance liquid chromatography (HPLC) (*14*), and characterized by ion-spray mass spectrometry (MS). The ligation reactions were carried out by mixing the fully unprotected amino- and carboxyl-terminal halves in 6 M guanidine hydrochloride (GuHCl), 0.1 M sodium phosphate buffer at pH 4.3 (*15*). The solubility of unprotected peptides and protein fragments in this buffer is extremely high (here we used 20 mg/ml, ~4 mM), thus eliminating completely the major drawback of the classical segment ligation approach to the chemical synthesis of proteins, the limited solubility of protected peptide fragments (*16*). The progress of ligation was followed by analytical HPLC (Fig. 3) as well as by a fluorogenic assay (*17*) for HIV-1 PR enzymatic activity (Fig. 4B). Neither peptide segment alone showed any detectable activity when tested separately for enzymatic activity under the same conditions.

The ligation of HIV-1 PR (1-50,Gly-αCOSH) with [$BrCH_2CO$(53-99)]HIV-1

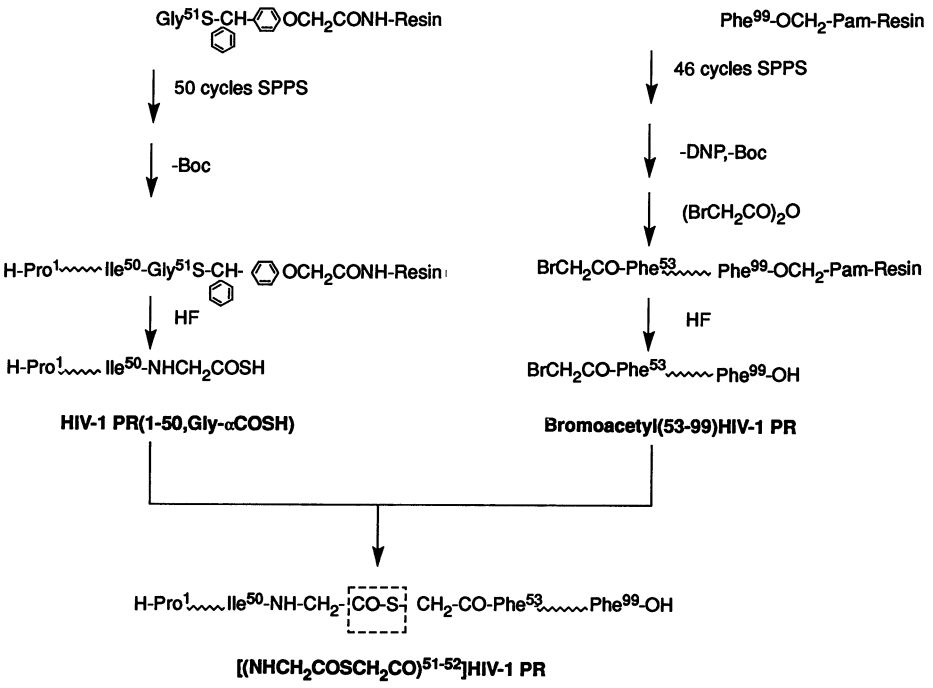

Fig. 2. Strategy for the total chemical synthesis of the HIV-1 PR analog (*23*). Functionalized, unprotected segments were prepared by stepwise solid-phase synthesis (*12, 13*). Bromoacetyl (*25*) was used as the functional group at the NH_2-terminus of the segment HIV-1 PR(53-99). In the other half of the enzyme molecule, HIV-1 PR(1-51), the Gly^{51} was replaced by a Gly α-thiocarboxylic acid (that is, -$NHCH_2COSH$) (*7*). HIV-1 PR(1-50,Gly-αCOSH) was reacted with [$BrCH_2CO$(53-99)]HIV-1 PR to yield [($NHCH_2COSCH_2$-CO)$^{51-52}$]HIV-1 PR 99-aa monomer. The boxed area represents the chemical structure of the thioester analog of the peptide bond Gly^{51}-Gly^{52} at the site where the ligation occurred.

PR occurred rapidly. Extensive product formation could already be detected after 45 min, and the reaction was essentially complete after 3 hours (Fig. 3). The ligated product showed the same retention time on a C_{18} reversed-phase column as native HIV-1 PR. The product peak was collected and characterized by ion-spray MS (Fig. 5); the protonated molecular ions had the mass expected for the HIV-1 PR analog with a thioester bond. The ([$NHCH_2COSCH_2$-CO]$^{51-52}$$Aba^{67,95}$)HIV-1 PR dimer showed the same specificity as the native enzyme in cleaving a synthetic peptide substrate analog of the GAG p24/p15 processing site (*18*) (Fig. 4A). Quantitative comparison of enzymatic activity in a fluorogenic assay showed that this HIV-1 PR analog had the same specific activity as the native enzyme (Fig. 4B).

The full activity of the backbone-engineered ([$NHCH_2COSCH_2CO$]$^{51-52}$-$Aba^{67,95}$)HIV-1 PR enzyme analog can be explained in terms of the high-resolution x-ray structure of the [$Aba^{67,95,167,195}$]HIV-1 PR complexed with a substrate-based inhibitor (Fig. 1, A and B). The peptide bond between Gly^{51}-Gly^{52} is on the outside of the flaps, at the surface of the protein and away from the substrate. Although the NH of Gly^{52} (which in this analog has been replaced by a sulfur atom) is potentially involved in a hydrogen bond to the carbonyl oxygen of Gly^{49} across the β turn at the end of the flap, the N · · · O distance observed (3.32 to 3.37 Å) is significantly greater than is

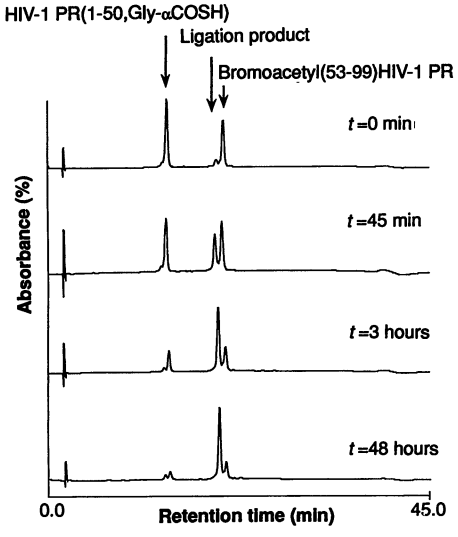

Fig. 3. HPLC analysis of the reaction of HIV-1 PR(1-50,Gly-αCOSH) with [$BrCH_2CO$(53-99)]HIV-1 PR. Samples (2 μl) of the ligation reaction mixture (*15*) were taken at t = 0, 45 min, 3 hours, and 48 hours and analyzed immediately. The progress of the ligation was followed by reversed-phase HPLC on a Vydac C_{18} column with a linear gradient of 30 to 60% buffer B (90% acetonitrile/0.09% trifluoroacetic acid) in buffer A (0.1% trifluoroacetic acid) over 30 min. The flow rate was 1 ml/min, and absorbance was monitored at 214 nm.

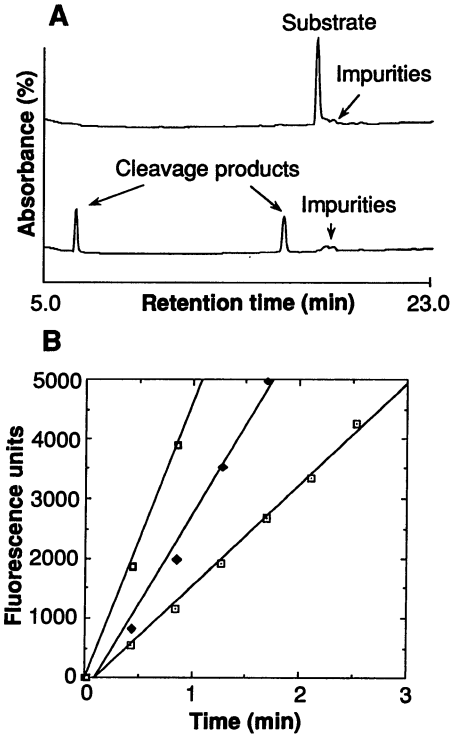

Fig. 4. Enzymatic activity of the product of the ligation reaction between HIV-1 PR(1-50,Gly-αCOSH) and [$BrCH_2CO$(53-99)]HIV-1 PR. (**A**) Specificity. The hexapeptide Ac-Thr-Ile-Nle-Nle-Gln-Arg-amide (1 mg/ml), an analog of the p24/p15 GAG viral processing site (*8*), was treated at pH 6.5 with an aliquot (taken at t = 3 hours) of the ligation reaction mixture: upper trace, peptide substrate before treatment; and lower trace; after 15-min treatment. Cleavage products were separated by reversed-phase HPLC (Vydac C_{18} column; linear gradient of 0 to 40% buffer B (90% acetonitrile/0.09% trifluoroacetic acid) in buffer A (0.1% trifluoroacetic acid) over 20 min; flow rate, 1 ml/min; absorbance monitored at 214 nm). Peptide products were identified by ion spray MS as (H)-Nle-Gln-Arg-amide (early eluting) and Ac-Thr-Ile-Nle-(OH) (late eluting). Minor impurities in the hexapeptide substrate were not cleaved and were revealed in the course of the reaction. (**B**) Kinetics. Aliquots of the reaction mixture taken at 45 min (dotted squares), 3 hours (diamonds), and 48 hours (open squares) were used in a fluorogenic assay (*26*). Data points shown were read from continuous chart recorder tracings. Quantitative comparison of ([$NHCH_2COSCH_2CO$]$^{51-52}$-$Aba^{67,95}$)HIV-1 PR dimer with an equal amount of ($Aba^{67,95,167,195}$)HIV-1 PR showed identical activities. Neither segment alone showed any activity (detection limit, <1 part in 1000).

typical for strong amide-N to carbonyl-O hydrogen bonds. In any event, other interactions in this region are clearly predominant in light of the observed full activity of the backbone-engineered HIV-1 PR analog. A similar approach could be used to systematically replace peptide bonds around the tip of the flap, where interactions mediated by peptide bonds appear to be functionally important, and along the underside of the flaps where functionally important hydrogen-bonding interactions with the substrate have been proposed (Fig. 1, A and B). Results of such experiments may help illuminate the quantitative contribution of interactions proposed based on crystal-structure data (*10*).

The chemical ligation reaction occurred rapidly and in high yield, giving a well-defined product (*19*). Purification of the synthetic protein in one step by HPLC was also straightforward. The segment ligation synthesis reported here was repeated several times and gave the same results. Typically, a complete synthesis of the HIV-1 PR analog by chemical segment ligation (including monitoring the reaction by HPLC, product isolation, assay of enzymatic activity, as well as the characterization of the ligation product by MS) could be performed in less than 1 day.

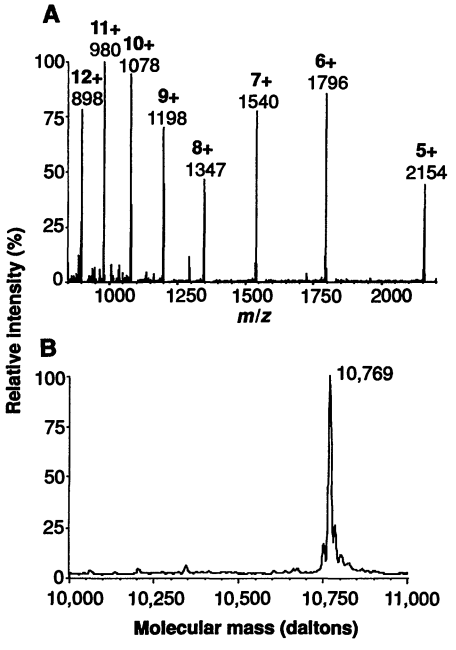

Fig. 5. Covalent characterization of the ligation product. (**A**) Ion spray mass spectrum of the HPLC-purified ([$NHCH_2COSCH_2CO$]$^{51-52}$-Aba^{67,95})HIV-1 PR 99-aa monomer formed by ligation of HIV-1 PR(1-50,Gly-αCOSH) and [$BrCH_2CO$(53-99)]HIV-1 PR. The labeled peaks represent a single molecular species differing in the number of excess protons. The observed molecular mass of the ligated product is 10,768.6 ± 1.1 daltons [Calculated: (monoisotopic) 10,763.9 daltons; (average) 10,770.8 daltons]. (**B**) Deconvoluted mass spectrum in which the raw data shown in (A) have been reduced to a single charge state. All data points in (A) were included in the calculation. No mathematical filtering was performed. The mass region 10 to 11 kD is shown for clarity. The ligated enzyme analog molecule is at 10,769 daltons. The minor peak toward low mass is −18 daltons and is presumably the sum of all dehydration products of the target polypeptide chain arising from the sum of trace impurities present in the purified segments used in the ligation reaction. The high-mass minor components are +16 and +32 daltons and may be the result of atmospheric oxidation of the methionines.

The chemical ligation approach is a versatile one. By replacing bromoacetic acid (an isosteric replacement for a Gly residue) with another α-bromo carboxylic acid (*20*), analogs of other amino acids can be introduced. Allowance must be made for the inversion of configuration that occurs in the S_N2 ligation reaction. Similarly, the Gly-αCOSH residue on the other side of the ligation site can be replaced by any amino acid simply by starting the synthesis of the corresponding peptide on an aminomethyl resin loaded with the corresponding 4-[(Boc-amino acyl)thiobenzyl]-phenoxyacetic acid (*21*). In addition, nonpeptidic elements bearing SH groups can be introduced to replace natural amino acids at the ligation site. Other combinations of unique, mutually reactive moieties (*22*) can also be used in similar chemical ligations of unprotected large synthetic peptide segments.

This selective chemical ligation approach to the preparation of protein analogs from large synthetic peptide segments offers a twofold advantage: improved syntheses and concomitant introduction of new structural features into the protein. In addition to the backbone engineering described in this report, this approach provides an efficient way to make a number of interesting and important modifications of protein structure, including the stereochemical engineering of proteins with D-amino acids, the incorporation of fixed elements of 3-D structure into the protein molecule (6), and the preparation by head-to-head condensation of novel species such as protein molecules with two amino- or carboxyl-terminals. The approach is also readily adapted to the synthesis of hybrid protein-nonprotein macromolecules.

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13. The machine-assisted chain assembly of the peptides was carried out in a stepwise fashion at a rate of 50 residues per 16 hours according to our in situ neutralization protocol for Boc (*tert*-butyloxycarbonyl) chemistry solid-phase peptide synthesis as described elsewhere (*12*). Briefly, Nα-Boc amino acids were coupled without prior neutralization of the peptide-resin salt formed by removal of the Boc group with neat trifluoroacetic acid (TFA). The following resins were used: Boc-Phe(4-carboxamidomethyl)benzyl ester resin [Boc-Phe-OCH_2Pam resin] for the synthesis of the carboxyl-terminal segment [$BrCH_2CO$(53-99)]HIV-1; and, 4-[α-(Boc-Gly-S)benzyl]-phenoxyacetamidomethyl resin for the synthesis of the amino-terminal segment HIV-1 PR(1-50,Gly-αCOSH). The latter resin was prepared as described (*8*). Boc amino acids were coupled as preformed *N*-hydroxybenzotriazol (HOBt) esters formed with benzotriazolyltetramethyluronium hexafluorophosphate (HBTU) plus diisopropylethlyamine (DIEA) as activating agent [R. Knorr, A. Trzeciak, W. Bannwarth, D. Gillessen, *Tetrahedron Lett.* **30**, 1927 (1989)]. Single 10-min coupling reaction times were used throughout (no double coupling). The following side chain protecting groups were used: D and E, cyclohexyl; N, xanthyl; Q and M, unprotected; K, 2-chlorobenzyloxycarbonyl (2ClZ); R, tosyl; Y, bromobenzyloxycarbonyl (BrZ); H, 2,4-dinitrophenyl (DNP); and T, benzyl. Abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; T, Thr; V, Val; W, Trp; and Y, Tyr. Side chain–unprotected W was used because the thiolysis conditions used to remove the formyl group caused a side reaction with the -αCOSH group of the HIV-1 PR(1-50,Gly-αCOSH) peptide. The bromoacetyl group was introduced at the NH_2-terminal of the carboxyl-terminal peptide, after removing the His(DNP) and Boc protecting groups, by manually coupling the preformed symmetric anhydride of bromoacetic acid as described (*25*) and was compatible with all subsequent deprotection and cleavage steps. Side chain–protecting groups were removed and the peptides cleaved from the resin by treatment with HF–10% *p*-cresol (0°C, 1 hour) after prior removal of the Nα-Boc group. The resulting crude polypeptide products were precipitated and washed with ether, dissolved in 50% acetic acid, diluted with water, and lyophilized.
14. Peptides were purified by reversed-phase HPLC on a semipreparative (1.0 cm by 25 cm) Vydac C_{18} column with linear gradients of 90% acetonitrile plus 0.09% TFA versus 0.1% aqueous TFA.
15. Peptides were separately dissolved in ligation buffer at a concentration of 20 mg/ml. After taking a sample of each stock solution for activity assay, equal volumes were mixed to give a final concentration of 10 mg/ml of each peptide in the ligation reaction.
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19. The HIV-1 PR analog containing a thioester bond replacement for the peptide bond at Gly^{51}-Gly^{52} was indefinitely stable (no detectable breakdown over several weeks) under the ligation reaction conditions at pH 4.3 (Fig. 3). However, model studies of the ligation reaction indicated that the thioester pseudopeptide bond was labile at higher pH. The ($[NHCH_2COSCH_2CO]^{51-52}Aba^{67,95}$)HIV-1 PR underwent hydrolysis forming HIV-1 PR(1-50,Gly-αCOOH) and $HSCH_2CO$(53-99)HIV-1 PR, with a half life of 2 hours at pH 7.5. Hydrolysis of thioesters is base catalyzed, and the rate of hydrolysis will be proportional to the concentration of hydroxide ion. The thioester analog of HIV-1 PR is thus quite stable over the pH range (pH 4 to 6) [A. D. Richards, R. Roberts, B. M. Dunn, M. C. Graves, J. Kay, *FEBS Lett.* **247**, 113 (1989)] and times (hours to days) normally used to study this enzyme. The HIV-1 PR is itself subject to autolytic digestion [J. E. Strickler *et al.*, *Proteins* **6**, 139 (1989)] and has a limited lifetime (hours to days) in solution. For this reason, we routinely store the native enzyme in 6 M GuHCl, conditions comparable to the ligation reaction conditions. Samples of enzyme stored in this way may be diluted directly into assay buffer for activity studies.
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23. Amino acid sequences of the peptide segments. Amino-terminal half, HIV-1 PR(1-50, Gly-αCOSH): (H)-P^1QITLWQRPL^{10} VTIRIGGQLK^{20} EALLDTGADD^{30} TVLEEMNLPG^{40} KWKPKMIGGI^{50} G^{51} (αCOSH); carboxyl-terminal half, ($BrCH_2CO$(53-99)]HIV-1 PR: ($BrCH_2$-CO)-F^{53}IKVRQYD^{60} QIPVEI Aba^{67} GHK^{70} AIGTVLVGPT^{80} PVNIIGRNLL^{90} TQIG Aba^{95} $TLNF^{99}$-(OH). Aba is L-α-amino-*n*-butyric acid and substitutes for the two Cys residues 67 and 95 in HIV-1 PR (SF2 isolate) (*24*).
24. The native sequence of HIV-1 PR contains Cys residues at positions 67 and 95 in each subunit. Previous studies in our laboratory have shown that replacement of the four Cys residues in the homodimeric enzyme by the noncoded, isosteric amino acid Aba resulted in a fully active enzyme analog [S. B. H. Kent *et al.*, in *Peptides 1990, Proceedings of the 21st European Peptide Symposium*, E. Giralt and D. Andreu, Eds. (ESCOM, Leiden, 1991), pp. 169–171]. Comparison of the crystal structure of the Cys-containing enzyme obtained by recombinant techniques [R. Lapatto *et al.*, *Nature* **342**, 299 (1989)] with the original structural data (*5*) generated with chemically synthesized [J. Schneider and S. B. H. Kent, *Cell* **54**, 363 (1988)] [$Aba^{67,95,167,195}$]HIV-1 PR showed that the four Cys residues in the recombinant enzyme are unpaired and that the Cys side chains have the same conformations as the Aba side chains in the chemically synthesized enzyme. In the studies reported here, the target sequence was [$Aba^{67,95,167,195}$]HIV-1 PR, avoiding the presence of Cys residues in the segments undergoing ligation. However, use of the sulfur nucleophile chemistry described here for the chemical ligation of large unprotected peptide segments is not contraindicated by the presence of Cys residues in the target protein molecule. In model studies, we find that the -αCOSH group carried out rapid nucleophilic attack on a bromoacetyl group even at low pH conditions where the alkyl-SH side chain of a Cys residue did not react (M. Schnölzer and S. B. H. Kent, unpublished results). Thus, the ligation reaction described in this work can be carried out in the presence of unprotected Cys residues.
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26. The assay was carried out by adding an aliquot of the ligation reaction mixture to a solution of 50 μM fluorogenic substrate in 100 mM MES buffer, pH 6.5. The sequence of the substrate is 2-aminobenzoyl-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-amide (*17*). Nle is L-norleucine.
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Cloning and Characterization of Inducible Nitric Oxide Synthase from Mouse Macrophages

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Nitric oxide (NO) conveys a variety of messages between cells, including signals for vasorelaxation, neurotransmission, and cytotoxicity. In some endothelial cells and neurons, a constitutive NO synthase is activated transiently by agonists that elevate intracellular calcium concentrations and promote the binding of calmodulin. In contrast, in macrophages, NO synthase activity appears slowly after exposure of the cells to cytokines and bacterial products, is sustained, and functions independently of calcium and calmodulin. A monospecific antibody was used to clone complementary DNA that encoded two isoforms of NO synthase from immunologically activated mouse macrophages. Liquid chromatography–mass spectrometry was used to confirm most of the amino acid sequence. Macrophage NO synthase differs extensively from cerebellar NO synthase. The macrophage enzyme is immunologically induced at the transcriptional level and closely resembles the enzyme in cytokine-treated tumor cells and inflammatory neutrophils.

Nitric oxide (NO) is a short-lived, gaseous radical that is the smallest biosynthetically derived secretory product of mammalian cells. Through oxidation of thiols, hemes, Fe-S clusters, and other nonheme iron prosthetic groups, NO regulates enzymes, alters vascular tone, platelet function, inflammation, neurotransmission, and lymphocyte proliferation, and mediates some of the cytotoxic action of murine macrophages against tumor cells and microbes (*1*).

In endothelium and neurons, transient synthesis of small amounts of NO is rapidly triggered by agonists that elevate Ca^{2+}. Increased intracellular Ca^{2+} alters the conformation of calmodulin, which binds to nitric oxide synthase (NOS) to activate NO production (*2*, *3*). In contrast, macrophages, hepatocytes, smooth muscle cells, fibroblasts, mesangial cells, and some tumor cells begin to produce NO several hours after exposure to cytokines and microbial products. These cells then release large quantities of NO for many hours by a

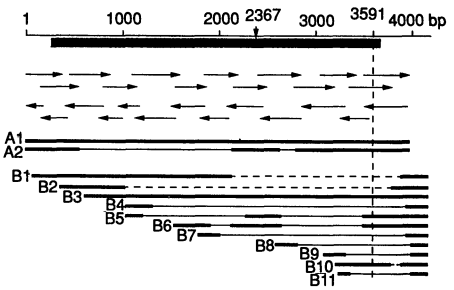

Fig. 1. Cloned iNOS cDNAs. cDNAs were cloned after immunoscreening with antibody to iNOS (*14*) and sequenced where indicated by the thick bars. Thin bars, regions not sequenced. Polyadenylated tails are not shown. The sequencing strategy was similar in each case and is illustrated for clone A1 by horizontal arrows. Clones A1, A2, and B1 included the ATG initiation codon within a consensus initiation sequence (GACATGG) (*15*). The dashed vertical line divides the nucleotide sequence into the region (base pairs 1 to 3591, numbered for clone A1) in which it was identical for all clones [except at position 2367 (vertical arrow)] and the remaining region (base pair 3592 to polyadenylate tails), where the A clones shared one sequence and the B clones shared an entirely different sequence. Black bar at top, longest coding region; horizontal dashes, deletions in the cDNA. GenBank accession number M87039.

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