ments with DNA as seen by EM and has ATPase and LexA repressor cleavage activities that are highly ssDNA dependent. RecA protein purified by this procedure does not form appreciable amounts of filaments when incubated with ATPγS and Mg^{2+}, a property we will show elsewhere is due to contaminating RNA and polynucleotide phosphorylase. There are other stages in the purification of RecA protein at which microcrystallization can be introduced, in particular very early. We have explored some of these schemes, and in time, it would be expected that even better purifications based on this phenomenon will be developed.

Registry No. ATPase, 9000-83-3; chloride, 16887-00-6; acetic acid, 64-19-7; sulfate, 14808-79-8; phosphate, 14265-44-2; spermidine, 124-20-9; spermine, 71-44-3; polymin P, 74913-72-7.

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Determination of the Relative Positions of Amino Acids by Partial Specific Cleavages of End-Labeled Proteins†

Rodney A. Jue‡ and Russell F. Doolittle*

Department of Chemistry, University of California, San Diego, La Jolla, California 92093

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ABSTRACT: We have developed a new method for obtaining information about protein sequences that uses an approach analogous to that used to determine DNA sequences. In essence, three steps are involved. First, a detectable label is attached exclusively to the amino terminus of a polypeptide. Next, the labeled chain is subjected to partial specific cleavage in a way that produces roughly equimolar amounts of fragments of different sizes. Cleavages for methionine, tryptophan, arginine, aspartyl-proline bonds, and asparaginyl-glycine bonds have been employed. Lastly, the labeled fragments are separated according to size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The distribution of target amino acids along the polypeptide chain can be deduced from the specific pattern of labeled bands by reading the "ladder" in the same way that DNA sequencing gels are read. Although the method can be conducted with a radioactive label, we have chosen to use a fluorescent label. We have applied the method successfully to the three subunit chains of two different fibrinogens.

Tremendous advances in genetics and biochemistry have been made possible by recent progress in DNA sequencing that depends on polyacrylamide gel electrophoresis. Many researchers use the biosynthetic incorporation of radioactive nucleotides, as introduced by Sanger et al. (1977), to generate a "ladder" that allows the direct reading of DNA sequences from polyacrylamide gels. Alternatively, the same result is achieved by the Maxam & Gilbert (1977) approach in which DNA that is exclusively labeled at one end is randomly fragmented at locations occupied by only one of the four bases. The development of an analogous sequencing procedure applicable to proteins must surmount a number of obstacles. In the first place, neither protein terminus easily lends itself to specific labeling, because the terminal amino group is similar

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‡ Present address: Hybritech, Inc., 9850 Distribution Avenue, San Diego, CA 92121.

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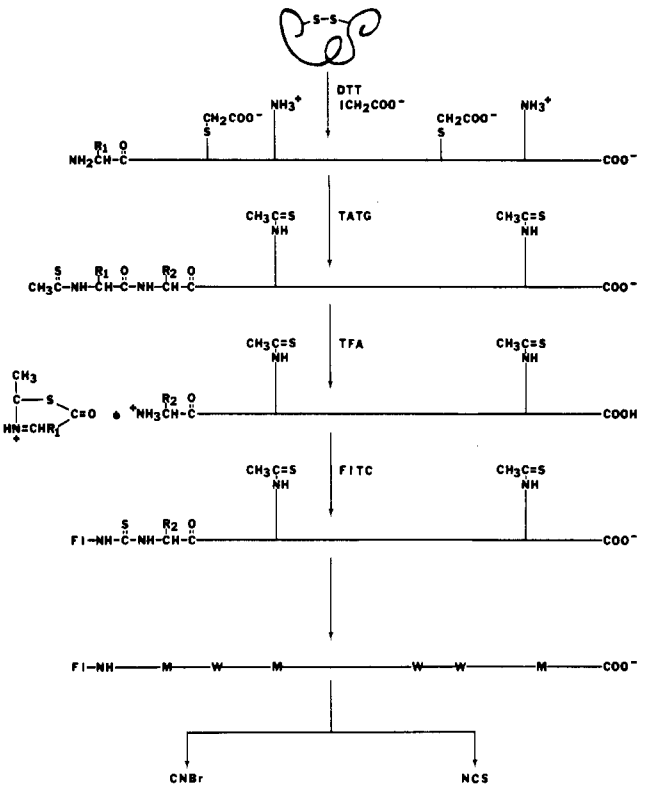
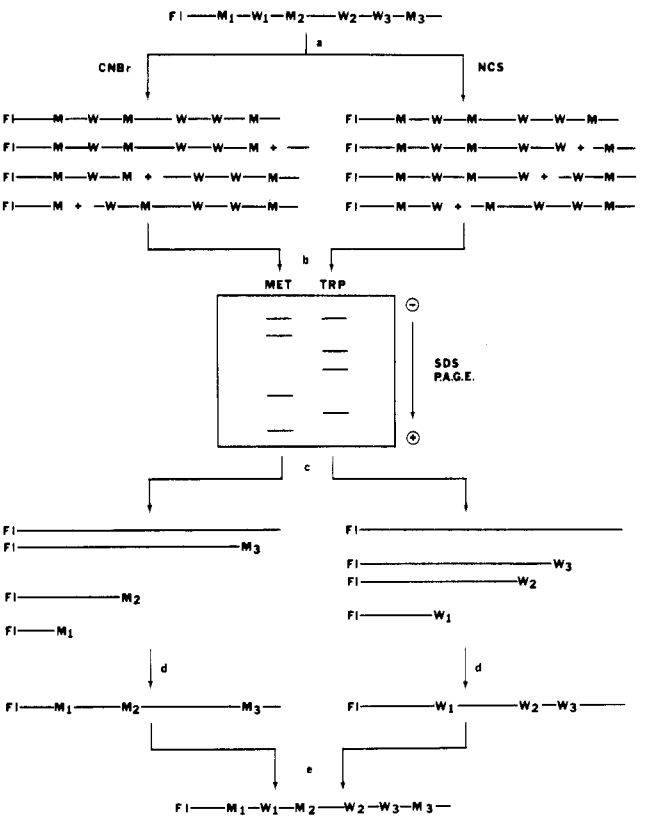
Scheme I



Scheme II



to the ϵ-amino group of lysine, and the terminal carboxy group is virtually indistinguishable from the carboxyl groups of glutamic acid and aspartic acid. Beyond that, specific cleavage procedures for all 20 amino acids are not available at this time. Finally, it is considerably more difficult to separate peptides differing in size by one amino acid residue than it is to separate polynucleotides differing in size by one nucleotide, partly because amino acids have different sizes and shapes and partly because peptides, in contrast to polynucleotides, are not uniformly charged. In spite of these formidable problems, we have developed a protein sequencing system analogous to the Maxam–Gilbert DNA sequencing method that yields valuable information about the primary structure of a protein quickly, simply, and inexpensively.

The method is based on three major operations. In the first of these, the amino terminus is exclusively labeled. This step is made possible by the stepwise removal of the first residue after chemical modification of all amino groups, followed by a labeling step with fluorescein isothiocyanate (FITC).[1] Ordinarily, the protein is first unfolded by reduction and alkylation (Scheme I). After all the amino groups are irreversibly modified by (thioacetyl)thioglycolic acid, the amino-terminal residue is removed by a single stepwise degradation with trifluoroacetic acid to expose the α-amino group of the penultimate residue (Mross & Doolittle, 1971). Subsequent reaction with FITC results in a protein labeled only at its amino terminus with a fluorescent group. Second, incomplete reaction of this protein by one of several specific cleavage procedures [e.g., CNBr cleaves at methionines (M) and NCS cleaves at tryptophan (W)] splits the molecule at one or more places and produces a mixture of peptides, some of which carry the fluorescent label (Scheme II, step a). Third, these peptides are separated according to size on a sodium dodecyl sulfate–polyacrylamide slab gel (Laemmli, 1970), and those with the amino-terminal label are detected by their fluorescence under ultraviolet light (Scheme II, step b). The distance from the amino terminus to the point at which cleavage occurs determines the size of a fluorescent peptide, and the size of a peptide determines its distance of migration (Scheme II, step c). Consequently, the relative position of a fluorescent peptide after electrophoresis indicates the relative position in the protein of the cleavage reaction that releases it (Scheme II, step d). The particular cleavage reaction used identifies the amino acid at the cleavage site. When the products of several selective cleavages are run next to each other on a slab gel, the relative positions of several amino acids can be determined by reading the ladder of fluorescent bands (Scheme II, step e). So far, we have successfully used cleavages at methionine by cyanogen bromide (Gross & Witkop, 1962), at arginine by trypsin (since all the lysines are thioacetylated), and at tryptophan by *N*-chlorosuccinimide (Shechter et al., 1976; Lischwe & Sung, 1977). In addition, we have cleaved aspartyl–proline bonds (Piszkiewicz et al., 1970) and asparaginyl–glycine bonds (Butler, 1969; Bornstein, 1969) by taking advantage of their unique sensitivities.

In our initial studies, we have applied this technique to the three nonidentical chains of the vertebrate fibrinogen molecule. In this regard, the known sequences of the human fibrinogen subunit chains, which have been characterized both by amino acid sequencing techniques (Lottspeich & Henschen, 1977; Henschen & Lottspeich, 1977; Watt et al., 1979; Doolittle et al., 1979; Henschen et al., 1979) and by DNA sequencing methods (Rixon et al., 1983; Chung et al., 1983a,b; Kant et al., 1983), were used to test the method. Lamprey fibrinogen sequences, which were largely unknown, were taken as a new challenge.

[1] Abbreviations: FITC, fluorescein isothiocyanate; DTT, dithiothreitol; TATG, (thioacetyl)thioglycolic acid; TFA, trifluoroacetic acid; CNBr, cyanogen bromide; NCS, *N*-chlorosuccinimide; $NaDodSO_4$, sodium dodecyl sulfate; CMC, (carboxymethyl)cellulose; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Gdn-HCl, guanidine hydrochloride; TEA-HCl, triethylamine hydrochloride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Fluorescent Labeling Procedure for Each Fibrinogen Chain

	human fibrinogen[a]				lamprey fibrin B		
treatment	γ	α	β	γ	α	β	γ
dansylcadaverine				+			+
reduction, alkylation, and chain isolation on CMC	+	+	+	+	+	+	+
ethyl acetimidate		+			+		
lamprey thrombin					+		
bovine thrombin		+	+				
(thioacetyl)thioglycolic acid	+		+			+	
trifluoroacetic acid	+		+			+	
fluorescein isothiocyanate	+	+	+		+	+	
$NaDodSO_4$ gel purification and electroelution				+			

[a] The human fibrinogen γ chain was labeled by two different procedures.

As it happens, the unique properties of the human and lamprey fibrinogen chains provided an opportunity to apply the strategy of the method in several different ways. For the initial test, the human γ chain was labeled by the general method (Table I). In the case of the human β chain, however, the amino terminus is blocked, rendering it inaccessible to labeling at the α-amino group. In this case, the block was overcome by treatment with thrombin to remove the blocked fibrinopeptide B (Table I) and expose a new and unique α-amino group. Both human and lamprey β chains were labeled by the general method after the removal of their fibrinopeptides B. A modified strategy was applied to the α chains (Table I), which were first amidinated (Hunter & Ludwig, 1962; Hand & Jencks, 1962) to conserve the positive charge on the modified lysines, and to increase their solubility. Subsequent treatment of the amidinated α chains with thrombin exposed a new accessible amino terminus, after which the α chains were labeled with FITC.

The difficulty presented by the lamprey γ chain with its blocked amino group was circumvented by exclusively labeling its cross-linking site with the factor XIII catalyzed incorporation of the fluorescent substitute donor dansylcadaverine (Lorand & Ong, 1966). Human γ chain, in which the cross-linking site is known to be situated very close to the carboxy terminus (Chen & Doolittle, 1970), was treated similarly (Table I). In line with the latter approach, it must be emphasized that the strategy of the fragmentation technique can be applied to polypeptide chains labeled at either the amino or the carboxy terminus.

Experimental Procedures

Materials. Human fibrinogen was purified from blood bank plasma by cold ethanol precipitation as described (Doolittle et al., 1967). Lampreys (*Petromyzon marinus*) were collected in various New England rivers and streams during spring spawning runs. Blood collection and fibrinogen preparation were carried out as previously reported (Doolittle, 1965; Cottrell & Doolittle, 1976). Methods for the preparation of dansyl-labeled fibrin (Lorand & Ong, 1966), synthesis of (thioacetyl)thioglycolic acid (Doolittle, L. R., et al., 1977), the isolation of lamprey thrombin (Doolittle, 1965), and isolation of the individual fibrinogen chains (Doolittle, R. F., et al., 1977) have been reported. The following were purchased from the vendor indicated: dithiothreitol, Aldrich; topical-grade bovine thrombin, Parke-Davis; fluorescein isothiocyanate, Calbiochem-Behring; cyanogen bromide, Matheson Coleman and Bell (MCB); *N*-chlorosuccinimide, Sigma; hydroxylamine, Allied Chemical Corp.; TPCK–trypsin, Worthington; sodium dodecyl sulfate, BDH Biochemicals; acrylamide and *N,N'*-methylenebis(acrylamide), Bio-Rad. Iodoacetic acid was obtained from Aldrich and used after recrystallization from ethanol.

Thioacetylation of Amino Groups. The procedure of Mross & Doolittle (1971) was modified in the following way. An alkylated protein (ca. 8 mg) was dissolved in 0.4 mL of 6 M guanidine hydrochloride (Gdn-HCl) and 0.5 M triethylamine hydrochloride (TEA-HCl), pH 9.5. TATG (40 mg/mL) was dissolved in 6 M Gdn-HCl and 0.5 M TEA-HCl, pH 9.5, and adjusted to pH 9.4–9.8 with TEA. The freshly prepared TATG solution (0.4 mL) was added to the protein in a 12-mL glass-stoppered Pyrex tube, flushed with nitrogen, stoppered, and incubated for 60 min at 40 °C. Unreacted reagents were removed by a series of extractions with acetone, 90% aqueous acetone, and 80% aqueous acetone (8 mL). A precipitate formed during the 90% aqueous acetone extractions, and the liquid phase was discarded. Excess acetone was evaporated from the precipitate with a stream of nitrogen. The coupling procedure was repeated a second time to ensure that all the amino groups were thioacetylated. After the second coupling, the precipitate was resuspended in 1.0 mL of distilled water and lyophilized.

Removal of the Thiazolinone. The thioacetylated protein was dissolved in 0.4 mL of TFA, flushed with nitrogen, stoppered, and incubated for 20 min at 40 °C. After the solution was cooled, the protein was precipitated by the addition of ethyl acetate (8 mL). The liquid phase was discarded and the precipitate washed 5 more times with 8 mL of ethyl acetate. Final traces of ethyl acetate were evaporated with a stream of nitrogen.

Amidination of α Chains. Human and lamprey fibrinogen α chains (ca. 8 mg) were dissolved in 0.8 mL of 6 M Gdn-HCl and 0.5 M TEA-HCl, pH 9.5. Ethyl acetimidate was added to a final concentration of 0.2 M (Hunter & Ludwig, 1962; Hand & Jencks, 1962). The pH was adjusted to 9.0 with 2.5 N sodium hydroxide (NaOH), and the reaction was allowed to proceed for 2 h at room temperature (22 °C), after which unreacted reagents were removed by a series of acetone and aqueous acetone washes. This reaction was repeated to ensure completion of amidination.

Thrombin Cleavage. Alkylated human β chains (5 mg/mL) or amidinated α chains (5 mg/mL) were resuspended in 1.6 mL of 0.005 M sodium phosphate and 0.3 M NaCl, pH 7.0. Solid thrombin (0.05 mg/mL) was added. The solution was stirred for 3 h at room temperature. Soluble thrombin, which amounted to less than 1% of the protein present, was removed by centrifuging the insoluble fibrinogen chains and discarding the supernatant. The chains were washed once with 1.6 mL of water.

Fluorescein Labeling. The TFA-treated thioacetylated protein was resuspended in 0.25 mL of cold (4 °C) pyridine and 0.35 mL of cold (4 °C) 6 M Gdn-HCl and 0.5 M TEA-HCl, pH 9.5. The tube was flushed with nitrogen, stoppered, and vortexed. If the protein did not dissolve, the tube was warmed for not more than 2 min at 100 °C, during which time solution usually occurs. FITC (70 mM) was dissolved in pyridine and adjusted with TEA to give a pH of 9.5 upon dilution with water. After the protein had dissolved, 0.1 mL of the 70 mM FITC solution was added. The tube was flushed with nitrogen, stoppered, vortexed, and incubated for 60 min at 40 °C. Unreacted reagents were removed by a series of acetone and aqueous acetone washes. The fluorescein-labeled protein was resuspended in 1 mL of water and lyophilized. Amidinated proteins were labeled in the same manner except that the 0.35 mL of 6 M Gdn-HCl and 0.5 M TEA-HCl, pH 9.5, was added before the pyridine.

The overall recovery of protein taken from the initial alkylation through labeling with fluorescein isothiocyanate was 60–75%.

Selective Amino Acid Cleavages. In order to achieve an even distribution of bands on the gels, it is necessary to perform the cleavages for varying lengths of time or with different amounts of cleavage reagent or enzyme. After the time course or reagent concentration series of each cleavage has been examined, the times or particular amounts of reagents that give the best distribution of bands are selected and rerun on a single gel comparing all of the cleavages. The following cleavage conditions may need to be modified to accomodate the unique properties of a particular protein.

For partial cleavage at methionine residues (Gross & Witkop, 1962), 200 μg of labeled protein was dissolved in 40 μL of 88% formic acid. The protein solution was adjusted to 70% formic acid by addition of 10 μL of water and cleavage initiated by adding 50 μL of CNBr (8 mg/mL) in 70% formic acid. This mixture was incubated at room temperature for 30–120 min. The reaction was terminated at 30-min intervals by adding 2 mL of water and freeze-drying. The dried peptides were dissolved in 100 μL of sample gel buffer consisting of 0.15 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, 0.1 M DTT, 4% $NaDodSO_4$, and 20% glycerol.

For cleavage at aspartyl–proline bonds (Piszkiewicz et al., 1970), 200 μg of labeled protein was dissolved in 114 μL of 88% formic acid. The solution was adjusted to 50% formic acid by adding 86 μL of water. The mixture was incubated at 40 °C for 20–30 h. After incubation, 2.5 μL of CNBr (8 mg/mL) was added, and the reaction was allowed to proceed for 3 min. The cleavage was terminated by adding 2 mL of water and freeze-drying. Dried peptides were dissolved in 100 μL of sample gel buffer.

Selective cleavage at tryptophan residues (Shechter et al., 1976; Lischwe & Sung, 1977) was achieved by dissolving 200 μg of labeled protein in 11.3 μL of 88% formic acid. Glacial acetic acid (80 μL) was added, followed by 8.7 μL of water and finally 100 μL of NCS (0.5–4 mg/mL) in 80% acetic acid. The reaction was allowed to proceed at room temperature for 30–60 min and was halted by adding 2 mL of 3 mM methionine and freeze-drying. Dried peptides were dissolved in 100 μL of sample gel buffer which also contained 0.1 M methionine.

Asparaginyl–glycine bonds were cleaved by treatment with hydroxylamine (Butler, 1969; Bornstein, 1969). Fluorescein-labeled protein (200 μg) was dissolved in 100 μL of 2 M hydroxylamine hydrochloride, 0.2 M Tris, and 8 M urea brought to pH 9.2–9.5 with 5 N NaOH. The cleavage was incubated at 40 °C for 2–8 h. After incubation, 100 μL of water was added, and the reaction was stopped by adding 2 mL of 100% acetone which precipitated the peptides. the precipitate was washed once with 90% aqueous acetone and dried with a stream of nitrogen. The dried peptides were dissolved in 114 μL of 88% formic acid, and 86 μL of water was added, followed by 2.5 μL of CNBr (8 mg/mL). The reaction was incubated for 3 min at room temperature and was stopped by addition of 2 mL of water and freeze-drying. The dried peptides were dissolved in 100 μL of sample gel buffer.

For partial proteolysis with trypsin, 200 μg of labeled protein was dissolved in 50 μL of 0.2 M Tris-HCl and 8 M urea, pH 8.2. Then 50 μL of trypsin (0.002–0.04 mg/mL) in 0.2 M Tris-HCl, pH 8.2, was added. The enzymatic cleavage was incubated for 30 min at 40 °C. The cleavage was stopped as described for the hydroxylamine cleavage procedure. The dried peptides were dissolved in 100 μL of sample gel buffer.

Polyacrylamide Gel Electrophoresis. Fluorescent fibrinogen chains (5–60 μg) subjected to partial cleavage, as well as fluorescent intact fibrinogen chains (1.5–5 μg), were analyzed on polyacrylamide/$NaDodSO_4$ gels (Laemmli, 1970). The running gels were either 22% or 25% polyacrylamide [1:50 *N,N'*-methylenebis(acrylamide):acrylamide] in 1 M Tris-HCl, pH 8.7, and 0.1% $NaDodSO_4$. The stacking gels were 5% polyacrylamide [also 1:50 *N,N'*-methylenebis(acrylamide): acrylamide] in 0.125 M Tris-HCl, pH 6.8, and 0.1% $NaDodSO_4$. The running buffer was 0.38 M glycine, 0.05 M Tris, and 0.1% $NaDodSO_4$, pH 8.5. The slab gels were run for 4–8 h at 20–40 mA. Since the fluorescent peptides could be observed during their migration with a 360-nm ultraviolet light, the electrophoretic run was simply terminated when the peptides had migrated a sufficient distance into the gel.

Fluorescent Photography. Slab gels were illuminated with an ultraviolet light (360 nm) and exposed to type 57 Polaroid Land film, 3000 ASA, for 15–60 s at $f = 4.5$ with a green Kodak 74 Wratten filter.

Gel Purification of Human Dansylated γ Chains. The factor $XIII_a$ preparation of dansylated human γ chains inadvertently generates a significant amount of labeled cross-linked γ–γ dimers (Chen & Doolittle, 1969). The non-cross-linked dansylated γ chains were separated from the dansylated γ–γ dimer chains on 7.5% polyacrylamide/$NaDodSO_4$ tube gels and the bands containing the dansylated γ chain excised. The gel pieces were put into a dialysis bag containing running buffer and placed in an electrophoresis chamber containing running buffer without $NaDodSO_4$. Electroelution was allowed to proceed for 3 h at 100 V. The electroeluted dansylated γ chains were dialyzed against water and freeze-dried.

RESULTS

Studies on Fluorescein-Labeled Human γ Chains. Human γ chains were labeled exclusively at the amino terminus with FITC (Table I) and subjected to the five partial specific cleavages described under Experimental Procedures. Since all the lysine side chains were thioacetylated, trypsin cleavage was limited to arginines. The intact chain (1.5 μg) and selective cleavage products (5–60 μg) were separated according to size on a 25% polyacrylamide/$NaDodSO_4$ slab gel (Figure 1). The various lanes contain intact labeled human γ chain (Hγ) and human γ chains cleaved at aspartyl–proline bonds (DP), methionines (M), asparaginyl–glycine bonds (NG), arginines (R), and tryptophans (W).

The sequence of these selected residues is determined by reading the ladder of bands in the same way that DNA sequencing gels are read, starting at the bottom of the gel and reading upward. Since the chain was labeled at the amino terminus, the bands at the bottom of the gel correspond to the sequence near the amino-terminal region. Thus, the smallest molecular weight band is in the methionine lane, and the next two are in the arginine lane. After that, there is a space on the gel where no bands occur; the fourth lowest molecular weight band corresponds to an asparaginyl–glycine bond. This is followed by a tryptophan, an arginine, two more tryptophans, and so on up the gel. In this case, the predicted sequence reads Met..Arg..Arg..Asn-Gly..Trp..Arg..Trp..Trp etc. The entire predicted sequence is shown to the right of the gel; each letter represents a band on the gel. Multiple arrows from a single letter indicate closely packed multiple cleavage sites that were not resolved on the gel. An arrow extending from a particular amino acid on the predicted sequence indicates which band

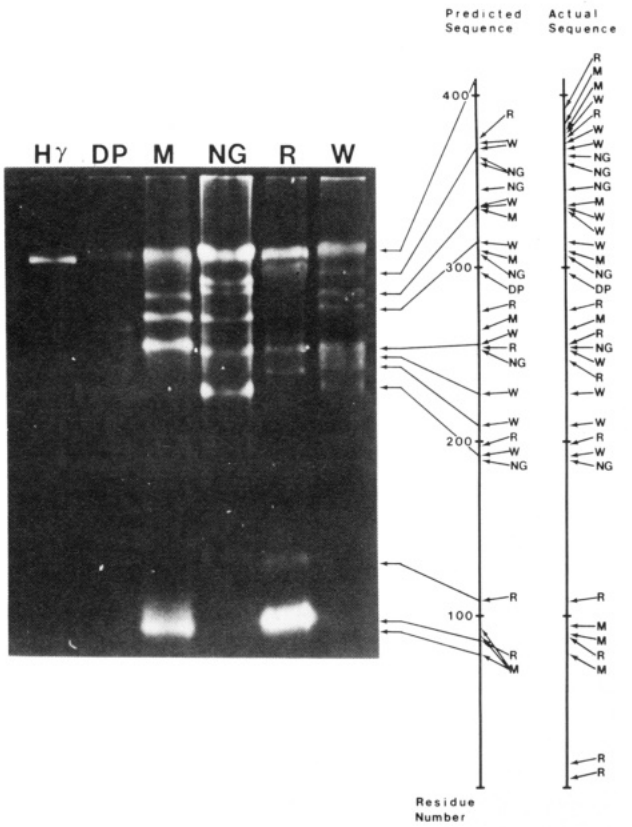


FIGURE 1: Photograph of a fluorescein-labeled human γ-chain sequencing gel. Labeling, selective cleavage, $NaDodSO_4$ gel electrophoresis, and photography were performed as described in the text. The lanes include intact human γ chain (Hγ) (1.5 μg) and human γ chains (5–60 μg) subjected to partial specific cleavage methods. Partial cleavages were selective for aspartylprolines (DP), methionines (M), asparaginylglycines (NG), arginines (R), and tryptophans (W). To determine the sequence of the human γ chain, begin at the bottom of the gel and read upward until the bands are not resolved. Since the chain was labeled at the amino terminus, reading upward gives a sequence from the amino terminus to the carboxy terminus. The predicted sequence is shown to the right of the gel. Each letter represents a band on the gel. Multiple arrows from a letter indicate multiple cleavage sites not resolved on the gel. An arrow extending from a particular amino acid on the predicted sequence indicates which band on the gel that particular amino acid corresponds to. The actual sequence is shown to the right of the predicted sequence.

on the gel that particular amino acid corresponds to. The actual sequence is shown to the right of the predicted sequence. In this case, the fragments corresponding to the two arginines closest to the amino terminus in the actual sequence were run off the gel in order to increase the separation between other fragments.

Overall, the sequence predicted for the human fibrinogen γ chain labeled at its amino terminus is in good agreement with the actual sequence. That the gel system had good resolution is evidenced by the correct prediction of residues 189, 191, 197, 208, and 227, which correspond to the amino acids Asn-Gly, Trp, Arg, Trp, and Trp, respectively. Similarly, residues 264, 275, 298, 308, 310, and 315, which correspond to the amino acids Met, Arg, Asp-Pro, Asn-Gly, Met, and Trp, respectively, were also correctly predicted. On the other hand, the methionine-cleaved fluorescent peptides that are 78, 89, and 94 residues in length appear as a single strong band on the gel.

Fluorescein-Labeled Human and Lamprey β Chains. Appropriately labeled human and lamprey fibrinogen β chains (Table I) were selectively fragmented by the five techniques described under Experimental Procedures. A 25% polyacrylamide/$NaDodSO_4$ gel was used to separate the intact chains (2 μg) and fragmented polypeptides (5–60 μg) according to size (Figure 2). The relative order of the human β-chain (Figure 2A) target amino acids observed was R..R..DP..R..(M, W, R)..R..R..R..M..R..M..(M, NG, W)..W..W..R..M..W.. M..(NG, M)..DP..(R, W, NG). The order of the amino acids in parentheses is listed as tentative because the respective cleavage bands migrated virtually the same distance on the gel. Since the bands at the bottom of the gel correspond to the amino-terminal region, the above sequence was read from bottom to top. In the gel shown, the bands could not be read past residue 414. The four bright bands at the top of the gel in the NG lane account for all three Asn-Gly sites (which occur at residues 246, 271, and 413) as well as the 461-residue intact chain. Only two of the three reported Asp-Pro sites were cleaved (residues 67 and 389). Seven methionine bands are clearly visible; in 3 of these, there are 2 methionine cleavage sites per band, accounting for all 10 methionines up to residue 414. Seven of the eight tryptophans up to residue 414 are clearly visible. The highest molecular weight tryptophan band contains the labeled fragments that are generated by cleavage at adjacent tryptophans (residues 402 and 403).

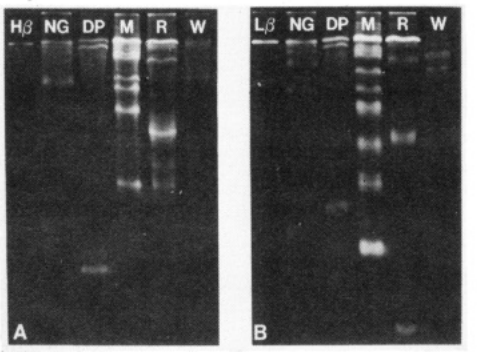


FIGURE 2: Sequencing gels of fluorescein-labeled human and lamprey β chains. (A) Intact human β chains (Hβ) (2 μg) and human β chains (5–60 μg) subjected to selective cleavages. (B) Intact lamprey β chain (Lβ) (2 μg) and selectively fragmented lamprey β chains (5–60 μg). Selective cleavage were specific for asparaginylglycines (NG), aspartylprolines (DP), methionines (M), arginines (R), and tryptophans (W). Since the chain was labeled at the amino terminus, the sequence reads up from the amino terminus to the carboxy terminus. Fluorescent fragmentation patterns were generated and analyzed as described in the text.

The lamprey β-chain (Figure 2B) amino acid alignment observed was R..M..M..DP..M..M..M..R..R..M..(NG, M).. M..W..NG..R..W..M..W..M..(NG, M)..DP..W..NG. There are 4 Asn-Gly sites, 2 Asp-Pro bonds, 11 methionines, and 4 tryptophans. As predicted from the amino acid composition (Doolittle et al., 1976), the lamprey β chain has more methionine bands and fewer tryptophan bands than the human β chain.

A side by side comparison was made of the human and lamprey β-chain fluorescent fragmentation patterns, the appropriate human (H) and lamprey (L) chain samples that had been subjected to the same cleavage method being loaded into adjacent gel lanes (Figure 3). In the NG pair of lanes, the human and lamprey β chains have been cleaved at their Asn-Gly bonds; they have three bands in common. Moreover, the topmost bands correspond to the intact chains, which appear to be the same size. The other two bands correspond to conserved Asn-Gly cleavage sites. The DP pair lanes reveals that the human and lamprey β chains each have two Asp-Pro bands. Only one of the Asp-Pro sites near the carboxy terminus is common to both, however. The other lamprey Asp-Pro fragment has a higher molecular weight than the

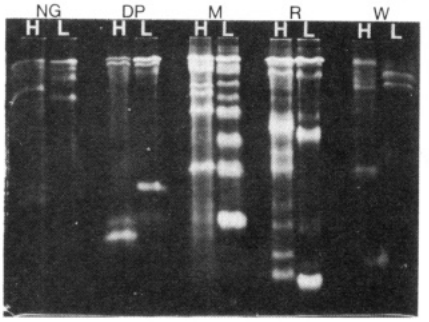


FIGURE 3: Comparison of human and lamprey β-chain fluorescent fragmentation patterns. Labeled human (H) and lamprey (L) β chains digested by the same selective cleavage method were compared side by side in adjacent gel lanes. The five pairs of lanes from left to right contain asparaginylglycine (NG), aspartylproline (DP), methionine (M), arginine (R), and tryptophan (W) cleavage products. See text for other details.

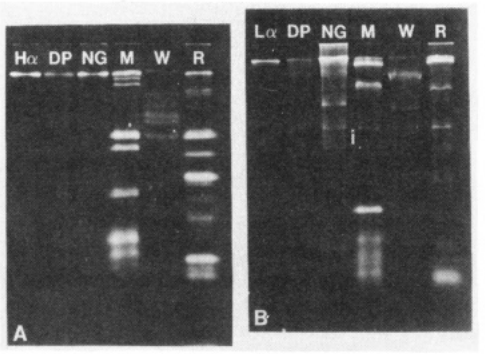


FIGURE 4: Sequencing gels of fluorescein-labeled human and lamprey α chains. (A) Intact human α chains (Hα) (2.5 μg) and human α chains (5–60 μg) subjected to selective cleavages. (B) Intact lamprey α chain (Lα) (5 μg) and selectively fragmented lamprey α chains (5–60 μg). Selective cleavages were specific for aspartylprolines (DP), asparaginylglycines (NG), methionines (M), tryptophans (W), and arginines (R). Since the chain was labeled at the amino terminus, the sequence reads up from the amino terminus to the carboxy terminus. The four bands marked "i" in panel B are minor impurities that were ignored during the reading of the lamprey sequence. Fluorescent fragmentation patterns were generated and analyzed as described in the text.

human Asp-Pro band, indicating that the latter is nearer to the amino terminus. The M pair of lanes shows that all but two of the methionine bands found in the human β chains are also found in lamprey β chains. The additional methionines in the lamprey chain are in the amino-terminal half of the molecule. The W pair of lanes shows that the tryptophan fragmentation patterns are similar. There is no indication of a tryptophan in the amino-terminal region of the lamprey β chain, however, in contrast to what is observed in the human sequence. Overall, the human and lamprey β chains are quite similar, especially in the carboxy third of the molecule.

Fluorescein-Labeled Human and Lamprey α Chains. FITC was used to label the amino termini of thrombin-treated amidinated human and lamprey fibrinogen α chains (Table I), and the five cleavage methods were applied. The intact chains (2.5–5 μg) as well as their cleavage products (5–60 μg) were resolved according to molecular weight by electrophoresis on 22% polyacrylamide/$NaDodSO_4$ slab gels (Figure 3). Since the chains were amidinated, trypsin cleavage was limited to arginines. Again, since the chains are labeled at the amino terminus, the sequence is read from the bottom to the top.

The distribution of target amino acids for the human α chain is easily derived from Figure 4A. Reading up the gel, the sequence we observed was W..W..R..M..M..R..R..M..R.. R..R..M..(W, M, R)..R..W..W..W..R..W..W..W..R..W..R..

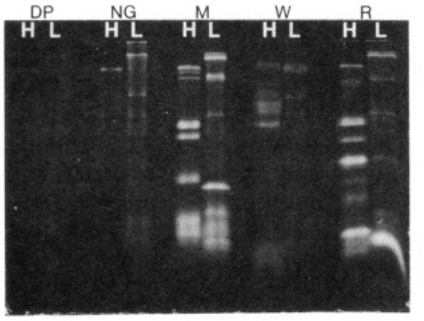


FIGURE 5: Comparison of human and lamprey α-chain fluorescent fragmentation patterns. Labeled human (H) and lamprey (L) α chains digested by the same selective cleavage method were compared side by side in adjacent gel lanes. The five pairs of lanes from left to right contain aspartylproline (DP), asparaginylglycine (NG), methionine (M), tryptophan (W), and arginine (R) cleavage products. See text for other details.

M..M..M. Of the 11 expected methionine bands, 9 are clearly shown on the gel. Only the region of residues 235–240, which corresponds to the sequence Met-Pro-Gln-Met-Arg-Met, was not resolved; it appears as a single very bright band on the gel. The gel resolved every one of the seven tryptophans in the tryptophan-repeat region. No bands corresponding to an asparagine–glycine cleavage are seen on the gel, nor were any bands corresponding to aspartyl–proline cleavage found. This is in agreement with DNA sequencing results (Rixon et al., 1983; Kant et al., 1983) which found residues 388–389 to be Asn-Pro and not Asp-Pro as reported for the protein sequence (Doolittle et al., 1979).

The lamprey α-chain (Figure 4B) amino acid alignment observed was R..W..M..M..NG..R..M..M..R..W..NG..R. M..M..W..W..M..M..R. The lamprey α chain has eight bands in the methionine lane, which is in good agreement with its amino acid composition (Doolittle et al., 1976). The tryptophan lane, on the other hand, has far fewer bands than expected on the basis of tryptophan analysis by a colorimetric procedure (Doolittle et al., 1976). It is possible that some of the tryptophans are closely grouped together and are not resolved on the gel; in this regard, the largest tryptophan fragment band is much brighter than the other tryptophan bands.

The human and lamprey α-chain fluorescent cleavage patterns were compared side by side (Figure 5). As has been reported (Doolittle et al., 1976), the lamprey α chain is 50% larger than the human α chain. Neither α chain has any aspartyl–proline bonds. On the other hand, the lamprey chain has two asparaginyl–glycine bonds whereas the human chain has none. The two low molecular weight tryptophan bands of the human α chain are clearly evident, partly because somewhat more material was loaded on this particular gel (Figure 5). In the amino-terminal region, the lamprey α chain has one tryptophan and one methionine in common with the human α chain. Interestingly, for the remainder of their lengths, the human and lamprey α chains have completely dissimilar methionine and tryptophan distributions.

Dansyl-Labeled Human and Lamprey γ Chains. In this special case, the human and lamprey γ chains were labeled near the carboxy termini with dansylcadaverine (Table I) and then subjected to the five selective fragmentation techniques. The intact γ chains (4–5 μg) and the fragmented γ chains (10–60 μg) were chromatographed on 25% polyacrylamide/$NaDodSO_4$ gels (Figure 6). In this situation, trypsin cleaved at both arginine and lysine, because the lysines were not modified during the labeling procedure.

The need for carboxy-terminal labeling arose because the lamprey chain has a blocked amino terminus. Carboxy-ter-

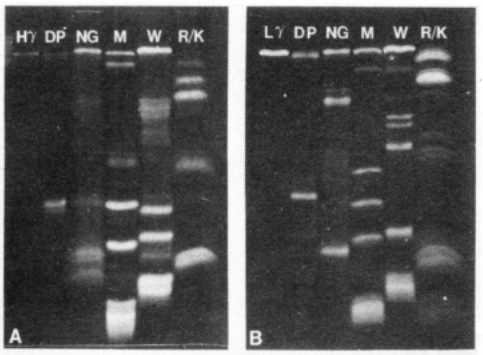


FIGURE 6: Sequencing gels of dansyl-labeled human and lamprey γ chains. (A) Intact human γ chains (Hγ) (5 μg) and human γ chains (10–60 μg) that were subjected to selective cleavages. (B) Intact lamprey γ chains (Lγ) (4 μg) and selectively fragmented lamprey γ chains (10–60 μg). Selective cleavages were specific for aspartylprolines (DP), asparaginylglycines (NG), methionines (M), tryptophans (W), and finally both arginines and lysines (R/K). Because the chain was labeled at the carboxy terminus, the sequence reads down from the amino terminus to the carboxy terminus. Fluorescent fragmentation patterns were generated and analyzed as described in the text.

minal labeling meant that the amino-terminal fragments are at the top of the gel. Accordingly, we read these patterns *down* the gel. The sequence of the human γ chain (Figure 6A) we observed reading down the gel was R/K..M..R/K..R/K..(NG, W)..W..W..W..M..R/K..DP..NG..M..W..W..M..NG..R/K..NG..W..W..M..M. In the DP lane, there is one band which represents the single Asp–Pro bond found in the human γ chain. There are four bands in the NG lane which account for five of the six expected Asn–Gly bonds. One band represents two Asn-Gly sites. Six methionine bands are visible, one of which represents three closely spaced cleavage sites. Eight tryptophan bands correspond to the 10 tryptophan sites in the molecule; in 2 of the bands, there are 2 tryptophan sites per band (Figure 6A). In one of these, the tryptophans are known to be adjacent to one another (residues 334 and 335).

Similarly, the lamprey γ-chain (Figure 6B) sequence observed was R/K..R/K..R/K..M..W..R/K..R/K..NG..R/K..(NG, W)..W..W..R/K..W..R/K..R/K..M..DP..M..W..M..R/K..NG..R/K..R/K..W..W..R/K..M..M. As in the human γ chain, the lamprey γ chain also has a single Asp-Pro site. Although three Asn-Gly bands are visible on the gel, recent DNA sequencing data from this lab have indicated that there are actually four Asn-Gly sites but two of these are adjacent to one another, thus accounting for the three bands seen on the gel. Six methionine bands are visible; one band represents two methionine sites. Although colorimetric tryptophan analysis suggested that the lamprey γ chain had fewer tryptophans than the human γ chain (Doolittle et al., 1976), we now find that the lamprey and human γ chains each have 10 tryptophans.

A side by side comparison of the human and lamprey γ-chain fluorescent fragmentation patterns was made (Figure 7). Although the human and lamprey carboxy-terminal regions have very similar patterns, the lamprey γ chain and its fluorescent fragments have migrated slightly faster than their human counterparts. This was taken into account in the amino acid alignment of the human and lamprey γ chains (Figure 8). The DP pair of lanes (Figure 7) shows that the single Asp-Pro site found in the human and lamprey γ chains has been conserved (residues 298 and 299). There are six Asn-Gly sites in the human chain and four Asn-Gly sites in the lamprey chain; only two of the Asn-Gly sites are in common, however. In the M pair of lanes, human and lamprey methionine

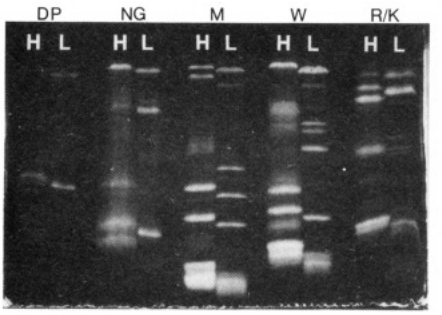


FIGURE 7: Comparison of human and lamprey γ-chain fluorescent fragmentation patterns. Dansyl-labeled human (H) and lamprey (L) γ chains digested by the same selective cleavage method were compared side by side in adjacent gel lanes. The five pairs of lanes from left to right contain aspartylproline (DP), asparaginylglycine (NG), methionine (M), tryptophan (W), and, lastly, arginine and lysine (R/K) cleavage products. See text for other details.

cleavage patterns are found, and in the W pair of lanes, tryptophan cleavage patterns are shown. Many of the methionines and tryptophans in the human and lamprey γ chains either are in the same position or are close to one another. Of 10 tryptophans, 9 are conserved between the lamprey and human. As in the case of the β chain, the human and lamprey γ chains are especially similar in the carboxy-terminal half of the molecule.

Discussion

As might be expected, the system described here still has some limitations, especially with regard to the separation of similarly sized peptides. For example, in the fluorescein-labeled human γ chain (Figure 1), the methionine-cleaved fluorescent peptides that are 78, 89, and 94 residues in length were not resolved and appear as a single strong band on the gel. It is possible that a longer gel might resolve these similarly sized peptides. Similarly, when the various types of cleaved residues occurred very close together, the gel system occasionally predicted an inverted sequence. The published sequence of residues 253–256 for the human γ chain is Trp-Asn-Gly-Arg, but the gel data (Figure 1) predicted the order to be Arg-Trp-Asn-Gly. In spite of these limitations, the gel system successfully predicted about 90% of all targeted residues in the three human fibrinogen chains (Figure 8).

The success of the $NaDodSO_4$ electrophoresis in giving the correct alignment is attributable to the fact that one is dealing with *nested* fragments. Thus, consider two end-labeled fragments that differ only by a few residues at their unlabeled end. Everything that contributes to the mobility of the two peptides is identical except for those few extra residues. It would be very unusual if the extra residues made the longer fragment move faster. Indeed, the limiting factor appears to be bandwidth, something that may be improved with radioactive labels and lower protein concentrations.

Sensitivity. Although we prepared relatively large amounts of each labeled protein for these exploratory studies (6–8 mg), it should be emphasized that each slab gel with five fragmentation lanes requires at most 300 μg of fluorescein-labeled protein. If the same labeling and fragmentation conditions described here are scaled down, it should be possible to take 1 mg of protein, run it through the labeling procedure, and have enough material for several gels. Indeed, as little as 0.03 nM fluorescein-labeled protein can easily be seen as a band on a gel. This corresponds to 2 μg of a 50 000-dalton protein. If on the average there are 50 bands per five cleavages, then 100 μg of the 50 000-dalton labeled protein is sufficient to perform all the cleavages. Dansyl-labeled materials need about

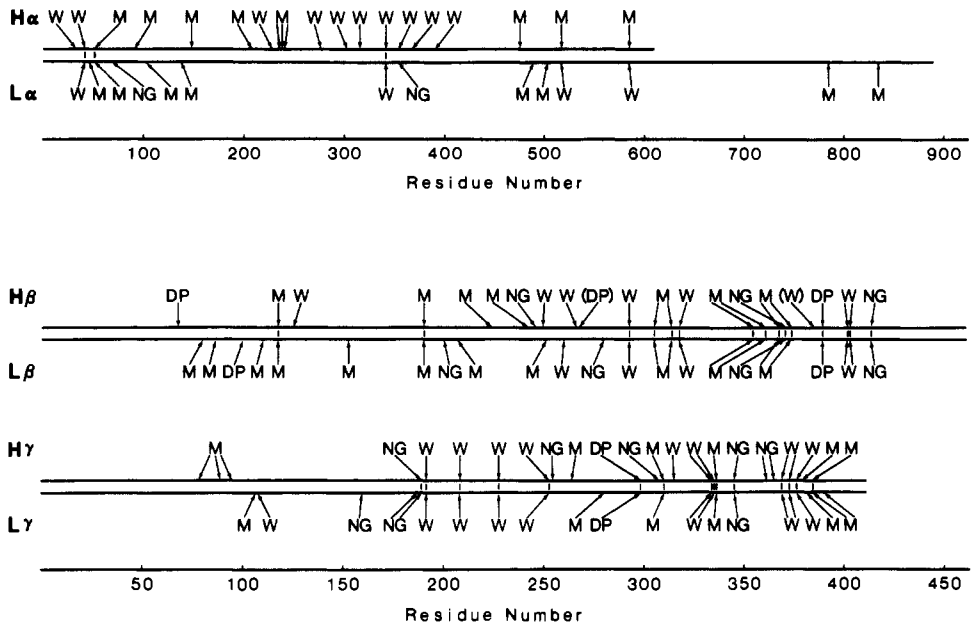


FIGURE 8: Schematic representation of human and lamprey fibrinogen α, β, and γ chains showing the locations of methionines (M), tryptophans (W), asparaginyl–glycine bonds (NG), and aspartyl–proline bonds (DP) that were predicted from the gel data. Each letter represents a band seen on the gel. Multiple arrows from a single letter indicate multiple cleavage sites seen as a single band on the gel. Letters in parentheses represent published cleavage sites not seen on the gel. In the case of the β chains, the bands could not be resolved past residue 414. The known fibrinogen sequence was used to establish the exact positions of the target amino acids on the diagram. Vertical lines between arrowheads indicate identical residues. Arginines (and lysines), which were also observed, have not been included in this figure.

3 times as much. It must be understood, of course, that sufficient protein must be derivatized so that a range of cleavage conditions can be explored.

Alternatively, the method can be applied to several proteins simultaneously in the following way. Several milligrams of a number of unpurified proteins is fluorescently labeled concurrently. They are then purified by separating them on $NaDodSO_4$ gels. Each part of the gel containing a desired fluorescent protein band is cut out and individually electroeluted from the gel slice, dialyzed, and lyophilized. Finally, any of these isolated proteins can then be analyzed by partial specific cleavage and subsequent $NaDodSO_4$ gel electrophoresis. In this manner, it should be possible to conduct this method on several hundred micrograms of each of these unpurified proteins. In fact, this approach was applied to the dansylated human γ chain in order to separate it from its labeled dimer. The same strategy can be applied to the case where a small amount of the protein of interest and a carrier protein are labeled simultaneously.

It is also certain that the sensitivity can be increased by the use of radioactive labeling agents. Although we tried both *N*-succinimidyl 3-(4-hydroxyphenyl)propionate (Bolton & Hunter, 1973) and methyl *p*-hydroxybenzimidate hydrochloride (Wood et al., 1975) as means of labeling the amino terminus with ^{125}I, in the end we settled on fluorescein isothiocyanate because of its ease of handling and long lifetime and because the progress of each electrophoresis could be followed in situ. Ultimately, a radioactive label may have to be used when this method is applied to microgram quantities of protein.

Other Degradative Blocking Groups. In some of our studies, we blocked the amino groups with several other sequencing reagents including methyl isothiocyanate, phenyl isothiocyanate, and 4-sulfophenyl isothiocyanate. None of these worked as well as TATG, because the protein either became less soluble or became harder to manipulate in other ways.

Other Cleavage Methods. Currently, several other fragmentation techniques are being tested for use with this method. In this regard, *Staphylococcus aureus* V8 protease (Houmard & Drapeau, 1972) in the presence of $NaDodSO_4$ (Cleveland et al., 1977) provides a way to preferentially cleave glutamic acid residues and to a lesser extent aspartic acid. We have also used the reagent 2-nitro-5-(thiocyano)benzoic acid, which cleaves at cysteines (Jacobson et al., 1973; Degani & Patchornik, 1974). In the case of fibrinogen, however, we had to use separate preparations in which the individual chains were isolated after sulfitolysis, since a free sulfhydryl is required for the procedure. In general, the results are not yet comparable to those fragmentations obtained with reduced and alkylated chains. Still, we have found that chemical cleavages react with more target amino acids and give a better distribution of bands on polyacrylamide/$NaDodSO_4$ gels than do enzymatic cleavages which preferentially cleave at only a few target amino acids, and we expect to pursue this cleavage route further.

The fragmentation strategy of the method can be applied to a protein labeled at either its amino terminus or its carboxy terminus. It may be that in some cases like the human γ chain, both procedures can be invoked. Because of the nature of $NaDodSO_4$/polyacrylamide gels, more extensive information can be obtained about the carboxy-terminal portion of the protein if it has a carboxy-terminal label. The inverse is true for the amino portion of the protein, and if used in conjunction, the two methods may yield a perfect arrangement.

Amino Acid Alignments. The results presented in this paper show that this method can provide data well beyond the mere ordering of CNBr or other fragments; indeed, it gives an arrangement of the selectively cleaved amino acids. It can be used to evaluate the evolutionary relationship of two proteins, as we have shown for the lamprey and human fibrinogens (Figure 8). In this regard, the lamprey γ-chain amino acid alignment results (Figure 8) are in excellent agreement with the known sequence established so far (Strong et al., 1985).

Fibrinogen Evolution. We have maintained a long-time interest in the evolution of vertebrate fibrinogen and have been characterizing lamprey fibrinogen in order to compare it with

its mammalian counterparts. Our studies here confirm that the β and γ chains of lamprey are similar to mammalian types, especially in the case of the carboxy-terminal halves. The α chains are more variable from species to species (Doolittle, 1973), and the information here is more interesting. Although very little of the lamprey α-chain sequence has been established by conventional procedures (Cottrell & Doolittle, 1976), it is known to have a very unusual amino acid composition that is quite different from its human counterpart (Doolittle et al., 1976). Although the positions of methionines and tryptophans are not very well conserved, it may be that an extra insertion in the longer lamprey α chain throws the alignment out of register. The amino acid alignment method confirms that the lamprey α chain has a molecular weight of about 100000 (Doolittle, 1973). Murtaugh et al. (1974) suggested that the larger lamprey α chains observed on gels might actually be the result of intramolecular cross-linking, perhaps by a γ-glutamyl-ϵ-lysine bond. Our results here show this not to be true. Upon careful analysis of the lamprey α-chain amino acid alignment (Figure 8) and the selective amino acid cleavage patterns (Figure 5), the possible existence of any molecular weight 100000 cross-linked α-chain dimer is excluded. In fact, the data show that the lamprey α chain is a single polypeptide of molecular weight 100000 consisting of approximately 900 amino acids and that it exhibits continuous selective cleavage positions along its entire length. The lamprey α chain is approximately 50% larger than its human counterpart.

In summary, we have developed a procedure for determining the arrangement of several key amino acids in a polypeptide chain. The method is quick, simple, reproducible, and inexpensive, and it yields a large amount of valuable information. As more selective cleavages are undertaken or become available, more primary protein structure information ought to be obtainable by this method. The procedure has usefulness in assessing the homology between two or more proteins, and it can be used to ensure that DNA-inferred sequences are in the proper frame and otherwise accurate. It also provides important information for the undertaking of an amino acid sequence by more traditional routes.

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Registry No. TATG, 17930-82-4; FITC, 27072-45-3; ethyl acetimidate, 1000-84-6; dansylcadaverine, 10121-91-2.

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