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Landmark mapping: A general method for localizing cysteine residues within a protein

(cysteine cleavage/peptide mapping/functional domains)

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ABSTRACT **We describe a general method to locate the positions of cysteine residues relative to the amino terminus of a protein, using a modified chemical cleavage of the polypeptide backbone at cysteine. The cleavage reaction introduces the carbon atom of ^{14}CN into the carboxyl-terminal fragment produced at each cleavage of the polypeptide chain. Peptides containing the amino terminus of the intact protein are not labeled; all other peptides are labeled at their amino termini. Partial cleavage of a protein followed by gel electrophoresis and autoradiography identifies a ladder of unlabeled peptides that maps positions of the cysteine residues relative to the protein amino terminus. To map individual proteins present in a complex mixture, the polypeptides are cyanolated in solution with ^{14}CN, and the modified proteins are separated by discontinuous SDS/PAGE. The gel is stained, and the desired protein is excised, cleaved at cysteine within the gel slice, and mapped in the second dimension by gel electrophoresis. These techniques are demonstrated with proteins of known sequence containing from zero to five cysteine residues. The cysteine "landmark map" should be particularly useful in locating protein modifications, in questions of protein similarity, and in mapping functional domains. A strategy is also presented for locating other residues in the polypeptide, for which specific cleavage methods exist.**

Protein mapping is a powerful biochemical tool frequently used to localize functional domains and modifications of proteins, to compare two or more similar polypeptides, and to aid protein sequencing. Mapping generally involves the specific cleavage of a protein into discrete peptides, the resolution of these peptides, and their localization within the protein sequence. Many specific methods for both cleavage of a protein and resolution of the resulting peptides exist. At present, at least three general methods locate some or all partial cleavage products of a protein within its primary structure. (*i*) The first method uses a series of reactions to label the protein at its amino terminus before cleavage and so is restricted to those proteins with unblocked amino termini (1). (*ii*) The second method employs a two-dimensional mapping technique to identify products of a partial CNBr cleavage of the protein at methionine residues (2). The complexity and resolution requirements of this technique limit its practical use to those proteins with few and simply distributed methionine residues, a relatively common amino acid. (*iii*) The third method compares by HPLC the partial cleavage products of the intact protein with a similar cleavage of a limited carboxypeptidase-digestion product of the protein (3); this requires extensive digestion of the protein by carboxypeptidase in the absence of any endopeptidase activity, which has generally proven difficult.

We used a modified cysteine cleavage reaction (4, 5) to generate an ordered map of the protein's cysteine residues, the most rare amino acid. Peptides thus identified can then be used to map cleavage sites other than cysteine within the protein and to identify partial cleavage products of the protein obtained from these sites. The entire procedure is simple and rapid, can be done with submicrogram amounts of material isolated by SDS/PAGE, and does not require an unblocked amino terminus.

METHODS

Chemicals and Proteins. Reagents of the highest purity commercially available were used. $K^{14}CN$ (50 mCi/mmol; 1 Ci = 37 GBq) was purchased from Amersham. Calmodulin from bovine brain and rabbit skeletal muscle actin were purified as described (6, 7); rabbit skeletal muscle creatine kinase was purchased from Sigma. The β subunit of spinach chloroplast coupling factor 1 ($CF_1\beta$) was the gift of M. Richter and R. McCarty (Cornell University).

Cysteine Cleavage in Solution and Primary Mapping. Pure protein was incubated in 100 mM Tris·HCl, pH 8.0/8 M urea/5 mM dithiothreitol at room temperature for 20 min to reduce cystine. This solution was made 15 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), pH 8.0, incubated for 15 min, dialyzed against 1000 vol of 50% (vol/vol) acetic acid at room temperature, and lyophilized to dryness. Protein solubility in 50% acetic acid may be a problem and so care must be taken to resuspend any protein precipitated within the dialysis bag. The lyophilized 2-nitro-5-thiobenzoic acid–cysteine-modified protein samples can be stored at −20°C for months with no discernible effect on cyanolation or cleavage.

To cyanolate and cleave the 2-nitro-5-thiobenzoic acid–cysteine-modified protein, the lyophilized protein was dissolved in cleavage buffer (100 mM sodium borate/500 mM glycylglycine/8 M urea, pH 9.0), made 1 mM $K^{14}CN$ (50 mCi/mmol) from a 100 mM aqueous $K^{14}CN$ stock solution, and incubated at 40°C for the indicated times. The reaction mixture was then made 100 mM dithiothreitol and incubated at 40°C for 15 min to remove any remaining ^{14}CN moieties from uncleaved cyanocysteine residues, effectively stopping the cleavage reaction. Peptide products of the cleavage reaction were resolved by SDS/PAGE (8, 9) and visualized by Coomassie blue R-250 (9) or silver staining (10). All stained gels were photographed after drying on a transparent, colorless membrane (Bio-Rad). To detect ^{14}C-labeled iminothiazolidine-4-carboxylyl ([^{14}C]ITC) peptides, Coomassie blue-stained gels were autoradiographed, and silver stained gels were fluorographed (11) at −70°C on Kodak XAR-5 film. Silver staining greatly decreases the sensitivity of ^{14}C detection by fluorography; to avoid this, duplicate samples can be run (one stained and the other fluorographed). Alternatively,

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Abbreviations: ITC, iminothiazolidine-4-carboxylyl; TIC, Tricine/imidazole chloride; $CF_1\beta$, the β subunit of chloroplast coupling factor 1.

the sample may be stained, photographed, destained (10), washed with water, and then fluorographed. In this case, however, a photograph of the wet gel is compared with the fluorograph of the dried gel, and nonuniform shrinkage of gradient gels during the drying process should be expected. When protein is detected by silver staining, gloves should be worn during preparation of all solutions and cleavage procedures to minimize keratin contamination.

Aqueous cyanide solutions are unstable. An initially colorless transparent solution turns yellow and eventually forms a brown precipitate upon storage at 4°C. We stored solutions at −70°C for several months with no apparent discoloration or adverse effect on cyanolation.

Cysteine Cleavage in a Gel Slice and Primary Mapping. The mixture of polypeptides in 100 mM imidazole hydrochloride, pH 7.2/1 mM dithiothreitol/2% SDS was incubated at 100°C for 1 min to denature the proteins and reduce any cystines. It was then made 5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (from a 20 mM stock at pH 7.2) and incubated a further 15 min at room temperature to form the 2-nitro-5-thiobenzoic acid–cysteine-modified peptides. The solution was then made 10 mM $K^{14}CN$ (50 mCi/mmol) from a 100 mM $K^{14}CN$ aqueous stock solution and incubated at room temperature for 15 min. The cyanolated proteins in the reaction mixture were resolved on either the continuous SDS/phosphate gel system (12) or the discontinuous Tricine/imidazole chloride (TIC) gel system (see below). When the SDS/phosphate gel system was used, the protein solution was made 10 mM sodium phosphate, pH 7.3/10% glycerol/0.02% bromophenol blue before running the gel. This gel system prepared as described (12) and prerun for at least 400 V·hr reduces cross-linking artifacts seen with some proteins. After replacing the reservoir buffer, the sample was loaded. The resolved cyanocysteine-modified proteins were visualized with Coomassie blue R-250. Absolute ethanol was used in place of reagent-grade methanol in both staining and destaining solutions to reduce protein crosslinking. The band containing the cyanocysteine-modified protein of interest was then excised, placed in a 1.5-ml Microfuge tube, and washed twice with distilled water for 10 min at room temperature with constant shaking to remove the destain solution. After equilibration with water, the stained cyanocysteine-modified protein bands can be stored for months at −70°C before cleavage.

For cleavage, the gel slice was placed in a 1.5-ml Microfuge tube and equilibrated three times for 10 min with cleavage buffer G (2 M glycylglycine·NaOH/100 mM sodium borate/8 M urea, pH 9.0) at room temperature with constant shaking. The buffer was then removed, and the gel slice was incubated at 40°C for 1–4 hr. The gel slice was then equilibrated three times for 15 min with sample buffer (8) with constant shaking at room temperature and loaded directly onto the resolving gel (8, 9). Good physical contact should be maintained between the gel slice and the top of the second-dimension gel to avoid loss of resolution or possible generation of artifactual bands. Once in place the gel slice is overlaid with a 1% agarose solution of the reservoir buffer. The resolved peptide products of the cleavage reaction are silver stained and fluorographed. Gloves should be worn during preparation of all solutions and throughout the cleavage procedure.

TIC Discontinuous Gel System. The standard 37:1 acrylamide/bisacrylamide ratio was used in all gels (8). The resolving gel was 750 mM imidazole hydrochloride/0.1% SDS/0.05% (vol/vol) *N,N,N′,N′*-tetramethylethylenediamine/0.05% wt/vol ammonium persulfate, pH 7.2, and the appropriate acrylamide concentration. The stacking gel was 135 mM imidazole·HCl/0.1% SDS/3% acrylamide/0.1% *N,N,N′,N′*-tetramethylethylenediamine/0.1% (wt/vol) ammonium persulfate, pH 5.4. Both stacking and resolving gel solutions were sterile-filtered before the addition of polymerization catalysts. Reservoir buffer was 190 mM Tricine/65 mM imidazole/0.1% SDS, pH 6.75. The samples were made 135 mM imidazole hydrochloride/10% glycerol/1% SDS/0.01% bromophenol blue, pH 6.0, and loaded on the gel. Use of a "shark-toothed" comb to form sample wells prevents the minor band distortion sometimes seen.

RESULTS AND DISCUSSION

Cysteine Mapping Strategy. The basic mapping strategy is shown schematically in Fig. 1. The hypothetical protein has two cysteine residues, which divide its sequence into three regions designated 1, 2, and 3. The first two steps lead to specific cyanolation and then partial cleavage of the protein at its cysteine residues with $K^{14}CN$. Cleavage at the amino peptide bonds of the modified cysteine residues occurs at elevated pH in an intramolecular, hydroxide ion-catalyzed, ring-closure reaction involving the cyanide carbon atom (4). Each cleavage produces two peptides, the amino- and carboxyl-terminal products. The carboxyl terminus of the amino-terminal product is unmodified, whereas the amino terminus of the carboxyl-terminal product is composed of an

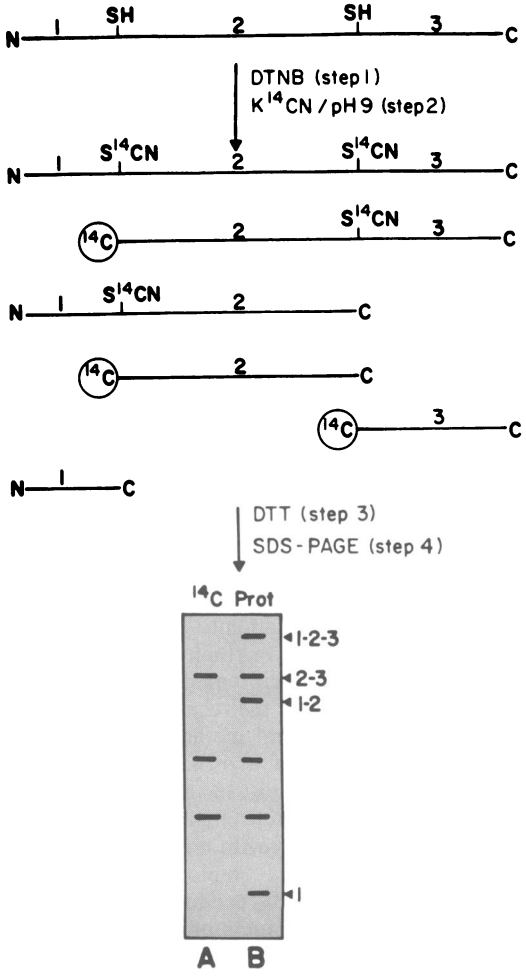

FIG. 1. Basic cysteine mapping strategy. A hypothetical protein containing two cysteine residues with the positions indicated by -SH is subjected to 5,5′-dithiobis(2-nitrobenzoic acid) activation (step 1) followed by $K^{14}CN$ cyanolation and cleavage (step 2). The resulting six partial cleavage products are shown: $S^{14}CN$ indicates the presence of cyanocysteine residues, and (14C) indicates the presence of an [14C]ITC group. The cyanocysteine residues are reconverted to cysteine by treatment with dithiothreitol (step 3). The resulting products are then resolved by SDS/PAGE (step 4), stained for protein (lane B), and autoradiographed (lane A). The identity of peptides for which this analysis provides unequivocal assignments are shown at right.

ITC moiety containing the ^{14}C atom. The ^{14}C atom has been introduced into the protein and its cleavage products in two forms: the CN moiety of cyanocysteine residues and the ITC group. The cyanide group on the cyanocysteine residues can be quantitatively removed by treatment with excess dithiothreitol, reforming cysteine and thereby stopping the cleavage reaction. By contrast, the ITC group is stable under these conditions (4, 5). After reduction, the family of partial cysteine cleavage products containing the amino terminus of the intact protein are *not* labeled, whereas *all* other peptides are *stoichiometrically* end-labeled on their amino terminus with [^{14}C]ITC (step 3). The reaction products are then resolved by gel electrophoresis, stained for protein, and autoradiographed (step 4). Comparison of the protein stain and autoradiograph identifies the ladder of unlabeled peptides that contains the amino terminus of the intact protein (peptides 1, 1–2, and 1–2–3). The molecular mass of each unlabeled polypeptide determines the position of the cysteine residue in the intact protein that was cleaved to produce it. The only other partial-cleavage product that can be immediately identified is the labeled peptide of highest molecular mass (2–3 in the example), which extends from first cysteine to carboxyl terminus.

Given a protein with P cysteine residues there are $(P + 1)(P + 2)/2$ possible partial cleavage products, including the intact protein. $P + 1$ of these peptides contain the amino terminus of the intact protein and are unlabeled. Ideally, both the total number of cleavage products and the number of unlabeled products indicate the number and position of all cysteine residues in the protein. But in practice, this derived number depends, among other things, upon relative peptide abundance and SDS/PAGE resolution and, therefore, should be taken as a minimum. Because cleavage at different cysteine residues in a protein can occur at different rates, it is prudent to perform a time course of the cleavage reaction to ensure that all resolvable amino-terminal fragments have been obtained. Even without a resolved amino-terminal fragment, the redundancy of information within the cysteine map can sometimes be used to deduce the existence and location of a cysteine residue, as demonstrated below.

Cysteine Mapping of Pure Proteins in Solution. To test our mapping strategy, proteins of known sequence with zero to five cysteine residues were selected. Attempts to employ existing cysteine cleavage protocols (4, 5) failed, as almost all peptide products were labeled to similar specific activities in a dithiothreitol-irreversible manner. We found that the presence of 500 mM glycylglycine during cyanolation and cleavage at pH 9.0 reduces this background labeling, yielding unambiguous cysteine maps. This result is illustrated in Fig. 2 for rabbit skeletal muscle creatine kinase, which has four cysteine residues, as indicated (13). A complete set of reduced partial cleavage products is expected to contain 15 different peptides, 10 of which should be labeled on their amino terminus with [^{14}C]ITC, and 5, containing the amino terminus of the intact protein, should not be labeled. Fig. 2, lanes A and B, compares the ^{14}C-labeling pattern obtained from a partial-cysteine cleavage with the standard two-step method (lane A) and our modified version of it (lane B). Reduction in background labeling is evident. A comparison of the ^{14}C-labeling pattern (lane C) with the protein stain (lane D) obtained using the modified reaction clearly identified the five peptides containing the amino terminus of creatine kinase and maps the cysteine residues of the protein. Rabbit skeletal muscle creatine kinase runs anomalously slowly on SDS gels, having an apparent mass 5 kDa greater than the value determined from its sequence. This fact is reflected in the derived map and the region of the protein giving rise to this anomaly is located between the third and fourth cysteine residue (region 4) (Fig. 2).

Actin is a 43-kDa polypeptide containing 375 amino acids (14), five of which are cysteines, as shown in Fig. 2. Their distribution presents a significant challenge to our mapping strategy. Thirteen peptides can be identified by SDS/PAGE of the partial cysteine cleavage products of actin, suggesting a minimum of four resolvable cleavage sites in the intact protein. Four cleavage sites produce a maximum of 15 peptides, five of which should not be labeled. Only four

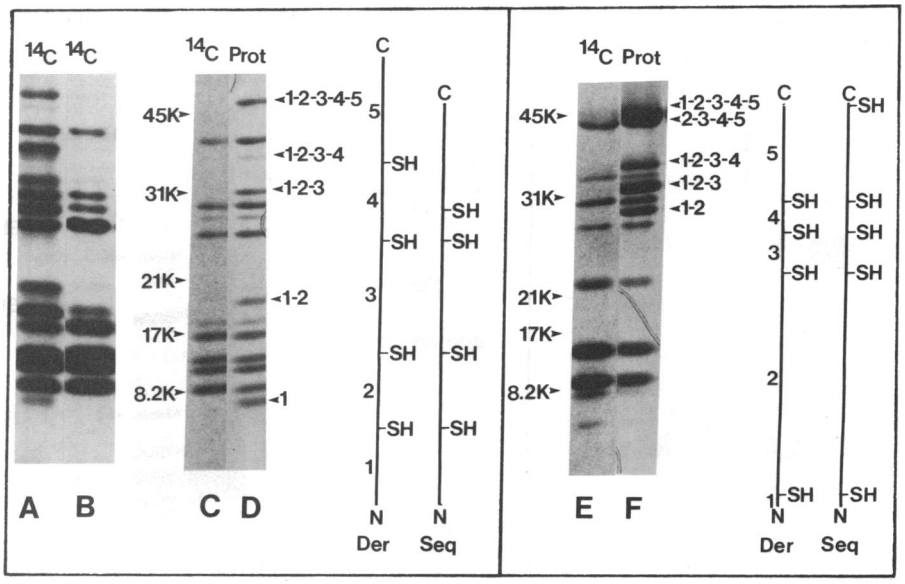

FIG. 2. Cysteine mapping of creatine kinase (lanes A–D) and actin (lanes E–F). Creatine kinase was partially cleaved at cysteine residues with K^{14}CN by either an earlier protocol (lane A) or as described here (lanes B–D). Lanes A and B compare the products from 16 μg of protein from each reaction, resolved on a 10–25% gradient gel (10), and autoradiographed for 100 hr. Lanes C and D compare the autoradiograph and protein stain, respectively, of 4 μg of creatine kinase resolved and autoradiographed as above. Ten micrograms of actin was partially cleaved, resolved on a 10–25% gel, stained for protein (lane F), and autoradiographed for 100 hr (lane E). Molecular mass standards run on the same gels are indicated at left in kDa (K). Also shown are the identity of amino-terminal peptide products and the map derived (Der) from analyzing the molecular masses of these peptides and the map determined from the sequence (Seq). For actin, the largest labeled peptide (2–3–4–5) is also indicated.

unlabeled peptides, including the intact protein, are evident, indicating either a lost or unresolved amino-terminal reaction product. The highest molecular mass-labeled peptide contains all the amino acids from the first cysteine residue to the carboxyl terminus. From the difference in molecular mass between the intact protein and this peptide we can estimate the molecular mass of the peptide from the amino terminus up to the first cysteine, peptide 1. For actin this difference is ≈1 kDa, clearly less than 23 kDa, the size of the smallest identified unlabeled peptide. The missing unlabeled peptide can therefore be identified as peptide 1, and the corresponding cysteine can be placed ≈10 amino acids from the amino terminus. The smallest unlabeled peptide on this gel is thus composed of the sequence from the amino terminus to the amino acid preceding the second cysteine residue. Positions of the four cysteine residues in the deduced map agree with the known sequence. Actin, however, has five cysteine residues, only four of which are mapped. The unmapped cysteine is the penultimate amino acid of the protein, and no cleavage products from this site can be resolved by SDS/PAGE.

Cysteine Mapping of Proteins Isolated by SDS Gel Electrophoresis. The simplicity and resolution of SDS/PAGE have made it the method of choice for separating complex mixtures of polypeptides. The ability to map proteins isolated by SDS/PAGE within gel slices greatly extends the potential applications of this analysis. Initially we attempted both to cyanolate and cleave polypeptides isolated within a gel slice. Inefficient cyanolation and high background labeling of the protein and its cleavage products made this approach impractical.

To avoid these problems, we denatured all components of a protein mixture and subjected them to the ^{14}C cyanolation reaction in solution at neutral pH before electrophoresis. Nearly quantitative cyanolation of cysteine residues occurs under these conditions. The cyanocysteine-modified proteins formed are not stable under the alkaline conditions used in Laemmli gels (8) but are stable at neutral and acidic pH and can be resolved by SDS/PAGE under those conditions. We developed a discontinuous TIC SDS/PAGE system to improve the resolution available in the pH range. In this system, proteins are stacked at pH 5.4, resolved at pH 7.2, and migrate with the characteristic linear relationship between relative mobility and the logarithm of molecular mass seen on all SDS/PAGE.

A mixture of creatine kinase (containing four cysteine residues), $CF_1\beta$ (containing a single cysteine residue) (15), and calmodulin (which lacks cysteine) (16) was cyanolated with ^{14}CN at neutral pH, and the reaction products were resolved on a 15% TIC gel. The protein stain and autoradiograph of the gel are shown in Fig. 3, lanes A and B. As expected, both creatine kinase and $CF_1\beta$ were labeled, and calmodulin was not.

Gel slices containing the cyanolated proteins were excised, and the proteins were cleaved *in situ* by incubation at pH 9. The peptide products of the cleavage reaction were then resolved by SDS/PAGE in the second dimension and visualized by silver stain (Fig. 3, lanes D, F, and H). The labeled products were identified by fluorography of an unstained duplicate sample (Fig. 3, lanes C, E, and G). The overall efficiency and background labeling of the cleavage reaction performed within a gel slice is comparable to the solution reaction, yielding clear and unambiguous cysteine maps. Proteins lacking cysteine, as exemplified by calmodulin, are neither labeled nor cleaved (lanes C and D). $CF_1\beta$ was cleaved to give one labeled product (lanes E and F). The expected unlabeled amino-terminal peptide was lost from the gel during staining. The map of creatine kinase was comparable to the map obtained from the solution reaction (lanes G and H). The maps obtained for the test proteins accurately predicted the positions of their cysteine residues.

Uses and Limitations of "Landmark Mapping." The identified peptide products of a partial cysteine cleavage reaction establish a series of landmarks within the primary structure of the protein. These peptides can be used to determine the positions in this map of other residues for which specific cleavage methods exist. Those methods that can cleave a protein and its cysteine cleavage products within a gel slice should prove particularly useful. Preliminary results suggest that all sites cleaved in the intact protein by CNBr at methionine (2), *N*-chlorosuccinimide at tryptophan (17), acid hydrolysis at aspartic acid–proline (18), and hydroxylamine

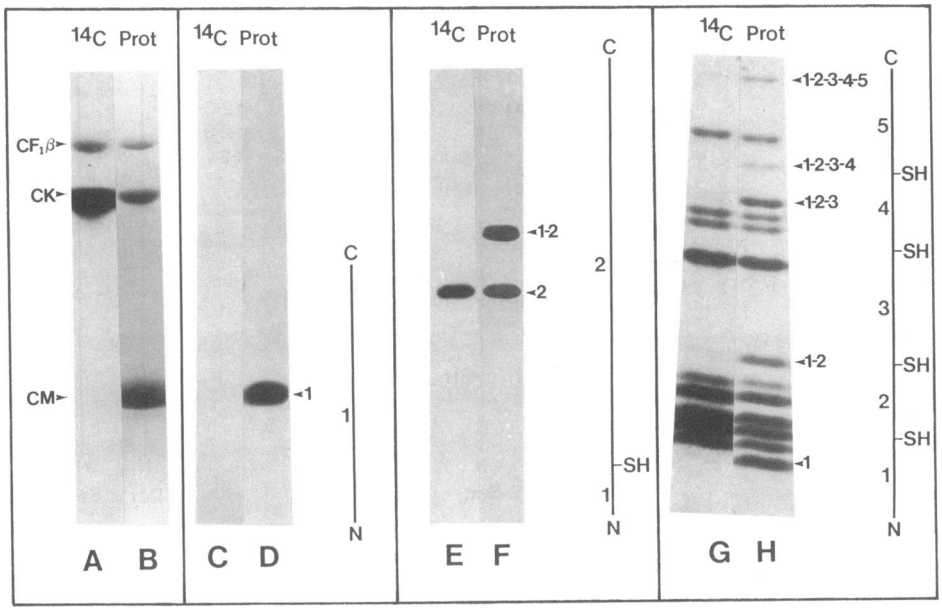

FIG. 3. Cysteine mapping of proteins from a gel slice. A solution containing $CF_1\beta$, creatine kinase, and calmodulin was cyanolated with $K^{14}CN$, resolved on a 15% TIC gel, stained (lane B), and autoradiographed (lane A). Duplicate samples containing 0.5 μg each of these proteins were cyanolated with $K^{14}CN$, isolated in a TIC gel slice, cleaved, and mapped at cysteine as described. One sample of each protein was silver stained (lanes D, F, and H), and the other was fluorographed (lanes C, E, and G), as indicated. Lanes C and D are calmodulin, lanes E and F are $CF_1\beta$, and lanes G and H are creatine kinase. The derived maps are shown to the right of the gel lanes.

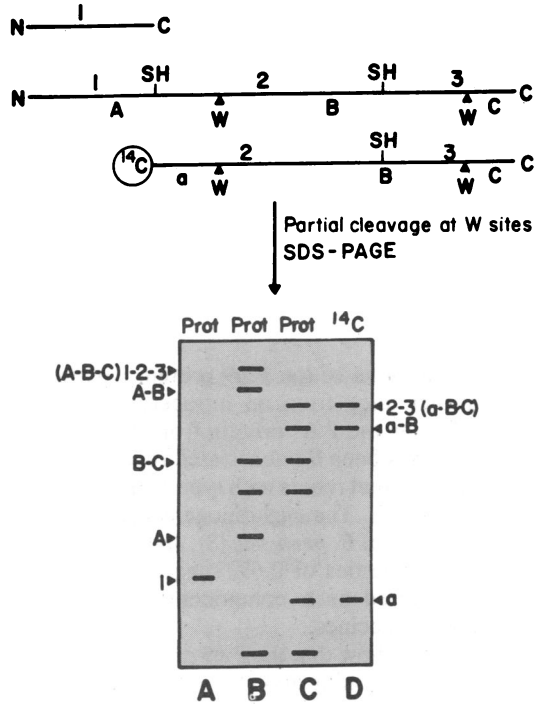

FIG. 4. Approach for localizing specific cleavage sites within the cysteine landmark map. This figure shows the basic strategy, as applied to a hypothetical protein with two cysteine residues (SH), which divide the protein into regions 1, 2, and 3, and two tryptophan residues (W), positioned as shown; the latter divides the protein into three other regions designated A, B, and C. The intact protein and its ^{14}CN–cysteine cleavage products, fragment 1 and fragment 2–3 isolated by SDS/PAGE, are subjected to a cleavage reaction specific for tryptophan residues within the gel slice. The products resulting from each reaction are resolved by SDS/PAGE in the second dimension. Lanes A, B, and C depict the cleavage products obtained from fragment 1, the intact protein 1–2–3, and fragment 2–3, respectively. Lane D is the predicted fluorograph of lane C. Fragments that can be immediately identified are indicated.

at asparagine–glycine (19) are cleaved in SDS/PAGE-isolated cysteine fragments. As an example, let us suppose we wish to locate the tryptophan residues within the hypothetical protein shown in Fig. 4. One useful approach, outlined in Fig. 4, is to compare the partial cleavage products of the intact protein with two peptides that span the complete sequence.

The intact protein, its largest labeled cysteine fragment (composed of the amino acids from the first cysteine to the carboxyl terminus), and the smallest cysteine peptide containing the amino terminus are excised from the gel, and each is subjected to partial chemical cleavage at tryptophan. The products are resolved with SDS/PAGE followed by silver staining and fluorography. Compare the products generated from the intact protein and both cysteine fragments (lanes A, B, and C). The peptides unique to the cleavage reaction of the largest labeled fragment share a common amino terminus, the [^{14}C]ITC group of the first cysteine residue, and terminate at each of the tryptophan residues in this cysteine fragment. The molecular mass of these peptides maps the distance between the first cysteine residue and their corresponding tryptophan. The same peptides can be identified by fluorography (lane D). The amino-terminal cysteine peptide chosen cannot be cleaved at tryptophan (lane A), indicating that no tryptophan residues lie between the amino terminus of the protein and its first cysteine. Thus, the peptides unique to cleavage products of the intact protein have the same amino terminus as the intact protein and one of the protein's resolvable tryptophan cleavage sites on their carboxyl terminus. The molecular masses of these peptides map the corresponding tryptophan cleavage sites relative to the amino terminus. If there are tryptophan cleavage sites within the amino-terminal peptide, the molecular masses of its unique partial cleavage products map these sites relative to the carboxyl terminus of the fragment (and thus to the corresponding cysteine residue in the protein). A comparison of the partial cleavage products obtained from other identified cysteine fragments of the protein can be used to confirm the map; the information gained is redundant and can usually be used to resolve ambiguities in the map.

Landmark mapping offers a simple and rapid method of localizing many protein modifications, such as phosphorylation, glycosylation, and proteolysis sites, and should prove useful in protein sequencing. Regions of heterogeneity between isoforms and antibody epitopes can be easily mapped. The mapping of protein–protein, protein–lipid, or protein–nucleic acid crosslinks should be possible. We developed this technique to map protein functional domains for which the technique serves well. We have found cleavage of native proteins with ^{14}CN can, in some cases, simultaneously generate and map functional protein fragments. The ^{14}CN cleavage and mapping approach described here has been successfully applied to mapping functional domains in the F-actin and calmodulin binding protein caldesmon (20).

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