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A Technique for the Detection of Deleted Immunoglobulin Heavy Chains†

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ABSTRACT: A method, "carboxymethylcysteine diagonal map," has been developed to detect immunoglobulins with structural defects. It is based on the comparison of all cysteine-containing peptides present in the molecule with their normal counterparts. Immunoglobulin molecules have a high content of cysteine residues which are strategically spread along the chains and can be used as markers for different domains and interdomain regions. Since they occupy a distinct position on the map, the absence of one or more peptides defines rather accurately the nature of the defect.

Amino acid sequence studies of several γ heavy-chain disease (γHCD)[1] proteins (Frangione and Milstein, 1969; Franklin and Frangione, 1971; Cooper *et al.*, 1972) and the heavy (H) chain of a myeloma protein (Fett *et al.*, 1973) have shown internal deletions ranging in size from 15 to 240 residues. These results have indicated (1) that these smaller synthetic products resulted from abnormalities of gene expression rather than degradation of intact molecules and (2) that codons GAA–GAG specifying glutamic acid at position 216 (γ1 numbering) (Edelman *et al.*, 1969) have special significance since in three instances reinitiation of normal synthesis after an internal deletion started at position 216 (Frangione and Milstein, 1969; Franklin and Frangione, 1971; Cooper *et al.*, 1972) and in one case the gap commenced at the same position (Fett *et al.*, 1973). Since the interpretation of these data is not yet clear and since studies of such proteins promise to be of great value in developing theories to explain the genetic mechanisms involved in the control of immunoglobulin synthesis, a method has been developed to detect deleted immunoglobulins. It is based on the comparison of all cysteine-containing peptides in the molecule with those in its normal counterpart. The method used employs "carboxymethylcysteine diagonal maps" and differs from classical diagonal maps (Brown and Hartley, 1966) in that the proteins are subjected to mild thiol reduction and labeling with iodo[^{14}C]-acetic acid prior to the performance of the diagonal map. This results in the selective labeling of the labile interchain disulfide bridges.

Materials and Methods

As previously described, 5 mg of purified protein (Frangione *et al.*, 1969a) was dissolved in 0.5 ml of 0.27 M Tris-HCl buffer (pH 8.2) and mildly reduced with 0.005 M dithiothreitol at room temperature under N_2. After incubation for 60 min at 37°, reduction was terminated by the addition of iodo[^{14}C]-acetic acid (0.01 M, specific activity 0.7 Ci/mol) and incubated at room temperature for another 60 min. The solution was then dialyzed overnight in 5% formic acid and digested with pepsin (Worthington, twice crystallized), enzyme–substrate ratio 1:50 (w/w) for 14 hr at 37°, freeze-dried, dissolved in 0.2 M ammonium bicarbonate (pH 8.3), and digested with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin (Worthington), enzyme–substrate ratio 1:50 (w/w) for 6 hr at 37°. The digest was dried, dissolved in 0.1 ml of water, and applied as a 3-cm band on 3MM Whatman paper and subjected to high-voltage paper electrophoresis in solvent-cooled tanks (pyridine–acetic acid–water, 1:10:190, v/v, pH 3.5) for 1 hr, 60 V/cm. A mixture of aspartic acid, glutamic acid, ϵ-Dnp-lysine, and glycylalanine was applied at the sides of the paper as markers. Peptides containing *S*-carboxymethylcysteine were detected by autoradiography using Kodak Royal Blue Medical X-Ray film.

The strip from this ionogram (without the markers) was incubated for 2 hr at room temperature in a desiccator con-

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[1] Nomenclature of immunoglobulin and their chains follow the recommendation of the World Health Organization (*W.H.O. Bull. 33*, 721 (1965); *35*, 953 (1966); *38*, 151 (1968); *41*, 975 (1969)). Myeloma proteins are designated by the first three letters of the patient's name.

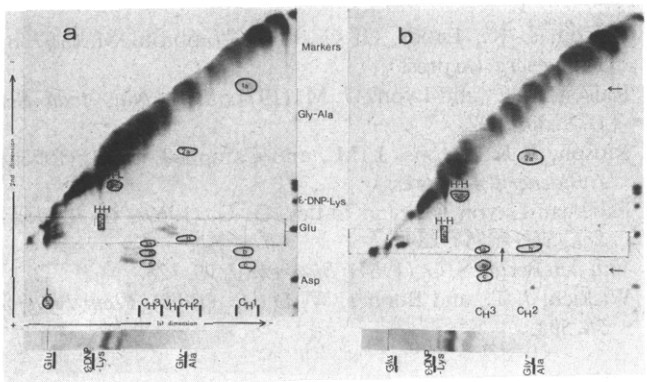

FIGURE 1: "Carboxymethylcystine diagonal electrophoretic maps" of partially reduced and labeled peptic–tryptic digests of (a) IgG k myeloma protein Car and (b) γl HCD protein Cra. Electrophoresis at pH 3.5 was carried out horizontally before oxidation and vertically after oxidation. At the botton autoradiographs after the first dimension. *S*-Carboxymethylcystine sulfone containing peptides lying off the diagonal are hatched. Major cysteic acid ninhydrin positive peptides lying off the diagonal and corresponding to constant H-chain domains (C_H1, C_H2, and C_H3) are circled. V_H: H-chain, variable domain. Group of peptides to the left of C_H3 belong to L chain: C_L + V_L domains. H–L: heavy–light: H–H: heavy–heavy; L: light. Hinge or interdomain: region containing the H–H disulfide bonds located between C_H1 and C_H2 domains. Arrows indicate absence of corresponding H chain domains.

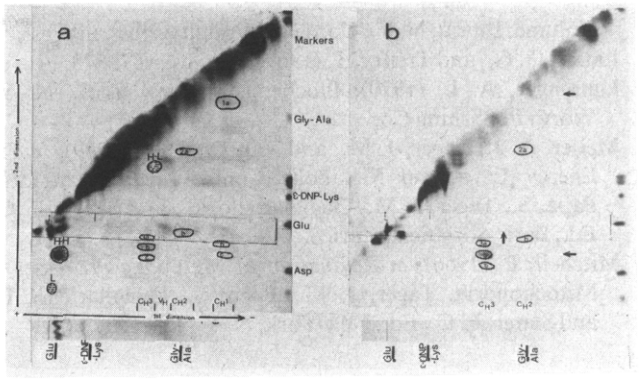

FIGURE 2: "Carboxymethylcystine diagonal electrophoretic maps" of partially reduced and labeled peptic–tryptic digest of (a) IgA4 k myeloma protein Wur and (b) γ4 HCD protein Hal. Same conditions as in Figure 1. Notice the complete lack of labeled peptides in protein Hal (absence of interchain bonds). Arrows indicate absence of C_H1 and V_H domain peptides. Cystine peptide corresponding to C_H1 in protein Wur is more anodic than the homologous one in protein Car (compare Figures 1a and 2a) due to a charged amino acid replacement: Asp for Asn (Tables I and II). Protein Wur shows cysteic acid peptides on the left of C_H3 and in the region between C_H1 and C_H2 which corresponds to the L chain (unpublished observations).

taining a freshly prepared mixture of 50 ml of 98% (v/v) formic acid and 2.5 ml of 30% (v/v) H_2O_2 in a Petri dish. (A convenient support for the strip is a spiral glass rod.) The oxidized strip was removed from the desiccator and left for 1 hr in a fume cupboard to allow evaporation of the formic and performic acid. It was then stitched with a sewing machine to another sheet of paper and subjected to high-voltage electrophoresis under the original conditions but at right angles to the original direction. Peptide maps were stained with cadmium–ninhydrin; this solution contained 14% (v/v) cadmium acetate solution (6 g of cadmium acetate in 30% (v/v) acetic acid) in 1% (w/v) ninhydrin in acetone. *S*-Carboxymethylcysteine sulfone peptides were detected by autoradiography. The map can be used also as a guide to the selective purification of each cysteic acid and *S*-carboxymethylcysteine sulfone peptide to permit further characterization. For this purpose, 50 mg of protein is required. The initial map is used then to identify selected sections containing cysteine peptide. Purification of peptides, amino acid analysis, Edman degradation and N-terminal analysis were the same as published (Wolfenstein *et al.*, 1971).

Results

To illustrate the method, the maps of four proteins, two belonging to the γ1 heavy-chain subclass and the other two to the γ4 subclass, are presented. Emphasis will be given to the heavy chains of immunoglobulin G, although this method can be used for light chains, different subclasses of γ chains, as well as α and μ HCD proteins (unpublished observations).

Figure 1 shows carboxymethylcysteine diagonal maps of (a) IgG1k myeloma protein Car and (b) γ1 HCD protein Cra. At the bottom of Figure 1 are the autoradiographs of partially reduced, labeled, and digested proteins obtained after first-dimensional electrophoresis at pH 3.5. Three radioactive bands (one very weak) containing *S*-carboxymethyl peptides are present in protein Car and two in protein Cra. Under the conditions used only interchain bridges were reduced and labeled (Frangione *et al.*, 1969a), and intrachain bonds remain intact. After performic acid oxidation and diagonal electrophoretic separation at pH 3.5, intrachain bonds have been converted into a pair of cysteic acid peptides, each with an extra negative charge. Consequently, they will lie off the diagonal but vertically below each other, thus allowing the identification of each pair of cysteic acid peptides which was originally disulfide bridged. In addition, *S*-carboxymethylcysteine peptides will also lie off the diagonal since the pK_a of the *S*-carboxymethyl group is lowered when it is oxidized to the sulfone form.

Table I shows the amino acid sequence, function and position of *S*-carboxymethylcysteine peptides and cysteic acid peptides obtained from proteins Car and Cra. It can be seen that the intrachain S–S bond present in each domain (Edelman *et al.*, 1969) or pseudosubunit (Frangione *et al.*, 1969b), especially those belonging to the constant region (C_H1, C_H2, C_H3), run in a specific position not only in the first but also in the second dimension. Thus the bridge corresponding to C_H1 runs ahead of the dipeptide glycylalanine in the first dimension and splits in two cysteic acid peptides after oxidation (1a and 1b) and a low yield peptide (1c) which is related to 1b (see Table I). The cystine peptide present in C_H2 domain runs with glycylalanine in the first dimension and gives two peptides after the second dimension (2a and b, Table I). The one corresponding to C_H3 domain runs between glycylalanine and ε-Dnp-lysine and after oxidation gives a number of cysteic acid peptides (3a, b, and c) running between glutamic and aspartic acid after the second dimension. The cystine bridge present in the V_H region of protein Car runs between C_H3 and C_H2 (see Figure 1). It was also present in the diagonal map of the separated H chain (not shown). A group of cysteic acid peptides near to those present in C_H3 belongs to the L chain (k type) and will be discussed elsewhere. As expected three *S*-carboxymethyl sulfone peptides were obtained. Two of these are present on the H chain (H–H and H–L) and one (L) comes from the carboxy end of the k chain (see Table I and Figure 1).

In the case of protein Cra only intrachain bonds coming from C_H2 and C_H3 domains were present as well as those

TABLE I: Sequence of Peptic–Tryptic Cysteine-Containing Peptides Obtained from IgG 1k Myeloma Protein Car and γ1 Heavy-Chain Disease (HCD) Protein Cra.[a]

Peptide		Sequence	Domain
		Protein CAR	
Cms	Cya	200	
	1a	Tyr-Ile-Cys-Asn-Val-Asn-His-Lys-Pro-Ser-Asn-Thr	
	1b	Leu-Gly-Cys-Leu	C_H1
		144	
	1c	Gly-Cys-Leu	
		321	
	2a	Cys-Lys	C_H2
	2b	Thr-Pro-Glu-Val-Thr-Cys-Val-Val	
		261	
		425	
	3a	Phe-Ser- Cys-Ser-Val-Met	
	3b	Leu-Thr-Cys-Leu	C_H3
		367	
	3c	Thr-Cys-Leu	
		226 229	
H–H		Thr-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu	Hinge (interdomain)
		220	
H–L		Ser-Cys-Asp-Lys	
		214	
L		Gly-Glu-Cys	C_L
		Protein CRA	
	2a	Cys-Lys	
	2b	Thr-Pro-Glu-Val-Thr-Cys-Val-Val	C_H2
	3a	Phe-Ser- Cys-Ser-Val-Met	
	3b	Leu-Thr-Cys-Leu	C_H3
	3c	Thr-Cys-Leu	
H–H		Thr-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu	Hinge
H–H[b]		Ser-Cys-Asp-Lys	

[a] Partial or complete sequence of some peptides have been published (Frangione *et al.*, 1969b; Franklin and Frangione, 1971). Numbering of protein Car was taken from proteins Eu (Edelman *et al.*, 1969); Cms: *S*-carboxymethylcysteine sulfone peptides; Cya: cysteic acid peptides. [b] Extra interheavy chain bond (see text).

involved in interchain binding (Table I and Figure 1). This molecule did not contain L chain (lack of peptide L) although it did have the H–L peptide which was shown to form a third interheavy chain bond (Franklin and Frangione, 1971). These findings are consistent with the detailed chemical studies, which proved an internal deletion of about 200 residues including the loops corresponding to V_H and C_H1 (Fd fragment) (Franklin and Frangione, 1971).

Figure 2 shows carboxymethylcysteine diagonal maps of (a) IgG 4k myeloma protein Wur and (b) γ4 HCD protein Hal. Autoradiographs disclosed three radioactive bands in protein Wur and none in protein Hal, thus showing that HCD protein Hal lacked interchain bridges. As a result it existed as a monomer and could not be typed chemically (Frangione *et al.*, 1969a). It could be placed in the γ4 subclass by studying the carboxyl end of the molecule (Frangione *et al.*, 1973), which is characteristic for the γ4 subclass (Prahl, 1967).

Performic acid diagonal electrophoresis maps disclosed only the presence of cysteine bridges present in C_H2 and C_H3. It was concluded that protein Hal lacks not only the loops present in V_H and C_H1 but also the regions responsible for interchain binding. Amino acid sequence studies (Frangione *et al.*, 1973) indicated that it contained a gap of about 240 residues, starting 10 residues from the N-terminal end, and that normal sequence apparently resumed beyond the hinge at a methionine residue (position 252).

The carboxymethylcysteine diagonal map of protein Wur (Figure 2) showed cysteic acid peptides coming from V_H, C_H1, C_H2, and C_H3 domains (those from L chain are not discussed here) and three *S*-carboxymethylcysteine sulfone peptides: H–H, H–L, and L. The H–L peptide was ninhydrin negative since proline is the N-terminal residue (Table II). The amino acid sequence of cysteine-containing peptides and function of proteins Wur and Hal, are shown in Table II. Note that the cystine peptide present in C_H1 of protein Wur is more anodic than the homologous one present in protein Car (Figure 1). This is due to an amino acid substitution in peptides 1a (Asp, instead of Asn, Tables I and II).

TABLE II: Sequence of Peptic–Tryptic Cysteine-Containing Peptides Obtained from IgG 4k Myeloma Protein Wur and ϕ4 Heavy-Chain Disease (HCD) Protein Hal.[a]

Peptide		Sequence	Domain
		Protein WUR	
Cms	Cya		
	1a	Tyr-*Thr*- Cys-Asn-Val-*Asp*-His-Lys-Pro-Ser-Asn-Thr	
	1b	Leu-Gly-Cys-Leu	C_H1
	1c	Gly-Cys-Leu	
	2a	Cys-Lys	
	2b	Thr-Pro-Glu-Val-Thr-Cys-Val-Val	C_H2
	3a	Phe-Ser- Cys-Ser-Val-Met	
	3b	Leu-Thr-Cys-Leu	C_H3
	3c	Thr-Cys-Leu	
H–H		Tyr-Gly-Pro-Pro-Cys-Pro-Pro-Cys-Pro-Ala-Ser-Glu-Phe	Hinge (interdomain)
H–L		Pro-Leu-Ala-Pro-Cys-Ser-Arg	C_H1
L		Gly-Glu-Cys	C_L
		Protein HAL	
		Cys-Lys	
	2a		
	2b	Thr-Pro-Glu-Val-Thr-Cys-Val-Val	C_H2
	3a	Phe-Ser- Cys-Ser-Val-Met	
	3b	Leu-Thr-Cys-Leu	C_H3
	3c	Thr-Cys-Leu	

[a] Partial sequence of H–H peptide from protein Wur was previously reported (Frangione *et al.*, 1969b). Italics indicates amino acid substitutions in homologous regions between γ4 and γ1 chains.

Discussion

During studies of the interchain disulfide bonds of myeloma proteins (Frangione *et al.*, 1969b, 1971; Frangione and Wolfenstein-Todel, 1972) it became apparent that the number and sequences around these bonds differed, and appeared to be unique to, and characteristic of, each class, subclass and type. Based on these differences, a simple method was developed for classifying all the currently known immunoglobulin chains (Frangione *et al.*, 1969a; B. Frangione and E. C. Franklin, 1972, unpublished results). Mild thiol reduction selectively breaks interchain bridges, which can then be labeled with iodo[^{14}C]acetic acid. High-voltage electrophoresis of digested proteins gave autoradiographic patterns which were characteristic for each type of heavy and light chain, and allowed classification by simple inspection, "chemical typing" (Frangione *et al.*, 1969a). However, this method was generally insufficient for studying the molecular defect present in HCD proteins and immunoglobulin molecules with other abnormalities. On the other hand, tryptic fingerprint analysis proved difficult to interpret because of the presence of variable peptides. An alternative approach was developed, taking advantage of the facts that a loop (or intrachain bridge) of about 60 residues is a fundamental pseudosubunit which is repeated six times in the heavy- and light-chain monomer of an IgG molecule and seven times in the heavy- and light-chain monomer of an IgM molecule. One such loop in the L chain, three in the γ chain and four in the μ chain (Frangione *et al.*, 1971) are essentially invariant within each class or subclass. Therefore, studies of interchain bridge peptides (chemical typing) combined with those of intrachain bridges can be extremely powerful tools in analyzing specific positions along the chains. Interchain sequences are recognized by the radioactive label technique and intrachain sequences by ninhydrin staining.

The first dimension of the map classifies the immunoglobulin chain type, class, or subclass and/or unusual structure (negative results in this step indicate the absence or sequences responsible for interchain binding and lack of dimerization). The second dimension allows studies of fragments along the chains since each domain contains a group of thiol-containing peptides with a distinct position on the map. Absence of one or more peptides defines rather accurately the nature of the defect. It is worth mentioning the possibility that reduction in 0.005 M dithiothreitol may not always distinguish so clearly between inter and intra bonds when examining abnormal proteins. However, no such difficulties were met with the protein examined here and others currently under study, and detailed chemical analysis, molecular weight determination and amino acid sequence (Franklin and Frangione, 1971; Frangione *et al.*, 1973) further corroborated the usefulness of the method. Studies of H chains possessing deletions in various segments of their chains and selected amino acid sequence on the variations found in the "hinge or interdomain" region (between C_H1 and C_H2) and in the so-called "switch" region (between V_H and C_H1) should aid in establishing the genetic

mechanisms which control the synthesis of immunoglobulin heavy chains.

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Syntheses and Properties of Flavine–Histidine Peptides†

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ABSTRACT: Flavinylhistidine peptides were synthesized by attaching the amino group of the histidine moiety through amide linkage to aliphatic carboxylic acid chains of varying length at the N-10 position of the isoalloxazine ring. The fluorescence properties of these models were measured in order to understand more fully flavine–histidine interactions, which can occur in certain flavoprotein systems. Measurements of fluorescence quenching indicate interaction between the isoalloxazine and nonprotonated imidazole ring of the histidine moiety. Very little interaction apparently occurs between flavine and protonated histidine. The effects seen when temperature and solvent are varied seem to indicate that there is greater ground-state association for the short-chain peptides, in which the histidine and flavine intramolecularly interact to form a dark, nonfluorescent complex. There is less complexing apparent in the case of the long-chain peptides and more fluorescence quenching due to increased collisions between the light-excited flavine and the histidine. Proton magnetic resonance measurements of these models in D_2O (37°) at various pD values suggest a weak intramolecular interaction between the imidazole ring of the histidine moiety and the benzenoid portion of the flavine. Such conformations are disrupted at higher temperatures.

Interactions of flavine coenzymes and aromatic molecules have been recognized for quite some time (Weber, 1950; Yagi *et al.*, 1959; Strittmatter, 1961; McCormick *et al.*, 1967). Recently, this has led to a study of the degree and type of molecular interactions of flavins with such aromatic amino acids as tryptophan, tyrosine, and phenylalanine. Flavine peptide models were synthesized by attaching the amino acid through amide linkage to the N-10 position of the isoalloxazine ring *via* aliphatic chains of varying length (Föry *et al.*, 1968). A fluorescence spectral investigation of the compounds showed that hydrophobic interactions probably cause complex formation in aqueous solution, while a dipole–dipole type of interaction is responsible for intramolecular association of some of the amino acid residues with the flavine portion in nonaqueous media (MacKenzie *et al.*, 1969). The spatial aspects of the intramolecular interactions of these flavine amino acids, both in aqueous and nonaqueous media, were examined by means of proton magnetic resonance spectroscopy (Föry *et al.*, 1970).

The FMN-dependent enzyme, pyridoxine (pyridoxamine) 5′-phosphate oxidase, is another case where molecular association occurs between the flavine coenzyme and an aromatic compound, in this case, a substrate. Synthesis of spectroscopic flavine-B_6 models (McCormick and Johnson, 1972) and examination of their fluorescence properties (McCormick, 1972) again suggested a significant interaction between such chromophores.

In the last few years, several flavine-containing enzymes have been shown to have a histidine residue at the active site.

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