Primary Structure of Human γ3 Immunoglobulin Deletion Mutant: γ3 Heavy-Chain Disease Protein Wis†

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ABSTRACT: The complete sequence of a γ3 heavy-chain disease (HCD) protein Wis is presented. The molecule is a dimer of a 289-residue chain linked by 12 disulfide bonds. Protein Wis has an unusual amino terminus, followed by a deletion of most of the V_H domain. After a small stretch homologous to the VC joining region, there is a second deletion which ends at the beginning of the quadruplicated hinge. Two carbohydrate groups are linked to Asn-6 and -140. The molecule has an extra interchain disulfide bridge at position 7 in addition to the 11 normally present in the quadruplicated hinge. The previously noted homology to the γ1 heavy chain is striking; from positions 224 to 234, protein Wis resembles γ1 Nei [Ponstingl, H., & Hilschmann, N. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1571–1604] except for a serine which replaces Asn at position 227. The results, taken together with studies of other immunoglobulin heavy-chain deletion mutants, support the suggestion that the different domains and interdomain regions of human H chains are coded for by different gene segments and that the deleted proteins reflect alterations in the recombination of different genes and/or the splicing of heterogeneous nuclear messenger ribonucleic acid (hn mRNA).

γ heavy-chain disease (HCD) proteins are immunoglobulins consisting of part of or the entire heavy (H) chain and devoid of light (L) chains. It is not known whether the L chains are not produced, not assembled, or rapidly degraded. Among the γ HCD proteins studied so far, there is an unexpectedly high incidence of proteins belonging to the γ3 subclass. This subclass is of particular interest since it has an unusually large hinge region in the middle of the heavy chain due to a quadruplication of a 15-residue segment (Michaelsen et al., 1977). Of the three γ3 HCD proteins studied previously, two were internally deleted (Frangione et al., 1969; Frangione, 1976), and one was probably the result of degradation (Adlersberg et al., 1975). However, the length of the deletion was not exactly the same since it stopped at the beginning of the hinge region in two (Omm and Chi) and at the start of the fourth of the quadruplicated hinge segments in the other (Zuc). A fourth internally deleted γ3 HCD protein Wis was recently reported (Franklin et al., 1979). Its partial amino acid sequence coupled with a partial nucleotide sequence obtained from cloned DNA containing the constant region of mouse γ1 and γ2b H chain genes (Sakano et al., 1979; Kataoka et al., 1979) suggested a gene rearrangement to explain the unusual deletion (Frangione & Franklin, 1979a,b). This report completes the primary structure of protein Wis and compares it to γ1 heavy chain and other γ3 HCD deletion mutants.

Experimental Section

Isolation of Myeloma Protein Wis. Protein Wis was isolated from the serum of a patient with HCD by zone electrophoresis carried out on starch (Kunkel, 1954). Purity was checked by immunoelectrophoresis and Ouchterlony double diffusion in agar by using antisera to γ chains, Fab, Fd, and Fc fragments, and light chains (Franklin & Frangione, 1971).

Reduction and Radioactive Alkylation. Protein Wis was totally reduced in 6 M guanidine, 0.6 M Tris, 0.001 M EDTA, and 10 mM dithiothreitol, pH 8.2, for 1 h at room temperature. Alkylation was performed by making the solution 22 mM in iodo[^{14}C]acetic acid (0.7 mCi/mmol, New England Nuclear). Molecular weights were determined in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate ($NaDodSO_4$) (Weber & Osborn, 1969).

Cyanogen Bromide (CNBr) Cleavage. Completely reduced and alkylated protein Wis as well as the Fc fragment obtained after papain digestion was dissolved at 40–50 mg/mL in 70% trifluoroacetic acid (F_3CCOOH) to which was added a threefold excess (w/w) of CNBr (Pierce Chemical Co.). The reaction was allowed to proceed at room temperature for 16–20 h and stopped by the addition of 10 volumes of distilled water, followed by lyophilization. Separation of the CNBr fragments was done on columns packed with Sephadex (Pharmacia Fine Chemicals) operated under acid conditions. The protein concentration was determined both by absorbance readings at 280 nm and by monitoring the radioactivity in a liquid scintillation counter (Beckman, Model LS-250).

Aspartic Acid–Proline Peptide Bond Cleavage. Limited acid hydrolysis of Asp–Pro peptide bonds was performed essentially as described by Mole et al. (1977). The protein was dissolved in 50% formic acid (Fisher, certified ACS) at 10 mg/mL in a 25 × 75 mm acid-washed sealed tube, incubated at 40 °C for 96 h at atmospheric pressure, and then applied directly to a Sephadex G-75 column in 10% formic acid to separate the peptides.

Urea–Pepsin (up) Digestion. Digestion with pepsin in the presence of urea and subsequent fractionation was carried out essentially as described by Parr et al. (1976a,b). The lyophilized protein sample was dissolved in 8 M urea and 0.1 M sodium acetate buffer, pH 4.3, which was brought to pH 3.6 by the addition of concentrated HCl prior to the addition of the enzyme. Pepsin (from hog stomach mucosa, twice crystallized and lyophilized, 3180 units/mg of protein, Sigma Chemical Co.) was then added to give an enzyme/substrate ratio of 1:100 (w/w). The reaction was allowed to take place at room temperature for 55 min with continuous slow stirring. At the end of this time, the reaction was halted by the addition of Tris base and 1 M NaOH to bring the pH to 9.0, and the digest was then separated by gel filtration on Sephadex G-150 equilibrated with 0.1 M sodium acetate buffer, pH 5.0, in 6 M urea.

Papain Digestion. Protein Wis (20 mg/mL) was digested with papain (Worthington Biochemical Corp.) at an enzyme/substrate ratio of 1:100 in 0.1 M phosphate buffer, pH

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0006-2960/80/0419-4304$01.00/0

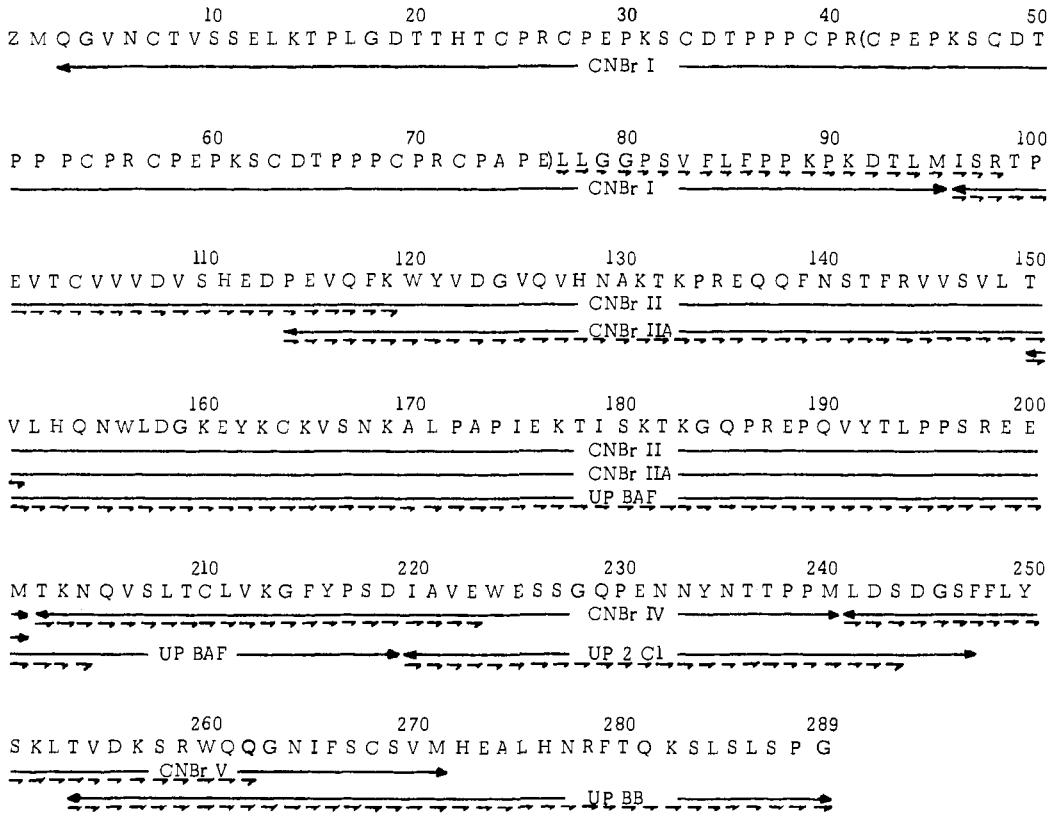

FIGURE 1: Amino acid sequence of deletion mutant protein Wis. CNBr and proteolytic fragments are indicated. Residues 1–95 were previously reported (Franklin et al., 1979). The sequence from residue 42 to residue 77 (in parentheses) was based on the sequence of a $\gamma 3$ myeloma protein (Michaelsen et al., 1977). The sequence from position 77 to position 98 was obtained from starch block fraction I (see the text). (→) represents sequenced positions. Amino acids are identified by the single letter code: A (Ala), C (Cys), D (Asp), E (Glu), F (Phe), G (Gly), H (His), I (Ile), L (Leu), M (Met), N (Asn), P (Pro), Q (Gln), R (Arg), S (Ser), T (Thr), V (Val), W (Trp), Y (Tyr), and Z (pyrrolidonecarboxylic acid); Glu, or Gln, not distinguished.

7.0, without reducing agents for 45 min at 37 °C. The digestion mixture was applied directly to a Sephadex G-150 column equilibrated in 0.1 M Tris-HCl, pH 7.6, 0.2 M NaCl, and 2 mM Na_2EDTA (Michaelsen & Natvig, 1973).

High-voltage electrophoresis (HVE) was done on Whatman chromatography paper at pH 2.1, 3.5, and 6.5 as described (Frangione & Milstein, 1968). Peptides were located by staining a guide strip of the paper with 1% ninhydrin in acetone containing 0.1% $Cd(OAc)_2$ or by autoradiography if they contained ^{14}C-labeled cystine. Peptides were eluted from the paper with 1% acetic acid.

Amino Acid Analysis. Protein and peptide samples were analyzed in a Durrum D-500 automatic amino acid analyzer after hydrolysis in 6 M HCl with 0.1% phenol for 16–78 h (Michaelsen et al., 1977).

NH_2-Terminal Amino Acid and Amino Acid Sequence Analysis. Amino-terminal analysis of peptides was performed either by the manual Edman degradation technique (Gray, 1972) using amino acid analysis to identify the cleaved derivatized amino acid or by the dansyl chloride method (Gray, 1967). Automated amino acid sequence analysis was performed in a Beckman 890 C sequencer with 0.1 M Quadrol buffer. For peptide up2C1, polybrene was added to the cup along with the peptide (Tarr et al., 1978; Klapper et al., 1978). Identification of phenylthiohydantoin (PTH) amino acids was generally done by three and a minimum of two methods including two-dimensional thin-layer chromatography on polyamide plates (Hartley, 1970), amino acid analysis after back-hydrolysis in 0.2 mL of 6 N HCl containing 5 μL of 5% $SnCl_2$ in vacuo (Lai, 1977), and high-pressure liquid chromatography with a Waters HPLC, Model ALC/GPC-204, equipped with a 3.9 mm × 30 cm μBondapak C_{18} column (Waters Associates, Milford, MA).

Results

(A) CNBr Cleavage. Starch block electrophoresis of serum Wis yielded two fractions. One, starch block fraction I (SBI), had a molecular weight of 25 000 which did not change after reduction. Automated amino acid analysis yielded the first 22 residues and placed the beginning of this fragment at position 77 which corresponds to position 234 in a $\gamma 1$ chain (Edelman et al., 1969) (Figure 1). The second fraction, SBII, had a molecular weight of 85 000 which fell to 43 000 after reduction. Its amino terminal was unreactive. Its amino acid composition is shown in Table I. Fraction II (250 mg) which contained five methionine residues (Table I) was cleaved with CNBr, completely reduced and radioactively alkylated, and applied to a Sephadex G-100 column in 30% formic acid (Figure 2). CNBr I which extends from position 3 to position 95 and the amino-terminal dipeptide (Figure 1) were studied previously (Franklin et al., 1979). CNBr II was lyophilized and recycled on a Sephadex G-75 column in 10% formic acid. On $NaDodSO_4$–polyacrylamide gel electrophoresis it was slightly contaminated by a lower molecular weight band probably resulting from acid hydrolysis of an Asp–Pro bond. Its amino terminus was Ile with traces of Pro. The sequence of the first 24 residues places them at positions 96–119 (corresponding to 253–276 in $\gamma 1$ numbering (Edelman et al., 1969)) (Figure 1). CNBr II was further subjected to partial acid hydrolysis and applied to the same Sephadex G-75 column in 10% formic acid. Two peaks were obtained (IIA and IIB). Fraction IIA had Pro at the amino terminus; its amino acid composition, as shown in Table I, corresponds to positions 114–201 (271–358 in $\gamma 1$), and it was sequenced 38 steps

Table I: Amino Acid Composition of Protein Wis and Its Fragments

amino acid[a]	position: Wis (γ3): Eu (γ1):	SBII[e] 1–289	CNBr IIA[g,h] 114–201 (271–358)	CNBr IV[g] 202–240 (359–397)	CNBr V[g] 241–271 (398–428)	up2C1[i] 220–247 (377–404)	upBB[i] 254–289 (411–446)	upBAF[i] 150–219 (307–376)
Cys[b]		14.9	2.1	0.8	1.0		1.1	1.8
Asp		27.3	6.0	5.1	4.0	5.4	3.1	4.5
Thr		23.3	6.0	3.6	0.8	1.7	1.9	5.3
Ser		26.1	4.6	3.9	5.3	3.1	5.2	4.5
Glu		34.2	12.7	5.2	2.3	4.2	4.2	9.4
Pro		37.0	8.2	4.0		3.5	1.0	6.2
Gly		12.1	3.0	2.3	1.9	2.1	2.1	2.8
Ala		6.3	2.9	1.0		0.9	1.0	2.1
Val		21.5	9.6	3.2	2.1	0.7	2.1	4.3
Met		5.1				1.0	1.0	1.2
Ile		4.4	2.2	1.0	1.0	0.8	0.9	2.0
Leu		19.0	5.2	2.1	3.1	1.1	3.1	6.0
Tyr		10.2[f]	4.7[f]	1.9	1.1	1.1		2.7
Phe		10.2	3.3	1.1	3.1	1.0	2.2	0.5
His		8.6	1.7				2.0	1.2
Lys		21.0	7.9	2.0	2.0		2.2	9.6
Arg		10.1	3.4		1.2		2.4	2.4
CHO[c]		+	+					
Hse[d]			+	+	+			
NH_2-terminal		neg	Pro	Thr	Leu	Ile	Thr	Thr

[a] Tryptophan was not determined. [b] Detected as cysteric acid in the intact molecule and as CM-Cys [(carboxymethyl)cysteine] in the fragments. [c] CHO: carbohydrate (+) present. [d] Hse: homoserine (+) present. [e] SBII: starch block II. [f] High value due to carbohydrate. [g] CNBr: cyanogen fragment. [h] CNBr IIA: Asp–Pro acid cleavage. [i] up: urea-pepsin cleavage.

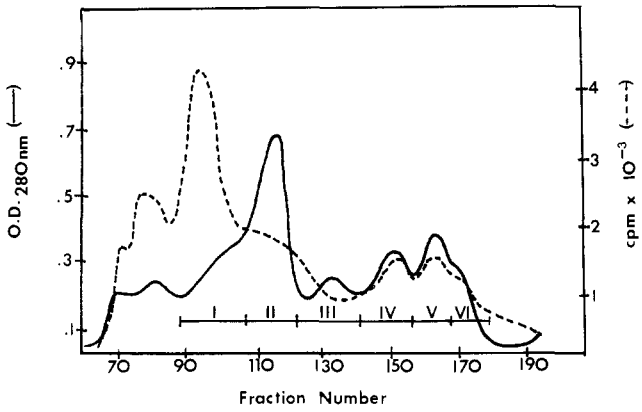

FIGURE 2: Fractionation of a completely reduced and alkylated CNBr digest on a 4 × 140 cm Sephadex G-100 column equilibrated in 30% formic acid. Fractions (4.5 mL) were collected, and 30-μL aliquots were counted in a liquid scintillation counter. Optical density was monitored in a Gilford spectrophotometer at 280 nm.

through position 151. Its high content of CM-Cys is unexplained. Fraction IIB had Ile at the amino terminus, and, although not further characterized, it corresponds to fragment 96–113.

The amino-terminal analysis of CNBr IV from Figure 2 yielded Thr and Leu. Though purification of this fragment was particularly difficult, an amount sufficient for amino acid analysis was obtained after purification employing HVE (pH 6.5, 3.5, and 2.1). Its amino acid composition corresponds to the region between position 202 and position 240. Attempts were made to increase the yield of this peptide by digesting intact protein Wis with papain. The resultant Fc fragment was cleaved by CNBr without reduction and separated on a Sephadex G-150 column in 10% formic acid (not shown). A good-yield fraction corresponding to the region between the third and fifth methionine residues (Figure 1) was obtained having Thr and Leu as amino-terminal residues. Although the fourth methionine at position 240 was cleaved, the two fragments were held together by an intact intrachain S–S bond. This fragment was then completely reduced, radioactively alkylated, and applied to a Sephadex G-50 column in 10% formic acid. A fraction having Thr at the amino terminus was shown by amino acid analysis (Table I) to correspond to residues 202–240 (359–397 in γ1). It was sequenced 22 residues to position 223.

CNBr V (Figure 2) was pooled as indicated. It contained a precipitate which was washed with 5% formic acid several times and then solubilized with 35% formic acid. This fragment was shown to have Leu as its amino terminus and to correspond to residues 241–271 (398–428 in γ1) (Table I). It was sequenced 22 steps to position 262 (Figure 1). CNBr peaks III and VI were heterogeneous and were not studied further.

(B) Urea–Pepsin Digestion of Protein Wis. Purified protein Wis (500 mg) was digested with pepsin in the presence of urea (Parr et al., 1976) and applied directly to a Sephadex G-150 column. Two peaks were obtained: B and C. Fraction C was purified by HVE (pH 6.5, 3.5, and 2.1). One of the peptides obtained (peptide up2C1) whose amino acid analysis is shown in Table I contained 28 residues corresponding to positions 220–247 (377–404 in γ1). It was sequenced in the presence of polybrene 24 steps to position 243 (Figure 1). The sequence of this peptide completes the sequence of CNBr IV and gives the overlap between CNBr IV and V. Fraction B was dialyzed against distilled H_2O, lyophilized, completely reduced and radioactively alkylated, and applied to a Sephadex G-50 column. Two components were obtained. Fraction BB contained 36 residues (Table I), and its sequence shows that it corresponds to the carboxyl end of the molecule (Figure 1). Fraction BA was applied to CM-Sephadex under the same conditions as described (Parr et al., 1976). A peak was obtained (BAF) whose amino acid composition is shown in Table I; its amino-terminal residue was Thr, and it was sequenced for 55 (positions 150–204) out of 70 residues. This sequence gives the overlap between CNBr fragments II and IV.

Discussion

Protein Wis is a dimeric human immunoglobulin γ3 H-chain variant with two chains linked by 12 S–S bonds, 11 in the hinge region (Michaelsen et al., 1977) and an extra one at position 7 near the amino terminus. It was typed as Gm

Protein	Subclass	Position
		381 391
Eu	$\gamma 1$	Trp-Glu-Ser-Asn-Asp-Gly-Glu-Pro-Glu-Asn-Tyr
Nei	$\gamma 1$	Trp-Glu-Ser-Asx-Gly-Glu-Pro-Glx-Asp-Asn-Tyr
Zie	$\gamma 2$	Trp-Glu-Ser-Asn-Gly-Glu-Pro-Glu-Asn-Asn-Tyr
		224 234
Wis	$\gamma 3$	Trp-Glu-Ser-Ser-Gly-Gln-Pro-Glu-Asn-Asn-Tyr

FIGURE 3: Comparison of the amino acid sequences from position 381 to position 391 of two $\gamma 1$ myeloma proteins [Eu, Edelman et al. (1969); Nei, Ponstingl & Hilschmann (1976)], a $\gamma 2$ protein Zie (Hofmann & Parr, 1979), and the homologous region of protein Wis. The sequences were lined up by homology. Numbering of the $\gamma 3$ chain is based on protein Wis. Precise alignment will have to await the complete sequence of a $\gamma 3$ chain.

b (5, 10, 11, 13) by Dr. Arthur Steinberg. The apparent molecular weight of the monomer is 43 000, instead of 60 000 reported for the normal $\gamma 3$ chain, due to two large internal deletions separated by a small stretch with striking homology to the VC joining region of the γ chain (positions 8–11, Figure 1). The first gap starts after an unusual seven-residue fragment, and the second ends at the beginning of the hinge region which is quadruplicated. The monomer of mutant Wis consists of 289 residues with a blocked amino terminus and contains two carbohydrate moieties attached to asparagine residues 6 and 140. A total of 253 residues were actually sequenced. The first 39 residues were obtained after deblocking by digestion with calf liver pyroglutamate aminopeptidase as reported (Franklin et al., 1979; Podel & Abraham, 1978). The sequence from position 40 to position 76 was deduced by amino acid composition of CNBr I, tryptic peptides, and comparison to the sequence of this fragment obtained from another $\gamma 3$ chain (Michaelsen et al., 1977). The remainder of the sequence (positions 77–289) was obtained from fragments derived by CNBr cleavage, acid hydrolysis, and digestion with pepsin in the presence of urea as indicated in Figure 1. A small proportion of Wis protein in the patient's serum was spontaneously digested and easily separated on starch block electrophoresis [starch block fraction I (SBI), see above]. Its amino terminal was Leu (position 77), and it was sequenced 22 residues giving the overlap between CNBr fragments I and II. Thus, the sequence of protein Wis from position 77 to position 289 (which corresponds to positions 234–446 in the $\gamma 1$ numbering), completes the primary structure of the CH2 and CH3 domains of the $\gamma 3$ H chain. The high degree of homology ($\sim$95%) as well as the few amino acid substitutions among the four human γ-chain subclasses has been repeatedly pointed out and will not be discussed further. However, a segment between Trp-381 and Tyr-391 ($\gamma 1$ numbering) present in the CH3 domain deserves clarification. Since there are some discrepancies in the sequences in the two reported $\gamma 1$ chains (Edelman et al., 1969; Ponstingl & Hilschmann, 1976) and the $\gamma 2$ chain Zie (Hofmann & Parr, 1979), this segment was sequenced repeatedly. As shown in Figure 3, protein Wis is more similar to $\gamma 1$ Nei and $\gamma 2$ Zie than to $\gamma 1$ Eu. The most important substitution is Ser ($\gamma 3$) for Asn ($\gamma 1$ and $\gamma 2$). The reason for the discrepancy between the two $\gamma 1$ proteins remains to be elucidated and may be technical; however, it is significant that this sequence has been reported to vary also between γ-chain subclasses in mice and guinea pigs (Melamed, 1976; Trischmann & Cebra, 1974). Its position appears to correspond to the beginning of the first hypervariable region of H and L chains, and its biological function and evolutionary relationship with V genes deserve to be investigated.

Comparison of the primary structure of protein Wis with another $\gamma 3$ deletion mutant Zuc (Frangione & Milstein, 1969; Wolfenstein-Todel et al., 1976) with the same allotypic markers indicated that from position 59 at the beginning of the fourth subunit of the hinge through the remainder of the CH_2 and CH3 domains (Figure 1) the sequences are similar or identical and that the amino-terminal end of both mutants is distinct. This is due to the fact that the V regions belong to different subgroups and to the extent of the deletion. In the case of mutant Zuc the deletion is longer, comprising not only part of the VH and CH1 regions but also the first three segments of the quadruplicated hinge region. It was proposed elsewhere (Frangione & Franklin, 1979a,b) that the different domains and interdomain regions of human H chains are coded by different gene segments. Recently, studies of mouse immunoglobulin H-chain genes have given strong support to this notion and, furthermore, have shown that untranslated DNA segments separate them (Sakano et al., 1979; Kataoka et al., 1979). In the case of the $\gamma 3$ chain, it has also been suggested that each duplicated stretch of the hinge region is coded by separate DNA fragments and that each one has "a signal" necessary for recombining different gene segments and/or splicing heterogeneous nuclear messenger ribonucleic acid (hn mRNA) (Frangione & Franklin, 1979a,b). Analysis of different $\gamma 3$ deletion mutants and cloning of human H-chain genes will clarify this problem.

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Multiple Binding of Antibodies to Antigens: Effect on Radioimmunoassay Binding Curves†

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ABSTRACT: Factors affecting the binding curves measured by radioimmunoassay are examined by comparing theoretical binding curves calculated for ideal populations of homogeneous antibodies and antigens in order to test the conclusions of Berzofsky et al. [Berzofsky, J. A., Curd, J. G., & Schechter, A. N. (1976) *Biochemistry 15*, 2113–2121] about the effect of multiple antigenic determinants on the shapes of such curves. When such ideal binding curves with the same midpoints and limiting values expected for univalent and multivalent antigens are compared, it is clear that multivalent antigens do *not* produce abnormally steep binding curves but rather tend somewhat to produce less steep curves. Under practical experimental circumstances, the binding curves for univalent and multivalent antigens are predicted to be indistinguishable in shape. Some alternative causes of abnormally steep binding curves are suggested.

Antibodies directed against proteins are extremely useful tools in the analysis of protein conformation in solution, especially since such analysis was given a quantitative basis by Sachs et al. (1972). Antibodies recognizing the native conformation of a protein have been used to measure the tendency of protein fragments to adopt that conformation (Sachs et al., 1972; Hurrell et al., 1977; Chavez & Scheraga, 1979), while antibodies against unfolded polypeptides have been used to probe the transient spontaneous unfolding of the native protein (Furie et al., 1975; Hurrell et al., 1977). Antibodies against both native and unfolded conformations have been used to characterize trapped intermediates in protein folding (Creighton et al., 1978; Creighton, 1979).

Although immunochemical analysis is simple in theory, the experimental measurements are made with a complex system. For example, antisera produced against even a simple molecule will generally contain a spectrum of antibody molecules, with varying affinities and in varying proportions. Furthermore, complex molecules such as proteins will elicit such heterogeneous antibody populations against each of several different antigenic sites on the molecule, some of which may overlap, while others are distinct and separate (Crumpton, 1974; Atassi, 1979). Protein antigens may then bind simultaneously two or more antibody molecules against nonoverlapping antigenic sites. Antibody molecules are also multivalent, with two identical binding sites per molecule for the most common IgG class but ten for the IgM class. Indeed, the common formation of an immune precipitate requires the simultaneous binding of at least three antibody molecules to at least some antigen molecules. Consequently, quantitative measurements of the interactions between antibodies and proteins may not be straightforward.

The effect on the radioimmunoassay method of multiple antigenic determinants was examined theoretically and experimentally by Berzofsky et al. (1976) in an attempt to explain an unexpectedly steep binding curve observed experimentally. They concluded that the shape of such a curve "is very sensitive to the multiplicity of determinants" (p 2113), that "as the number of sites n on the antigen molecule increases, the steepness of the slope increases rapidly" (p 2115), and that "classical Scatchard analysis will lead to meaningless numbers" (p 2120) for the affinity between antibodies and a multivalent antigen. This analysis was used recently by Chavez & Scheraga (1979) as one method of estimating the number of antigenic sites on ribonuclease. However, comparison of comparable binding curves calculated for univalent and multivalent antigens shows the primary conclusion of Berzofsky et al. (1976) to be inappropriate. This will be demonstrated here by comparing radioimmunoassay binding curves calculated for antigens with single antigenic sites to those calculated for antigens with up to 10 independent antigenic sites, using the equations of Berzofsky et al. (1976) and the most simple, ideal case where all the populations of antibody molecules have the same concentrations and affinities for their respective antigenic sites. It will be shown by comparing appropriate binding curves that the presence of multiple antigenic sites generally has very little effect on the shape of the curve and that where there is an effect, it is *opposite* to that predicted by Berzofsky et al. (1976).

Radioimmunoassay of a Single Antigenic Site by Homogeneous Antibodies. Binding curves measured by radioimmunoassay express the degree of binding of a small, constant amount of radioactive antigen by a constant amount of an-

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0006-2960/80/0419-4308$01.00/0