Partial Sequence of the Variable Region of an Anti-*p*-azobenzoate Antibody Light Chain: a One Solvent Sequenator Program†

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ABSTRACT: The partial amino acid sequence of the variable region of a rabbit anti-*p*-azobenzoate antibody was determined. The sequence determination was facilitated by a modified dimethylallylamine sequencer program in which 1-chlorobutane is used as both the S_1 and S_3 solvents. To establish the primary structure of virtually the entire light-chain variable region, two different sequencer runs were sufficient: a double cleavage run of 66 cycles on the intact light chain and a single cleavage run of 40 cycles on a peptide isolated from a tryptic digest of the succinylated light chain. Within the limits of the methods used, the light chain appears completely homogeneous. The heavy chains could be more heterogeneous, since the antibody preparation gives at least 18 bands on isoelectric focusing.

The antibody molecule is composed of two identical heavy and two identical light chains. The genes encoding for the synthesis of heavy chains are genetically unlinked to those encoding for light chains. This finding is consistent with more recent reports of more than one heavy-chain sequence paired with the same light-chain sequence or more than one light chain paired with the same heavy chain (Waterfield et al., 1972; Friedenson et al., 1973; Cesari and Weigert, 1973; de Preval and Fougereau, 1975; Trischmann et al., 1975).

The significance of this phenomenon in a typical immune response or in the generation of antibody diversity is still unclear. Numerous studies on reassociation of heavy and light chains have usually shown a preference by a given heavy chain for its original (autologous) light chain. These studies probably rule out a completely random pairing process between heavy and light chains. Nonetheless, there have been exceptions (e.g., de Preval and Fougereau, 1975), and, when the classes or subgroups of the L chains being compared were restricted, the preference of a heavy chain for its autologous light chain has been much less marked (Stevenson and Mole, 1974).

We have approached this problem by attempting to determine, as rigorously as possible, the number of different heavy and light chain sequences present in the same antibody preparation. The approach rests heavily on the use of the automated sequencer, since this instrument allows for a rigorous test of molecular homogeneity or heterogeneity (Hermodson et al., 1972). To facilitate sequence analysis, we have developed a modified dimethylallylamine sequencer program which seems to be adequate. We report here its application in establishing the partial sequence and the homogeneity of a rabbit anti-*p*-azobenzoate antibody light chain.

Materials and Methods

Antibody. Antibody Xp-1 was raised in a rabbit in response to intravenous injections of a conjugate of diazotized *p*-aminobenzoic acid and bovine γ-globulins. Serum was collected each week with injection, following each bleeding. From about 700 ml of serum, 1.8 g of Xp-1 antibody was isolated with the aid of a solid immunoadsorbent (Onoue et al., 1965). On immunoelectrophoresis using goat antibody to whole rabbit serum, Xp-1 gave only an IgG arc. Indirect quantitative radioprecipitation assay for the heavy chain a1 or the light chain b4 allotype (Gilman et al., 1964) showed that 100% of the Xp-1 molecules had the antigenic determinants recognized by either the anti-a1 or the anti-b4 antisera.

Preparation of Light Chains. Antibody Xp-1 was separated into heavy and light chains by mild reduction, followed by alkylation, as previously described (Fleishman et al., 1973). The amount of light chain used for all sequence determinations was determined by amino acid analysis.

Isoelectric Focusing. Isoelectric focusing of the Xp-1 antibody preparation and the Xp-1 light chain was conducted in gel tubes 25-cm long. Focusing was with an ampholine gradient of pH 3.5–10 (Wrigley, 1971).

Sequence Determination. Automated Edman degradations were conducted using a Beckman sequenator equipped with a refrigerated fraction collector, undercut cup, "N_2 flush kit" (Hermodson et al., 1972), and a heavy gauge-restricted vacuum line. Dimethylallylamine buffer (DMAA) was used throughout.

Sequenator Program. The sequenator program used is based on Beckman sequencer program no. 111374, which uses an HFBA[1] delivery system similar to that of Brauer et al. (1975). A brief summary of our modifications is given below and we would be glad to send the details to those interested.

Coupling was with DMAA[2] buffer (R_2) delivered under a pressure of 160 psi. After the first coupling stage, a cup speed of 1200 rpm was used throughout. Drying of the coupling buffer was initiated by a restricted vacuum plus fraction collector vacuum step (700 s) and was completed by an extended (700 s) "fine vacuum-N_2 flush" step. *S_1 extraction* was with 1-chlorobutane (350 s). All post-solvent delivery delays were extended to 90 s and the ensuing N_2 drying steps were omitted. *Cleavage* involved a 1-s R_5 (HFBA) delivery which had been adjusted by lowering the appropriate N_2 pressure. A 120-s

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[1] Abbreviations used are: HFBA, heptafluorobutyric acid; DMAA, dimethylallylamine; PTH, phenylthiohydantoin; DFP, diisopropyl phosphorofluoridate; TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; DEAE, diethylaminoethyl.

[2] DMAA which was noticeably yellow was, in general, less satisfactory than colorless DMAA.

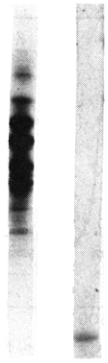

FIGURE 1: Isoelectric focusing of anti-*p*-azobenzoate antibody preparation Xp-1 (left) and Xp-1 light chain. The pH gradient was from pH 3.5 (bottom) to 10 (top).

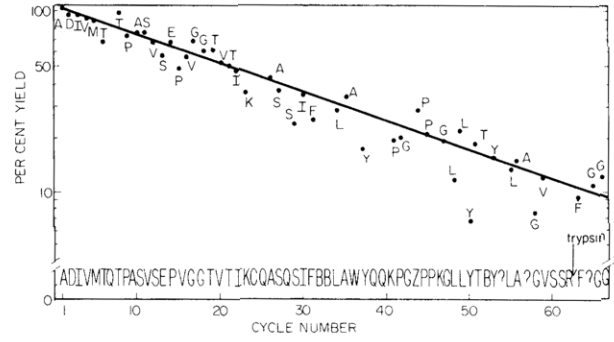

FIGURE 2: Semilog plot of percent yields of PTH-amino acids from a sequenator run using 250 nmol of light chain. The sequence deduced from this experiment and five other runs is shown below the graph in the single letter code for amino acids: (A) alanine; (B) aspartic acid or amide; (C) half-cystine; (D) aspartic acid; (E) glutamic acid; (F) phenylalanine; (G) glycine; (I) isoleucine; (K) lysine; (L) leucine; (M) methionine; (P) proline, (Q) glutamine; (R) arginine; (S) serine; (T) threonine; (V) valine, (W) tryptophan; (Z) glutamic acid or amide; (?) unidentified residue. Glutamine and tryptophan were not quantitated. Tryptophan-36 was identified on a single cleavage run. The half-cystine residue appeared as a blank in the sequence and was inserted by homology with other immunoglobulin light chains, since peptide Ts2′-contained one half-cystine (Table I). Identification of every residue was attempted by gas chromatography of the PTH amino acid and its trimethylsilyl derivative. These yields are shown. In addition, residues 1–40 were subjected to thin-layer chromatography and residues 28–66 were hydrolyzed with HI and subjected to amino acid analysis. The average repetitive yield was about 97%. The arrow indicates the site of tryptic cleavage of the succinylated light chain. Serines at steps 60 and 61 and arginine at step 62 were identified primarily by carboxypeptidase A and B digestion of peptide Ts2′ (see text).

"fine vacuum-N_2 flush" step was added to the drying steps following the cleavage reaction. On some runs, a second cleavage cycle was incorporated. *S_3 extraction* (240 s) was with 1-chlorobutane to which 1,2-butanedithiol (Aldrich) had been added (50 μl/l.). The S_3 precipitation step was omitted (Niall, 1973). On double-cleavage runs, both S_3 extracts were collected in the same fraction collector tube.

Identification of PTH–Amino Acids. Attempts were made to identify every PTH-amino acid by at least two of the following methods: thin-layer chromatography (Summers et al., 1973), gas chromatography (Pisano and Bronzert, 1969), and HI hydrolysis followed by amino acid analysis. HI hydrolysis was conducted using 0.05 ml of HI as described by Smithies et al. (1971). After hydrolysis, the samples were dried under vacuum, redissolved in 0.2 N sodium citrate, pH 2.2, and extracted with CCl_4 to remove traces of residual iodine.

Succinylation and Tryptic Digestion of Light Chains. The light chain was succinylated at pH 9.5 with a 100-fold excess of succinic anhydride (Klotz, 1967), totally reduced and alkylated (Friedenson et al., 1973) and the succinylation reaction was repeated in the presence of 7 M guanidine. Tryptic digestion was with TPCK-treated trypsin (Worthington) at an enzyme/substrate ratio of 1:50 (w/w), pH 9.0, 25 °C, 100 min, and was followed by monitoring the uptake of standardized NaOH. The digest was then applied to a column of Sephadex G-75 (0.9 × 200 cm) and eluted with 0.02 M NH_4OH.

Carboxypeptidase Digestions. DFP-treated carboxypeptidases A and B were obtained from Worthington. Digestion was conducted as described by Narita (1970).

Amino Acid Analysis. Amino acid analysis was with the aid of a Beckman Model 120C amino acid analyzer converted to a 2-h single-column run using a column of Beckman AA-20 resin (0.9 × 31 cm) at a temperature of 50.5 °C. The reaction coil was cut to a length of about 38 ft and all connecting tubing from the bottom of the column to the inlet of the spectrophotometer was made as short as possible. Tryptophan was determined by amino acid analysis after hydrolysis with *p*-toluenesulfonic acid containing 3-(2-aminoethyl)indole (Liu, 1972).

Results

Figure 1 shows the results of isoelectric focusing of the untreated antibody preparation. Eight major and 10–15 minor bands are present. To determine whether all of these bands represent anti-*p*-azobenzoate antibodies, a portion of the whole Xp-1 antibody preparation was immunoprecipitated with a conjugate of diazotized *p*-aminobenzoic acid and human γ-globulins. The washed precipitate was dissolved in 0.3 M *p*-nitrobenzoate and reisolated by DEAE-cellulose chromatography (Keuttner et al., 1972). The product gave an isoelectric focusing spectrum which appeared to be identical to that shown in Figure 1. Thus, all of the isoelectric focusing bands given by the Xp-1 antibody preparation represent antibodies which are capable of binding the *p*-azobenzoate determinant.

Also shown in Figure 1 are the results of isoelectric focusing of the light chain. A single major band appears to be present.

The isolated light chain was applied to the sequenator and Figure 2 shows the yields obtained by gas chromatography of the PTH-amino acids or their silylated derivatives for the N-terminal 66 residues in the Xp-1 light chain. The sequence is given at the bottom of the figure and is based on data from a total of six runs and additional data given below.

Wherever possible, the yields in Figure 2 were calculated from the results of gas chromatography after subtracting the contribution to the yield from background amino acids. The yield of a given PTH-amino acid, which appeared in the next or subsequent steps, was not added to the yield shown in Figure 2 as the "in-step" contribution.

On a successful run, the background amino acids were barely detectable until cycle 8. From this point on, it became necessary to subtract their contributions. At step 8 (Thr), this amounted to a correction of about 3%, and the correction gradually rose until, at cycle 30 (Ile), a background correction of 20% was necessary. At cycles near the end of the run, e.g., steps about 50–66, corrections of 40–60% were used in some cases. Thus, the yields shown in Figure 2 are most reliable for approximately the first 30 steps and their reliability decreases thereafter because of the larger background corrections. To illustrate the highest overall background (i.e., at the very end of the run), Figure 3 shows the data obtained on gas chromatography of steps 63–66 for the run shown in Figure 2. The "background levels" of most PTH-amino acids are high, but

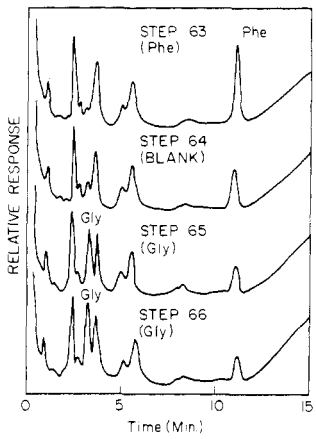

FIGURE 3: Gas chromatographic tracings of steps 63-66 of the run shown in Figure 2. Ten percent of the sample was injected in each case. The column was held at an initial temperature of 180 °C for 4.8 min and then programmed to rise to 270 °C over 8 min. Attenuation, 16; range, 100.

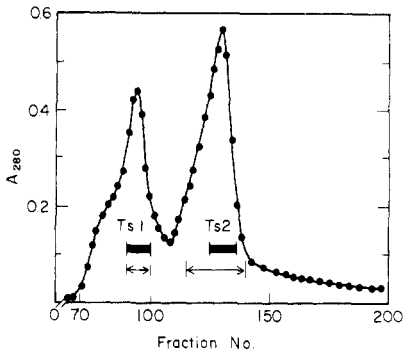

FIGURE 4: Gel filtration of tryptic digest of succinylated Xp-1 light chain on a column of Sephadex G-75 (0.9 × 200 cm) eluted with 0.02 M NH_4OH. At a flow rate of about 2 ml/h, fractions of 1 ml each were collected. Two runs were made (see text). Fractions indicated by horizontal arrows were pooled and applied to the sequenator. On a second run, fractions indicated by horizontal bars were pooled. Peptide Ts2′ was isolated from these as described in the text.

the major sequence present is still discernible and, in any case, was verified below. In addition, the sequence in Figure 2 agreed with the amino acid composition of a peptide representing the same region (see below).

Most residues were also identified by HI hydrolysis and amino acid analysis. The HCl solution and ethyl acetate extracts remaining after gas chromatography were recombined, dried, and hydrolyzed with HI. Because the losses due to handling and hydrolytic destruction necessitated additional correction factors, quantitation of the yields by this procedure was not done. Nonetheless, identification or confirmation by back-hydrolysis proved invaluable and the results were always consistent with those obtained by gas and thin-layer chromatography.

The initial yield obtained (Figure 2) was essentially quantitative. This is based on the amount of light chain applied to the sequenator as determined by amino acid analysis. An average repetitive yield of about 97% was obtained. In determining this value the yields (minus background) of the more stable PTH-amino acids (Figure 2) were visually weighted more heavily.

On two runs of the intact light chain, it was apparent that hydrolytic cleavage had occurred at the acid-labile Asp-Pro bond between positions 109-110 in the constant region (Fraser et al., 1972). Thus, the sequence Pro-Val-Ala-Pro-Thr-Val-Leu-Ile-Phe-Pro- was obtained in a yield of 2-5% of the major sequence. It is possible that a small amount of this acid splitting occurs at every cleavage step in a cycle and contributes to the background observed.

Amino acid analysis of the intact light chain showed 2.0 arginine residues per mole of light chain. The constant region contains 1 arginine residue, which is located at position 207 (Chen et al., 1975); the variable region contains the second arginine residue at position 62 (below). Thus, tryptic digestion of the succinylated light chain should release peptides representing residues 1-62, 63-207, and 208-210.[3] These peptides should be separable by gel filtration on Sephadex G-75.

Figure 4 shows the results of this experiment. Two major peaks, labeled as Ts1 and Ts2 in order of their elution from the column, were found. Fractions corresponding to Ts1 and Ts2 were pooled as shown by the horizontal arrows in Figure 4.

Fraction Ts2 (Residues 1-62, 63-136, and 137-207). Fraction Ts2 was subjected to 30 cycles of Edman degradation and gave two sequences, each in an overall yield of about 12% based on the number of nanomoles of light chain applied to the Sephadex G-75 column.

One sequence in the Ts2 fraction agreed with the sequence reported for the light-chain constant-region sequence beginning with residue 137 (Chen et al., 1975). The sequence starting at residue 137 began with a lysine residue, which could be identified by gas chromatography, and, hence, was not succinylated. Therefore, one of the peptides in fraction Ts2 results from an anomalous enzymatic split between Asn-136 (Chen et al., 1975) and Lys-137. Chymotrypsin may cleave proteins at the carboxyl group of Asn residues and the presence of a neighboring basic amino acid enchances cleavage at weakly susceptible bonds (reviewed by Kasper, 1970). Thus, we conclude that the split between residues 136 and 137 was due to chymotryptic activity in the TPCK-treated trypsin used.

The second sequence in the Ts2 fraction is derived from a tryptic peptide containing residues 63 to about 136, based on an estimate of the molecular weight of Ts2 by gel filtration, the finding of a peptide beginning at residue 137, and the fact that the N-terminal sequence agreed with that found for peptide Ts1 (residues 63-207; discussed below).

Fraction Ts2 was also shown to contain the peptide representing residues 1-62 (peptide Ts2′). Using another portion of Xp-1 light-chain preparation, the succinylation reaction and trypsin digestion steps were repeated, as before, except that tryptic digestion was conducted in 0.2 M NH_4HCO_3 at pH 8. Gel filtration, as before, gave results virtually identical to those in Figure 4. Amino acid analyses of individual fractions from the ascending and descending portions of the Ts2 peak were substantially different and indicated that the split after residue 136 had again occurred.

Fractions were pooled as shown by the horizontal bars in Figure 4 and further purification of peptide Ts2′ was accomplished by three passages through a column of Sephadex G-50 (0.9 × 220 cm) in 0.02 M NH_4OH. The amino acid compositions of the products after the second and third G-50 chromatography steps were virtually identical and this was taken as evidence that the product (peptide Ts2′) was pure. Carboxypeptidase A and B digestion of peptide Ts2′ gave Arg as the C terminal residue and established the C-terminal sequence of peptide Ts2′ as -Ser-Ser-Arg (Figure 2). The amino acid composition of peptide Ts2′ (Table I) agreed with the N-ter-

[3] The peptide representing residues 208-210 was not detected under the conditions used here.

Table I: Amino Acid Analysis of Peptide Ts2'.

Amino Acid	Found by Analysis[a]	Integer	Found by Sequence
Asp	3.8	4	4
Thr	5.5	6	5[c]
Ser	6.9	7	6[c]
Glu	7.3	7	7
Pro	5.3	5	5
Gly	4.9	5	5
Ala	5.1	5	5
Val	5.2	5	5
Met	0.8	1	1
Ile	2.8	3	3
Leu	3.6	4	4
Tyr	2.8	3	3
Phe	1.0	1	1
Lys	3.1	3	3
His	0.0	0	0
Arg	0.9	1	1
Trp	0.7	1	1
Cys	0.5[b]	1	1

[a] As residues per peptide. Values are uncorrected for hydrolytic losses and are averages of three determinations. [b] As *S*-(carboxymethyl)cysteine plus *S*-(carboxymethyl)cysteine sulfone. [c] The blanks at steps 54 and 57 account for the discrepancy between amino acid analysis and the sequence.

minal 62 residues of the sequence in Figure 2. This agreement was taken as evidence that the sequence in Figure 2 is correct.

Peptide Ts1 (Residues 63–207). Peptide Ts1 was then subjected to automated Edman degradation, and the sequence and the yields obtained are shown in Figure 5. The N-terminal sequence of Ts1 established an overlap with steps 63–66 in the N-terminal sequence of the light chain (Figure 2). Thus, Ts1 represents residues 63–207 of the light chain.

When the recovery of the sequence for residues 63–136 in the crude Ts2 fraction (indicated by arrows in Figure 4) was added to the recovery of Ts1 (residues 63–207), the sum (corrected for losses in handling) was in good agreement with the amount of light chain used. Thus, the tryptic cleavage at Arg-62 was essentially complete.

The amino acid sequence of the variable region of light chain Xp-1 is summarized in Table II. For comparison, the other anti-*p*-azobenzoate antibody, for which the light chain variable region sequence is known (Appella et al., 1973), is also included. Both sequences are numbered and aligned according to Margolies et al. (1975). Positions 0–97C constitute the variable region and the constant region begins with phenylalanine 98.

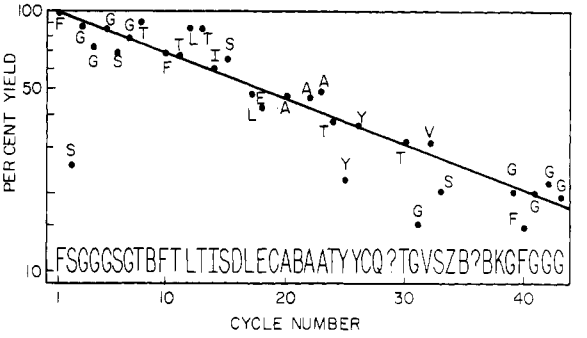

FIGURE 5: Semilog plot of percent yields of PTH-amino acids from a single cleavage sequenator run using 150 nmol of purified peptide Ts1. The sequence deduced from this experiment and one other run is shown below the graph in the single letter code (see legend of Figure 2). Identification of every residue was attempted by gas chromatography of the PTH-amino acid and its trimethylsilyl derivative. In addition, every sample was hydrolyzed with HI and subjected to amino acid analysis. Half-cystine residues appeared as blanks in the sequence and were inserted by homology with other rabbit light chains, since the Xp-1 light chain contained close to the expected number of half-cystine residues (expected: 7; found: 6.6). Cycles 1–4 establish an overlap with the sequence at the bottom of Figure 2. The average repetitive yield was about 96%.

Discussion

We have used the modified sequencer program described above for proteins with molecular weights similar to the light chains reported here. In several cases, the sample possessed no known amino acid sequence homology and no homology which became obvious as the run progressed. The results were essentially equivalent to those reported here for the Xp-1 light chain in terms of the length of the sequencer runs, the repetitive yields, and the clarity of the results (Bach et al., 1976).

The data in Figure 1 shows that the light chain gives a single major band in high yield on isoelectric focusing. The data in Figures 2 and 3 show that every residue we identified in the amino acid sequence of the light chain is present in a yield which is consistent with a homogeneous amino acid sequence. Moreover, we were unable to find any position in the sequence which contained more than one amino acid.

The intact heavy chain from the same Xp-1 preparation is about 90% blocked, but we isolated a CNBr fragment in about 10% yield which contained the N-terminal region of the heavy chain and which was unblocked. The amino acid sequence of this fragment contained Asp as the N-terminal residue (rather than pyrrolidonecarboxylic acid as in all other rabbit heavy chains) and was derived from heavy chains belonging to the V_{HIII} subgroup (Wang et al., 1975). Thus, at least two different heavy-chain sequences are probably present in the Xp-1 antibody preparation.

Table II: Amino Acid Sequences of Light Chains Xp-1 and 2717.

	0	1					10						20					a						30	31	32	32A	32B
Xp-1	Ala	Asp	Ile	Val	Met	Thr	Gln	Thr	Pro	Ala	Ser	Val	Ser	Glu	Pro	Val	Gly	Gly	Thr	Val	Thr	Ile	Lys	Cys	Gln	Ala	Ser	Gln	Ser	Ile	Phe	Asx	Asx	----------		
2717[b]		Val	Glu	—	Leu	————				Ser	Pro	——	Ala	Ala	————									Ser	——	Ser	Thr	Lys	——	Tyr	Asx	Asx	Asx	Tyr		

	33						40						50			c		c	60																
Xp-1	Leu	Ala	Trp	Tyr	Gln	Lys	Pro	Gly	Glx	Pro	Pro	Lys	Gly	Leu	Leu	Tyr	Thr	Asx	Tyr(	)	Leu	Ala(	)	Gly	Val	Ser	Ser	Arg	Phe	Ser	Gly	Gly	Gly	Ser	
2717	————								Gln	————			Ala	—	Ile	————			Ala	Ser	Ser	———		Ser	——		Pro	————			Thr	—	Ser	————			

		70							80a								a	90							97	97A	97B	97C	98		100	101
Xp-1	Gly	Thr	Asx	Phe	Thr	Leu	Thr	Ile	Ser	Asp	Leu	Glu	Cys	Ala	Asx	Ala	Ala	Thr	Tyr	Tyr	Cys	Glu(	)	Thr	Gly	Val	Ser	Glx	Asx(	)	Asx	Lys	Gly	Phe	Gly	Gly	Gly
2717	———	Glx	————			Leu	——	Val	Glx	—	Asp	Asp	————								Gly	Gly	Ala	Asp	Thr	Thr	Gly	Tyr	Ser	----------						

[a] Inserted by homology (see text). [b] Data of Appella et al. (1973); a solid line indicates identity of sequence. [c] Either Thr or Ser (see Table I).

It is, of course, possible that this minor population of V_{HIII} heavy chains might be associated with a minor light-chain sequence which was overlooked by the sequencing, isoelectric focusing, and allotyping procedures. By isoelectric focusing of the untreated antibody preparation (Figure 1), we have been able to identify at least 18 distinct bands due to charge heterogeneity of the heavy chains. However, these bands do not necessarily reflect biosynthetically different heavy-chain sequences, since Awdeh et al. (1970) showed that isoelectric heterogeneity could be induced in a homogeneous mouse myeloma protein by exposing it to serum.

Thus, the number of biosynthetically different heavy-chain sequences in the Xp-1 antibody preparation might be as few as two or, conceivably, as many as 18 or more. Quantitative allotypic data have shed no light on this question. Even though the antibody appeared to be 100% a1 allotype, the demonstration of subpopulations of a1 IgG molecules (Horng et al., 1976) coupled with sequence data (Jaton et al., 1973) show that the quantitative presence of an allotype does not preclude sequence heterogeneity.

Of the approximately 100 variable region positions shown in Table II for antibodies Xp-1 or 2717, at least 37 are different. Fourteen of these differences are concentrated within the two hypervariable regions which are clearly involved in antigen binding, i.e., positions 30–32B and 90–97C (Poljak et al., 1974).

Tyrosine-96 in the 2717 light chain is involved in antigen binding (Roholt et al., 1973; Appella et al., 1973). All the tyrosine residues found by amino acid analysis of Xp-1 can be accounted for by the sequence of the variable region and the one reported for the constant region (Chen et al., 1975). Thus, despite the blanks at position 90 and 97, the hypervariable regions of Xp-1 are devoid of tyrosine.

This may mean that Xp-1 and 2717 bind the same antigen by different mechanisms, as might be suggested by the poor homology found between the hypervariable regions of Xp-1 and 2717. Alternatively, if the combining sites of the two antibodies do utilize the same binding mechanism, the Xp-1 heavy chains would then have to contribute a tyrosine residue to the Xp-1 combining site.

Except for a threonine residue at position 50, which is also found in 2717, positions 50–52 in the Xp-1 light chain are different from all other published rabbit light-chain sequences (Jaton, 1974a,b; Chen et al., 1975; Margolies et al., 1975; Thunberg and Kindt, 1976). Positions 50–56 are hypervariable in the light chains of other species (Wu and Kabat, 1970). In the rabbit, significant variability has not been found in this region (Margolies et al., 1975), which does not appear to be involved in antigen binding (Poljak et al., 1974). Nonetheless, it may turn out that, as additional data becomes available, the variability in this region may become comparable to that noticed by Wu and Kabat (1970) for the light chains of human and mouse myeloma proteins.

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