Mouse Immunoglobulin Chains. Pattern of Sequence Variation among κ Chains with Limited Sequence Differences†

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ABSTRACT: Partial amino acid sequences of two κ light chains from the BALB/c mouse, M63 and T124, are compared with two previously published mouse sequences, M70 and M321. All four κ chains have nearly identical amino-terminal sequences and a four-residue insertion at or near position 27 when compared with two other mouse κ chains, M41 and M21. M321 and T124 differ from one another by three residues, all in the hypervariable regions. M63 differs from M321 and T124 by eight residues, while M70 differs from the other three proteins by 20–21 residues. The implications of these data with regard to the genetic mechanism of antibody diversity are discussed.

Two general types of amino acid sequence variation were observed in comparing the amino-terminal 23 residues of the variable regions from 44 mouse κ chains (MV_κ regions) (Hood *et al.*, 1973). Certain proteins were identical or nearly identical over this stretch of sequence, whereas others differed by 3–16 amino acid residues. Peptide map comparisons of the first group (identical or nearly identical) suggested that these proteins fall into two groups: (a) those with variable (V) regions which appeared identical and (b) those with variable regions exhibiting limited sequence differences (one to four distinct peptides). Immunologists generally agree that V regions with extensive sequence differences (*e.g.*, from different branches on the V region genealogic tree or different subgroups) are probably encoded by distinct germ line genes whereas each set of V regions with an identical sequence appears to be encoded by a single germ line (Cohn, 1971; Gally and Edelman, 1970; Hood and Prahl, 1971). However, a controversy exists as to whether similar but not identical V regions (those on the same branch of the V region genealogic tree or in the same subgroup) are encoded by a single germ line V gene with sequence diversification produced by somatic mutation (somatic theory) or whether these limited sequence differences are encoded by distinct germ line genes (germ line theory). In order to approach this question we present the pattern of variation which occurred among four light chains with limited sequence differences (MOPC 321, MOPC 63, TEPC 124 and MOPC 70).[1] The partial sequences of M70 (Gray *et al.*, 1967) and M321 (McKean *et al.*, 1973) have been previously reported. This paper employs the methodology used in analyzing M321 and presents the partial variable region sequences of M63 and T124. We describe the patterns of amino acid substitutions which exist among these proteins and discuss the implications of these observations vis-à-vis genetic mechanisms of immunoglobulin diversity.

Materials and Methods

Proteins. The isolation and purification procedures for M63 and M124 proteins were identical with those of M321 (McKean *et al.*, 1973). The respective plasma cell tumors produced approximately 200–500 mg of urinary light chain from 20 mice during one tumor generation. These studies were carried out on less than 500 mg of each protein.

Because of the structural similarities of M124 and M63 to M321, the methods used for amino acid analysis, cyanogen bromide cleavage, and separation of the corresponding peptide

TABLE I: Amino Acid Composition of M321, T124, and M63.[a]

	M321	T124	M63
Asp	26 (25)	24 (24)	26 (26)
Glu	20 (21)	20 (22)	22 (22)
Thr	20 (20)	22 (19)	18 (19)
Ser	28 (30)	29 (30)	22 (29)
Pro	16 (12)	15 (12)	12 (12)
Ala	13 (13)	15 (14)	14 (14)
Gly	13 (12)	13 (12)	14 (13)
Val	11 (11)	12 (11)	12 (11)
Met	2 (2)	2 (2)	2 (2)
Ile	10 (10)	10 (10)	8 (9)
Leu	15 (13)	15 (13)	14 (14)
Tyr	10 (8)	9 (8)	8 (8)
Phe	8 (9)	8 (9)	8 (9)
Lys	12 (12)	10 (11)	10 (11)
His	3 (2)	3 (2)	3 (2)
Arg	9 (9)	10 (9)	8 (8)

[a] Values reported are amino acid residues from 24-hr hydrolysates of native light chain rounded off to the nearest integral number except for Ser and Thr values which are based on a linear extrapolation of 24- and 48-hr hydrolysis values to zero time. Cys and Trp were not quantitated.

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[1] Mouse immunoglobulin chains MOPC 321, MOPC 63, TEPC 124, MOPC 70, MOPC 41, and MOPC 21 are abbreviated M321, M63, T124, M70, M41, and M21, respectively.

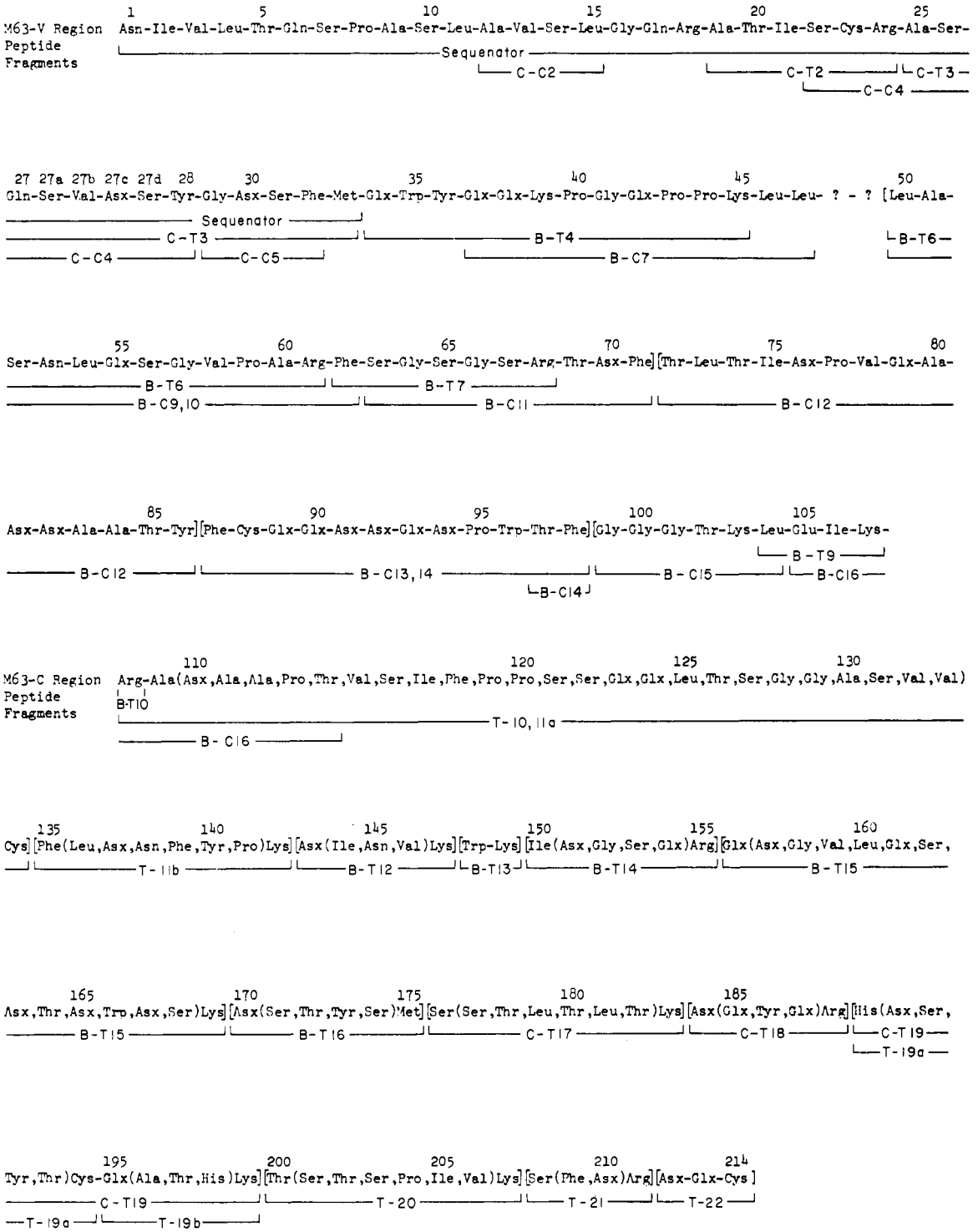

FIGURE 1: Peptide fragments from M63. The peptides of the methionine-cleaved chain are numbered from the amino terminus. Peptides labeled a and b indicate fragments derived from tryptic digests after aminoethylation of the half-cystine residues. The nomenclature is discussed in the text. Briefly for peptide C-T2, C indicates peptide 2 was derived from a trypsin (T) digest of cyanogen bromide peak C. Brackets enclose those peptides (or groups of peptides) whose position in the chain has not been established directly by sequence overlap. These are assigned an order by homology with other chains. Parentheses enclose residues whose sequence has not yet been determined. "Sequenator" indicates that the amino-terminal sequence was determined by the automatic sequenator.

fragments, peptide isolation, enzymatic cleavage, and the dansyl-Edman procedure were identical with those previously described (McKean *et al.*, 1973).

Peptide Nomenclature. Peptides for each light chain are numbered from the amino terminus as shown in Figure 1 and 2. For peptide designations, number of the tumor (124 or 63) is given first; then is given the enzyme which produced the fragment (T = trypsin; C = chymotrypsin); and finally is given the peptide number. CN designates that the corresponding fragment was derived from a larger peptide fragment produced by cyanogen bromide cleavage. The amino acid numbering is that of Gray *et al.* (1967).

TABLE II: Amino Acid Compositions of Tryptic Peptides from CN-C.[a]

	C-T2		C-T3		C-T17		C-T18		C-T19	
	M63	T124	M63	T124	M63	T124	M63	T124	M63	T124
Tyr			0.8 (1)	0.8 (1)			0.9 (1)	0.8 (1)	0.7 (1)	1.0 (1)
Phe			1.0 (1)	1.1 (1)						
Lys					1.2 (1)	1.0 (1)			0.9 (1)	1.0 (1)
His									1.7 (2)	1.8 (2)
Arg	1.1 (1)	1.0 (1)					0.8 (1)	1.0 (1)		
Asp			2.3 (2)	1.8 (2)			1.0 (1)	1.1 (1)	1.3 (1)	1.2 (1)
Glu			1.3 (1)	0.9 (1)			2.2 (2)	2.2 (2)	1.3 (1)	1.1 (1)
Thr	1.1 (1)	1.1 (1)			3.0 (3)	2.9 (3)			1.9 (2)	2.0 (2)
Ser	0.8 (1)	0.9 (1)	4.8 (5)[b]	3.5 (4)[b]	1.8 (2)	1.9 (2)			1.0 (1)	1.0 (1)
Pro										
Ala	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)					0.9 (1)	0.9 (1)
Gly			1.1 (1)	1.0 (1)						
Val			1.0 (1)	1.0 (1)						
CMCys	0.6 (1)	0.7 (1)							0.6 (1)	0.7 (1)
Ile	1.0 (1)	0.9 (1)								
Leu					2.0 (2)	2.1 (1)				
Trp				+[d]						
Total residues	6	6	13	13	8	8	5	5	11	11
Yield (%)[c]	57	26	16	32	12	17	9	30	27	27

[a] Values reported are amino acid residues. Amino acids present at a level of less than 0.2 residue are omitted. Values in parentheses represent the nearest integral number of residues. [b] Includes homoserine as well as serine as the zinc ligand amino acid analysis system would not separate these residues. The low value probably reflects the fact that a portion of the cyanogen bromide

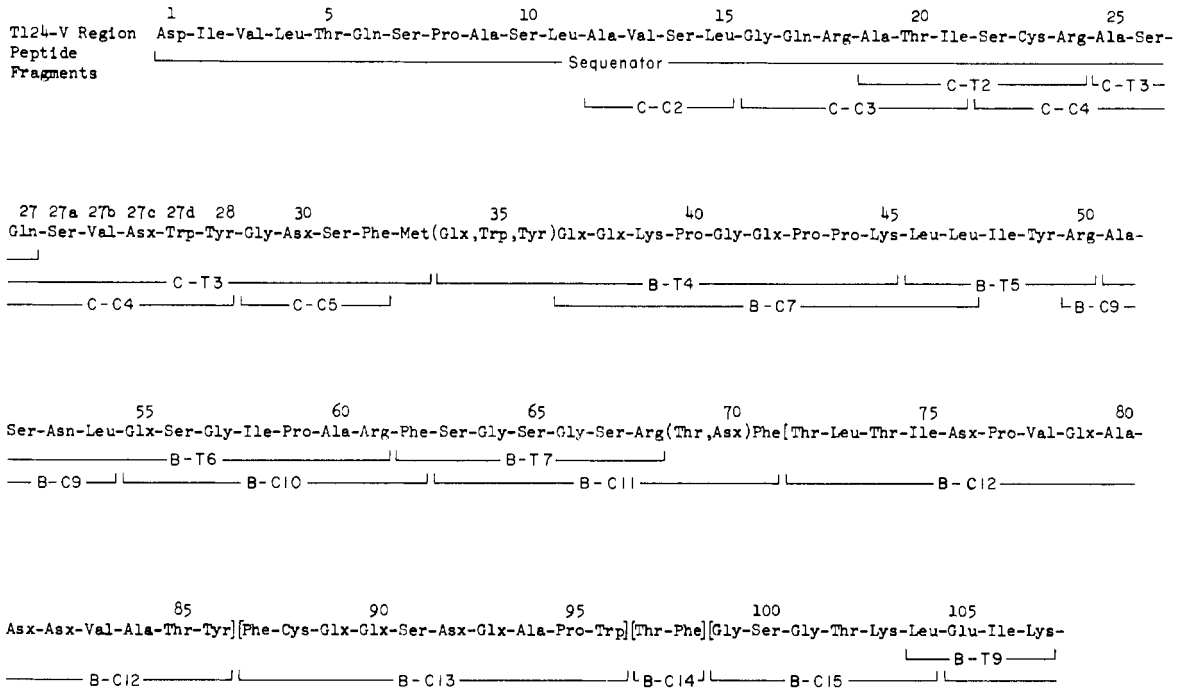

FIGURE 2: Peptide fragments from M124. See legend to Figure 1 for explanation. The C region isolated peptide fragments are identical with those of M63 (see Figure 1).

Results

Location of Methionyl Residues. The amino acid analyses of M63 and M124 gave 2 mol of methionine/mol of protein (Table I). The overall amino acid compositions of M63, M124, and M321 are remarkably similar. Furthermore, the amino-terminal 23 residues for each of the three proteins are identical apart from an Asp ↔ Asn interchange at position 1 (Hood *et al.*, 1973). Accordingly, it seemed likely that the methionine residues in T124 and M63 might be placed at positions homol-

	C-T20		C-T21		C-T22	
	M63	T124	M63	T124	M63	T124
Tyr						
Phe			1.1 (1)	1.1 (1)		
Lys	1.0 (1)	1.1 (1)				
His						
Arg			1.1 (1)	0.9 (1)		
Asp			1.1 (1)	1.2 (1)	0.9 (1)	1.0 (1)
Glu					1.1 (1)	1.1 (1)
Thr	2.1 (2)	2.0 (2)				
Ser	2.1 (2)	1.9 (2)	0.8 (1)	0.8 (1)		
Pro	0.8 (1)	1.1 (1)				
Ala						
Gly						
Val	0.9 (1)	1.0 (1)				
CMCys					0.6 (1)	0.5 (1)
Ile	1.0 (1)	0.9 (1)				
Leu						
Trp						
Total residues	8	8	4	4	3	3
Yield (%)[c]	59	25	29	14	7	8

cleavage product of methionine is present as basic homoserine lactone. [c] Yields are based on micromoles of peptides isolated compared with micromoles of fragment originally digested with trypsin. [d] Tryptophan determined by Ehrlich's stain.

ogous to those in M321. Thus efforts were directed at isolating and characterizing the three cyanogen bromide fragments from T124 and M63.

Isolation of Cyanogen Bromide Peptides. Native M63 and M124 were cleaved with cyanogen bromide, reduced and S-carboxymethylated. After gel filtration on G-75 Sephadex, three peaks, CN-A, CN-B, and CN-C, were eluted at volumes identical with those of their M321 counterparts (McKean *et al.*, 1973, see Figure 3B). The characterization of fragments CN-C and CN-B was carried out as described for M321 (McKean *et al.*, 1973). These analyses verified that the methionines for both M63 and T124 were positioned at residues 33 and 175. Thus for both proteins CN-C contained two cyanogen bromide fragments, L_1 and L_3, extending from positions 1 to 33 and 176 to 214, respectively, and CN-B contained one fragment, L_2, extending from positions 34 to 175 (see Figure 2 in McKean *et al.*, 1973). These fragments were identical in size with those of M321.

Light-Chain M63. The amino-terminal 37 residues of this protein were characterized on the sequenator and are given in Figure 1. The yield data were similar to that of M321. The characterizations of intact peaks CN-B and CN-C by dansyl analysis and by carboxypeptidase A were identical with those analyses for the corresponding peaks in M321 (McKean *et al.*, 1973) and are again given in Figure 1. Eight tryptic and three chymotryptic peptides were isolated from CN-C and their compositions are given in Tables II and IV and their sequences in Tables III and V, respectively. Ten tryptic and nine chymotryptic peptides were isolated from CN-B and their compositions are given in Tables VI and VII and their sequences in Tables VIII and IX, respectively. The compositions and sequences of four tryptic peptides isolated from aminoethylated light chains are given in Tables X and XI.

Various peptide overlaps shown in Figure 1 permit us to order the variable region peptides into six groups, residues 1–33, 34–47, 50–71, 72–86, 87–98, and 99–107. The order of

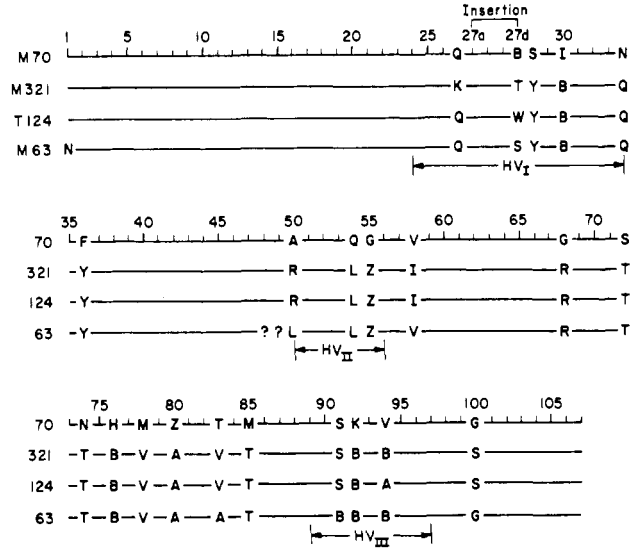

FIGURE 3: Amino acid sequence comparison of M70, M321, T124, and M63. The one letter amino acid code of Dayhoff (1969) is used: D-aspartic acid; N-asparagine; Q-glutamine; K-lysine; B-aspartic acid or asparaginine; T-threonine; W-tryptophan; S-serine; Y-tyrosine; I-isoleucine; F-phenylalanine; A-alanine; R-arginine; L-leucine; V-valine; G-glycine; Z-glutamic acid or glutamine; H-histidine; and M-methionine. HV_I, HV_{II}, and HV_{III} indicate the three hypervariable regions as defined by Wu and Kabat (1970). In M63 residues homologous to positions 48–49 were not isolated.

TABLE III: Amino Acid Sequence of Tryptic Peptides from CN-C.

Peptide	M63	T124
C-T2	Ala-Thr-Ile-Ser(Cys,Arg)	Ala-Thr-Ile-Ser(Cys,Arg)
C-T3	Ala-Ser-Gln-Ser-Val-Asx-Ser-Tyr-Gly-Asx-Ser-Phe-Met	Ala-Ser-Gln-Ser-Val-Asx-Trp-Tyr-Gly-Asx-Ser-Phe-Met
C-T17	Ser(Ser,Thr,Leu,Thr,Leu,Thr,Lys)	Ser(Ser,Thr,Leu,Thr,Leu,Thr,Lys)
C-T18	Asx(Glx,Tyr,Glx,Arg)	Asx(Glx,Tyr,Glx,Arg)
C-T19	His(Asx,Ser,Tyr,Thr,Cys,Glx,Ala,Thr,His,Lys)	His(Asx,Ser,Tyr,Thr,Cys,Glx,Ala,Thr,His,Lys)
C-T20	Thr(Ser,Thr,Ser,Pro,Ile,Val,Lys)	Thr(Ser,Thr,Ser,Pro,Ile,Val,Lys)
C-T21	Ser(Phe,Asx,Arg)	Ser(Phe,Asx,Arg)
C-T22	Asx(Glx,Cys)	Asx(Glx,Cys)

TABLE IV: Amino Acid Composition of Chymotryptic Peptides from CN-C.[a]

	C-C2		C-C3		C-C4		C-C5	
	M63	T124	M63	T124	M63	T124	M63	T124
Tyr					0.9 (1)	1.0 (1)		
Phe							1.2 (1)	1.1 (1)
Lys								
His								
Arg				0.9 (1)	1.0 (1)	1.0 (1)		
Asp					1.0 (1)	1.2 (1)	1.2 (1)	1.2 (1)
Glu				1.1 (1)	1.1 (1)	1.1 (1)		
Thr				1.1 (1)				
Ser	1.0 (1)	0.9 (1)			3.8 (4)	3.2 (3)	0.9 (1)	0.9 (1)
Pro								
Ala	0.9 (1)	1.0 (1)		1.1 (1)	1.1 (1)	1.0 (1)		
Gly				0.8 (1)			1.0 (1)	0.9 (1)
Val	0.9 (1)	1.0 (1)			0.9 (1)	0.9 (1)		
CMCys					0.7 (1)	0.5 (1)		
Ile				1.0 (1)				
Leu	1.2 (1)	1.1 (1)						
Trp						0.7 (1)		
Total residues	4	4		6	11	11	4	4
Yield (%)[c]	40	25		18	23	20	13	26

[a,c] See legend to Table II.

TABLE V: Amino Acid Sequence of Chymotryptic Peptides from CN-C.

Peptide	M63	T124
C-C2	Ala-Val-Ser-Leu	(Ala,Val,Ser,Leu)
C-C3		(Gly,Glx,Arg,Ala,Thr,Ile)
C-C4	Ser(Cys,Arg,Ala,Ser,Glx,Ser,Val,Asx,Ser,Tyr)	Ser(Cys,Arg,Ala,Ser,Gln,Ser,Val,Asx,Trp)Tyr
C-C5	Gly-Asx-Ser-Phe	(Gly,Asx,Ser,Phe)

these groups has been assigned by homology with proteins M70 and M321.

All of the tryptic peptides from the constant region were isolated and their compositions were identical with those of M321. No attempt was made to isolate overlap peptides because of the probable identity of this constant region to that of M321 and correspondingly their order was determined by homology to M41 (Gray *et al.*, 1967) and M321.

Three additional points are worthy of note. First, the amino-terminal residue of CN-B (L2) gave no result with the dansyl chloride procedure. As with M321 the presumed glutamine residue at this position probably cyclized to a pyrroli-

donecarboxylic acid. Second, the Lys–Pro bond between positions 39 and 40 did not cleave with trypsin digestion, in contrast to the results obtained with protein M321. Finally, residues homologous to 48–49 in M321 were not isolated. Possibly there is an Ile-Tyr sequence at these positions with the Tyr extremely susceptible to chymotrypsin cleavage. Tryptic (B-T6) and chymotryptic peptides (B-C9, 10) were isolated with their amino terminus at position 50. It may be that the hypothetical peptide Leu-Leu-Ile-Tyr was lost in the chromatographic dimension of the fingerprint isolation procedure.

Light-Chain T124. The amino-terminal 27 residues of this protein were characterized on the sequenator as indicated in Figure 2. Again the yield was similar to that of M321. The characterizations of intact CN-C and CN-B peaks by dansyl chloride and carboxypeptidase A were identical with those of M321. The tryptic and chymotryptic peptides are characterized in Tables II–XI as indicated for M63.

The peptide overlaps shown in Figure 2 permit us to order the variable region peptides into six groups, 1–33, 34–71, 72–86, 87–96, 97–98, and 99–107, whose order is assigned by homology to M70 and M321.

The constant region tryptic peptides were identical in composition with their counterparts from proteins M63 and M321. No overlap peptides were isolated from the C region and their order was determined by homology to M41 and M321.

Discussion

Immunoglobulins of the BALB/c mouse offer an excellent system for a study of the mechanism responsible for antibody diversity. These animals are highly inbred and accordingly their immunoglobulin sequence variations should reflect the genetic mechanism responsible for immunoglobulin diversity without a "noise" contribution due to the genetic polymorphisms of an outbred population such as man. The question of interest is whether a single mouse V_κ (MV_κ) germ line gene can give rise by somatic mutation to V_κ regions with limited sequence differences, or whether a germ line MV_κ gene must be postulated for each distinct sequence. To approach this question we have characterized three V_κ regions with limited sequence differences over their amino-terminal 23 residues (M63, M321, and T124) and compared them with a fourth similar κ chain (M70) already in the literature.

The variability which exists among four MV_κ regions with limited sequence differences is illustrated in Figure 3. Two light chains, M321 and T124, have three amino acid differences (Table XII). M63 differs from M321 and T124 by eight residues, whereas M70 differs from the other three by 20–21 residues. A majority of the substitutions resulting from pairwise comparisons of these four light chains can be explained by single nucleotide substitutions (67/77–88%).

The interrelationships of these MV_κ sequences can be best analyzed in terms of a genealogic tree which depicts the relationships of the MV_κ regions with regard to the minimum genetic events that are required to produce the corresponding diversity (Figure 4) (see discussion in Hood *et al.*, 1973). One can ask whether these genetic events could most reasonably occur during phylogenetic evolution (germ line mechanism) or whether during ontological development (somatic mechanism). The distance between the M70 branch and the other three proteins is as great as that found between certain human κ subgroup branches (Smith *et al.*, 1971). This suggests that the germ line gene encoding M70 is distinct from those encoding M321, T124, and M63. It is however not at present possible to determine whether the three sequences M321, T124, and M63 are products of three distinct germ line MV_κ genes or result from somatic modification of a single MV_κ gene. With regard to a somatic mutation mechanism it is not clear what selective forces might exist to produce, for example, the eight mutational events which separate the M63 and M321 immunocyte clones, nor how many successful mutations can be fixed in a V gene during the lifetime of a normal mammal (see Jerne, 1971, and Cohn, 1971, for speculation in this regard).

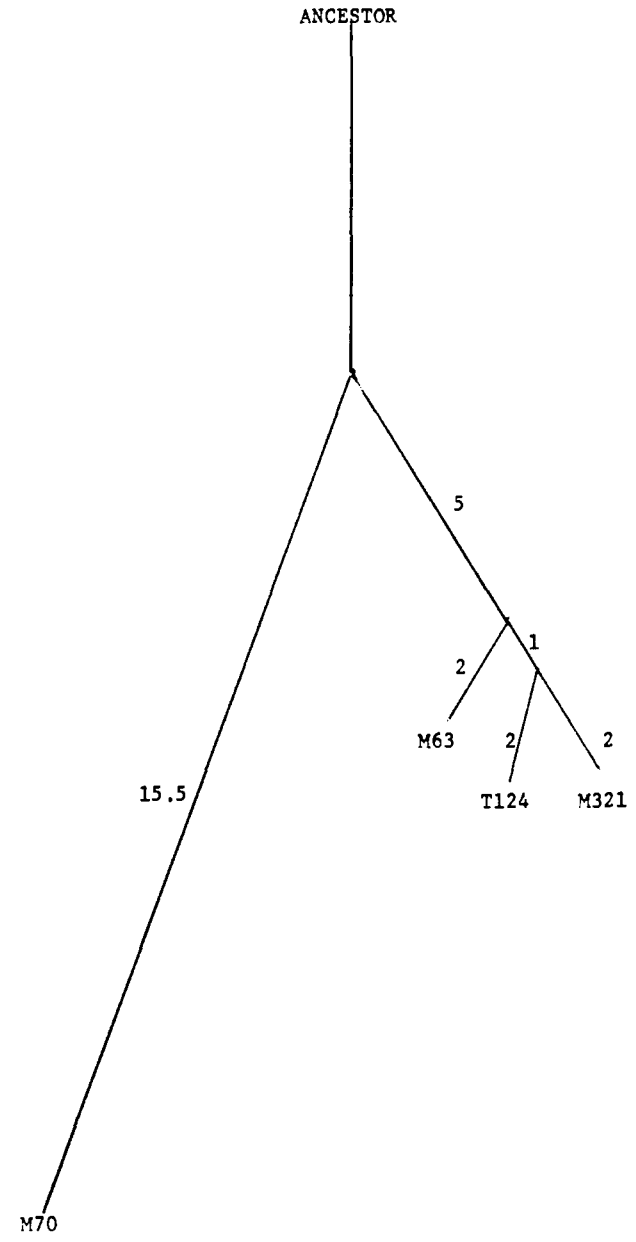

FIGURE 4: A genealogical tree of four mouse κ chains with "limited" sequence differences (see text). Numbers indicate the minimum number of base changes which separate the proteins from their nearest common nodal ancestors. Kindly prepared by Dr. G. Smith according to Smith *et al.* (1970).

These results are particulary interesting when analyzed in terms of the "hypervariable regions." Wu and Kabat (1970) analyzed the complete and partial sequence data available on 77 Bence-Jones proteins and immunoglobulin light chains from man (λ and κ) and from mouse (κ) and found three

TABLE VI: Amino Acid Composition of Tryptic Peptides from CN-B.[a]

	B-T4		B-T5		B-T6		B-T7		B-T9	
	M63	T124	M63	T124	M63	T124	M63	T124	M63	T124
Tyr	0.7 (1)	0.8 (1)		1.1 (1)						
Phe							0.9 (1)	1.0 (1)		
Lys	2.1 (2)	1.9 (2)							1.0 (1)	0.9 (1)
His										
Arg				1.0 (1)	1.2 (1)	1.0 (1)	1.0 (1)	1.1 (1)		
Asp					1.1 (1)	1.0 (1)				
Glu	4.2 (4)	4.2 (4)			1.3 (1)	1.1 (1)			1.0 (1)	1.0 (1)
Thr										
Ser					1.7 (2)	1.9 (2)	2.9 (3)	2.9 (3)		
Pro	2.8 (3)	3.1 (3)			0.9 (1)	1.0 (1)				
Ala					1.9 (2)	2.0 (2)				
Gly	0.9 (1)	1.0 (1)			0.9 (1)	1.0 (1)	2.0 (2)	1.9 (2)		
Val					0.8 (1)					
CMCys										
Ile				0.9 (2)		1.0 (1)			1.0 (1)	1.1 (1)
Leu				2.0 (2)	1.9 (2)	1.1 (1)			0.9 (1)	1.0 (1)
Trp	+[d]	+[d]								
Total residues	12	12		5	12	11	7	7	4	4
Yield (%)[c]	18	6		9	13	23	50	19	54	42

[a–d] See legend to Table II.

TABLE VII: Amino Acid Composition of Chymotryptic Peptides from CN-B.[a]

	B-C7		B-C9		B-C10		B-C11	
	M63	T124	M63	T124	M63	T124	M63	T124
Tyr								
Phe					1.0 (1)	1.0 (1)	0.9 (1)	0.9 (1)
Lys	2.2 (2)	1.9 (2)						
His								
Arg				1.1 (1)	0.9 (1)	1.1 (1)	1.0 (1)	1.0 (1)
Asp			1.0 (1)	1.0 (1)	1.1 (1)		1.0 (1)	1.1 (1)
Glu	2.9 (3)	3.0 (3)			0.9 (1)	0.7 (1)		
Thr							1.3 (1)	0.9 (1)
Ser			1.0 (1)	0.8 (1)	1.9 (2)	1.0 (1)	3.0 (3)	2.9 (3)
Pro	2.8 (3)	2.9 (3)			1.0 (1)	1.0 (1)		
Ala			1.0 (1)	1.0 (1)	1.9 (2)	1.1 (1)		
Gly	0.9 (1)	1.1 (1)			1.0 (1)	1.1 (1)	1.8 (2)	2.1 (2)
Val					1.1 (1)			
CMCys								
Ile						1.0 (1)		
Leu	2.0 (2)	2.1 (2)	2.1 (2)	1.1 (1)	1.8 (2)			
Trp								
Total residues	11	11	5	5	13	8	9	9
Yield (%)[c]	8	20	19	20	8	15	50	32

[a, c] See legend to Table II.

regions of hypervariability extending from residues 24 to 34, 50 to 56, and 89 to 97 (see Figure 3). These observations were extended by the findings of Weigert *et al.* (1970) and Appella (1971) who examined a total of 11 mouse λ chains. From compositional analyses of peptides and sequence analysis they concluded that seven λ chains were identical and that the remaining four showed variation only in the hypervariable regions. In comparing M321 and T124 all three amino acid

B-T10		B-T12		B-T13		B-T14		B-T15		B-T16	
M63	T124	M63	T124	M63	T124	M63	T124	M63	T124	M63	T124
										1.0 (1)	1.2 (1)
		1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)			1.0 (1)	1.0 (1)		
1.0 (1)	1.0 (1)					0.8 (1)	0.9 (1)				
		2.1 (2)	1.9 (2)			1.2 (1)	1.0 (1)	3.7 (4)	3.6 (4)	1.2 (1)	1.1 (1)
						1.2 (1)	1.1 (1)	2.1 (2)	2.2 (2)		
								1.0 (1)	1.1 (1)	1.2 (1)	0.9 (1)
						1.0 (1)	1.0 (1)	1.9 (2)	1.9 (2)	2.5 (3)[b]	2.8 (3)[b]
						1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)		
		0.9 (1)	1.0 (1)					1.0 (1)	0.9 (1)		
		1.0 (1)	1.0 (1)			0.9 (1)	0.9 (1)				
								0.9 (1)	1.0 (1)		
				+[d]	+[d]			+[d]	+[d]		
1	1	5	5	2	2	6	6	14	14	6	6
23	16	46	21	6	15	33	38	17	11	13	8

B-C12		B-C13		B-C14		B-C15		B-C16	
M63	T124	M63	T124	M63	T124	M63	T124	M63	T124
0.9 (1)	0.9 (1)								
		2.0 (2)	0.8 (1)	1.1 (1)	1.1 (1)				
						0.8 (1)	1.1 (1)	0.8 (1)	1.0 (1)
								1.0 (1)	1.0 (1)
3.0 (3)	3.3 (3)	3.2 (3)	1.1 (1)					1.0 (1)	1.0 (1)
0.8 (1)	1.0 (1)	2.9 (3)	2.9 (3)					1.2 (1)	1.0 (1)
2.9 (3)	2.9 (3)	0.9 (1)		1.0 (1)	0.9 (1)	1.0 (1)	1.1 (1)	0.9 (1)	1.0 (1)
			1.1 (1)				0.9 (1)		
1.0 (1)	1.0 (1)	0.9 (1)	1.1 (1)					0.9 (1)	1.0 (1)
3.2 (3)	2.2 (2)		1.0 (1)					3.1 (3)	2.9 (3)
						3.0 (3)	2.0 (2)		
1.1 (1)	2.0 (2)								
		0.6 (1)	0.7 (1)						
1.1 (1)	0.9 (1)							1.0 (1)	1.1 (1)
1.2 (1)	1.0 (1)					1.2 (1)	1.0 (1)		
		0.6 (1)	0.6 (1)						
15	15	12	10	2	2	6	6	10	10
11	17	8	13	16	17	34	20	37	21

substitutions are in the hypervariable regions (see Figure 3 and Table XII). M63 has all but one of its substitutions (an amide which may or may not be a true difference, see Hood *et al.*, 1973) in or near these hypervariable regions when compared to M321 and T124. M70 has more than 40% of its substitutions in the hypervariable regions when compared with M321, T124, or M63. This is of particular interest since the hypervariable regions constitute about 25% of the variable

TABLE VIII: Amino Acid Sequence of Tryptic Peptides from CN-B.

Peptide	M63	T124
B-T4	(Glx,Trp,Tyr,Glx,Glx,Lys,Pro,Gly,Glx,Pro,Pro,Lys)	(Glx,Trp,Tyr,Glx,Glx,Lys,Pro,Gly,Glx,Pro,Pro,Lys)
B-T5		Leu-Leu-Ile-Tyr-Arg
B-T6	(Leu,Ala,Ser,Asx,Leu,Glx,Ser,Gly,Val,Pro,Ala,Arg)	Ala-Ser-Asx-Leu-Glx-Ser-Gly-Ile-Pro-Ala-Arg
B-T7	Phe-Ser-Gly-Ser(Gly,Ser)Arg	Phe-Ser-Gly-Ser-Gly-Ser-Arg
B-T9	Leu-Glu-Ile-Lys	Leu-Glu-Ile-Lys
BT-10	Arg	Arg
B-T12	Asx(Ile,Asx,Val,Lys)	Asx(Ile,Asx,Val,Lys)
B-T13[a]	(Trp,Lys)	(Trp,Lys)
B-T14	Ile(Asx,Gly,Ser,Glx,Arg)	Ile(Asx,Gly,Ser,Glx,Arg)
B-T15[a]	Glx(Asx,Gly,Val,Leu,Glx,Ser,Asx,Thr,Asx,Trp,Asx,Ser,Lys)	Glx(Asx,Gly,Val,Leu,Glx,Ser,Asx,Thr,Asx,Trp,Asx,Ser,Lys)
B-T16	Asx(Ser,Thr,Tyr,Ser,Met)	Asx(Ser,Thr,Tyr,Ser,Met)

[a] Tryptophan was determined by Ehrlich's stain.

TABLE IX: Amino Acid Sequence of Chymotryptic Peptides from CN-B.

Peptide	M63	T124
B-C7	Glx-Glx-Lys-Pro-Gly-Glx-Pro-Pro-Lys-Leu-Leu	Glx-Glx-Lys-Pro-Gly-Glx-Pro-Pro-Lys-Leu-Leu
B-C9	(Leu,Ala,Ser)Asn-Leu	Arg-Ala-Ser-Asn-Leu
B-C10	Leu-Ala-Ser-Asn-Leu-Glx-Ser-Gly-Val-Pro-Ala-Arg-Phe	(Glx,Ser,Gly,Ile,Pro,Ala,Arg,Phe)
B-C11	Ser-Gly-Ser-Gly-Ser-Arg-Thr-Asx-Phe	(Ser,Gly,Ser,Gly,Ser,Arg,Thr,Asx)Phe
B-C12	Thr-Leu-Thr-Ile-Asx-Pro-Val-Glx-Ala-Asx-Asx-Ala-Ala-Thr-Tyr	Thr-Leu-Thr-Ile-Asx-Pro-Val-Glx-Ala-Asx-Asx-Val-Ala-Thr-Tyr
B-C13	Phe-Cys-Glx-Glx-Asx-Asx-Glx-Asx-Pro-Trp(Thr,Phe)	Phe-Cys-Glx-Glx-Ser-Asx-Glx-Ala-Pro-Trp
B-C14	Thr-Phe	Thr-Phe
B-C15	Gly-Gly-Gly-Thr-Lys-Leu	Gly-Ser-Gly-Thr-Lys-Leu
B-C16	Glx(Ile,Lys,Arg,Ala,Asx,Ala,Ala,Pro,Thr)	Glx(Ile,Lys,Arg,Ala,Asx,Ala,Ala,Pro,Thr)

TABLE X: Amino Acid Sequence of Tryptic Peptides from Aminoethylated Light Chain.

Peptide	M63	T124
T11a	Arg-Ala(Asx,Ala,Ala,Pro,Thr,Val,Ser,Ile,Phe,Pro,Pro,Ser,Ser,Glx,Glx,Leu,Thr,-Gly,Gly,Ala,Ser,Ser,Val,Val,Cys)	Arg-Ala(Asx,Ala,Ala,Pro,Thr,Val,Ser,Ile,Phe,Pro,Pro,Ser,Ser,Glx,Glx,Leu,Thr,-Gly,Gly,Ala,Ser,Ser,Val,Val,Cys)
T11b	Phe(Leu,Asx,Asx,Phe,Tyr,Pro,Lys)	Phe(Leu,Asx,Asx,Phe,Tyr,Pro,Lys)
T19a	His(Asx,Ser,Tyr,Thr,Cys)	
T19b	Glx(Ala,Thr,His,Lys)	

TABLE XI: Amino Acid Composition of Tryptic Peptides from Aminoethylated Light Chains.[a]

	T11a		T11b		T19a		T19b	
	M63	T124	M63	T124	M63	T124	M63	T124
Tyr			0.9 (1)	0.9 (1)	1.1 (1)			
Phe	1.3 (1)	0.9 (1)	2.2 (2)	1.9 (2)				
Lys			0.9 (1)	1.0 (1)			0.9 (1)	
His					0.9 (1)		1.0 (1)	
Arg	0.8 (1)	0.9 (1)						
Asp	1.2 (1)	1.1 (1)	2.0 (2)	2.2 (2)	0.9 (1)			
Glu	2.3 (2)	2.1 (2)					1.0 (1)	
Thr	1.8 (2)	2.0 (2)			1.2 (1)		1.0 (1)	
Ser	4.8 (5)	4.7 (5)			0.9 (1)			
Pro	2.7 (3)	3.2 (3)	1.0 (1)	1.1 (1)				
Ala	4.0 (4)	4.1 (4)					1.1 (1)	
Gly	2.2 (2)	2.0 (2)						
Val	3.1 (3)	3.0 (1)						
CMCys	1 (1)[d]	1 (1)[d]			1 (1)[d]			
Ile	0.8 (1)	1.1 (1)						
Leu	1.0 (1)	1.0 (1)	1.1 (1)	0.9 (1)				
Total residues	27	27	8	8	6		5	
Total (%)[c]	19	7	5	23	6		9	

[a,c] See legend to Table II. [d] Value of AECys estimated by peak height.

regions. When two other previously sequenced mouse κ chains with even more extensive amino acid substitutions, M41 and and M21 (Milstein and Svasti, 1971) are compared (Table XII) about 40% of the sequence differences are located in the hypervariable regions.

The three hypervariable regions appear to comprise a part of the active (antigenic) site of the antibody molecule in that affinity labels have been attached to each of these three regions (Eisen, 1971). Accordingly, amino acid sequence variation in these regions is required to produce a diverse series of active sites. It will be of interest to determine whether or not there is some special feature of the respective immunoglobulin genes or chromosome structure that permits mutation in these regions to occur at an accelerated rate (Benzer, 1961; Okada *et al.*, 1972).

Weigert *et al.* (1971) have argued from the mouse λ sequence data cited above that the effective mutations for changing antibody specificity are somatically produced and occur only in the hypervariable regions. They suggest that only the mutations within the hypervariable regions confer a positive selective advantage on a given immunocyte clone, and that any light chain with an amino acid substitution *outside* a hypervariable region must be encoded by an additional germ line V gene. When this criterion is applied to the mouse V_κ sequences presented in the first paper of this series (Hood *et al.*, 1973), at least 32 germ line genes are required for the 44 MV_κ amino-terminal sequences examined. In addition our study of the nearly complete sequences of four similar MV_κ sequences add yet another V_κ gene to this list (one for M70, one for M63 and one for the M321–M124 pair). Thus according to the hypothesis put forward by Weigert and coworkers, 33 MV_κ genes must encode the first 44 mouse κ chains examined (almost one gene per protein). This diversity at the amino terminus of mouse κ chains has shown no tendency to saturate, thus suggesting an even greater number of unique MV_κ sequences and accordingly a large number of germ line V_κ genes. A summary of the nearly complete MV_κ sequences of the BALB/c mouse now available is given in Figure 5.

A crude genealogic comparison of MV_κ regions M70, M321, T124, and M63 with human V_κ regions can be carried out by determining the ancestoral sequence for the set of four related

TABLE XII: Amino Acid and Nucleotide Substitutions among V_κ Regions of the BALB/c Mouse.

Light Chains Compared	Amino Acid Substitutions[a]	Amino Acid Substitutions in Hypervariable Regions[b]		Nucleotide Substitutions[c]	Nucleotide Substitutions in Hypervariable Regions[c]	
		No.	%		No.	%
M321-T124	3	3	100	4	4	100
M321-M63	8	4	50	8	4	50
T124-M63	8	4	50	8	4	50
M321-M70[d]	21	10	48	24	12	50
T124-M70[d]	20	9	45	25	13	52
M63-M70[d]	20	10	50	22	11	50
M41-M70[d]	45	15	33			
M41-M21[d]	44	19	43			
M70-M21[d]	42	17	40			

[a] Glx and Asx comparisons against the acid or amide were treated as identities. [b] Hypervariable regions constitute about 24.4% of the variable region and are defined in Figure 4. [c] Minimum nucleotide substitutions. [d] The sequences for these proteins were taken from Gray *et al.* (1967) [M41 and M70] and Milstein and Svasti (1971) [M21].

```
                                                    10                                                  20
MOPC 70      Asp Ile  Val Leu Thr Gln Ser  Pro Ala Ser  Leu Ala Val Ser  Leu Gly Gln Arg Ala Thr Ile  Ser Cys Arg Ala
MOPC 321     Asp Ile  Val Leu Thr Gln Ser  Pro Ala Ser  Leu Ala Val Ser  Leu Gly Gln Arg Ala Thr Ile  Ser Cys Arg Ala
TEPC 124     Asp Ile  Val Leu Thr Gln Ser  Pro Ala Ser  Leu Ala Val Ser  Leu Gly Gln Arg Ala Thr Ile  Ser Cys Arg Ala
MOPC 63      Asp Ile  Val Leu Thr Gln Ser  Pro Ala Ser  Leu Ala Val Ser  Leu Gly Gln Arg Ala Thr Ile  Ser Cys Arg Ala
MOPC 41      Asp Ile  Gln Met Thr Gln Ser  Pro Ser Ser  Leu Ser Ala Ser  Leu Gly Glu Arg Val Ser Leu  Thr Cys Arg Ala
MOPC 21      Asn Ile  Val Met Thr Gln Ser  Pro Lys Ser  Met Ser Met Ser  Val Gly Glu Arg Val Thr Leu  Thr Cys Lys Ala

                27  27a 27b 27c 27d 28       30                                                  40
70       Ser Gln Ser Val Asx Asx Ser Gly Ile  Ser Phe Met Asn Trp Phe Glx Glx Lys Pro Gly Glx Pro Pro Lys[Leu Leu
321      Ser Lys Ser Val Asn Thr Tyr Gly Asn  Ser Phe Met Glx Trp Tyr Glx Glx Lys Pro Gly Glx Pro Pro Lys Leu Leu
124      Ser Gln Ser Val Asx Trp Tyr Gly Asx  Ser Phe Met(Glx Trp Tyr)Glx Glx Lys Pro Gly Glx Pro Pro Lys Leu Leu
63       Ser Glx Ser Val Asx Ser Tyr Gly Asx  Ser Phe Met(Glx Trp Tyr)Glx Glx Lys Pro Gly Glx Pro Pro Lys Leu Leu
41       Ser Gln(Asx Ile  Gly  —   —   —   —)Ser Leu Ser  Asx Trp Leu Glx Glx(Gly Pro Asx Thr Glx)Ile  Lys Arg Leu
21       Ser Glu Asn Val Val  —   —   —   —  Thr Tyr Val  Ser Trp Tyr Gln Gln Lys Pro Glu Gln Ser Pro Lys Leu Leu

                    50                                                                              70
70       Ile Tyr Ala Ala Ser  Asn Gln Gly Ser  Gly Val Pro  Ala Arg Phe Ser  Gly Ser  Gly Ser  Gly Thr Asp Phe Ser  Leu
321      Ile Tyr Arg Ala Ser  Asn Leu Glx Ser  Gly Ile  Pro  Ala Arg Phe Ser  Gly Ser  Gly Ser  Arg(Thr Asx)Phe Thr  Leu
124      Ile Tyr Arg Ala Ser  Asn Leu Glx Ser  Gly Ile  Pro  Ala Arg Phe Ser  Gly Ser  Gly Ser  Arg(Thr Asx Phe Thr  Leu
63        ?   ? [Leu Ala Ser  Asn Leu Glx Ser  Gly Val Pro  Ala Arg Phe Ser  Gly Ser  Gly Ser  Arg Thr Asx Phe Thr  Leu
41       Ile Tyr Ala Thr Ser  Ser Leu Asx Ser  Gly Val Pro  Lys Arg Phe Ser  Gly Ser  Arg Ser  Gly Ser  Asp)Tyr Ser  Leu
21       Ile Tyr Gly Ala Ser  Asn Arg Tyr Thr  Gly Val Pro  Asp Arg Phe Thr  Gly Ser  Gly Ser  Ala Thr Asp Phe Thr  Leu

                                    80                                                  90
70       Asn Ile  His Pro Met Glx Glx Asx Asx  Thr Ala Met  Tyr Phe Cys Glx Glx Ser  Lys]Glu Val Pro Trp Thr Phe Gly
321      Thr Ile  Asx Pro Val Glx Ala Asx Asx  Val Ala Thr  Tyr Phe Cys Glx Glx Ser  Asx Glx Asx Pro Trp Thr Phe Gly
124      Thr Ile  Asx Pro Val Glx Ala Asx Asx  Val Ala Thr  Tyr]Phe Cys Glx Glx Ser  Asx Glx Ala Pro Trp Thr Phe Gly
63       Thr Ile  Asx Pro Val Glx Ala Asx Asx  Ala Ala Thr  Tyr Phe Cys Glx Glx Asx  Asx Glx Asx Pro Trp Thr Phe Gly
41       Thr/Ile  Ser Ser Leu/Glu Ser Glu Asp  Phe Val Asp/  Tyr  —  Cys Leu Gln Tyr  Ala Ser Ser Pro Trp Thr Phe Gly
21       Thr Ile  Ser Ser Val Gln Ala Glu Asp  Leu Ala Asp  Tyr His Cys Gly Gln Gly  Tyr Ser Tyr Pro Tyr Thr Phe Gly

         100
70       Gly Gly Thr Lys Leu Glu Ile  Lys
321      Ser Gly Thr Lys Leu Glu Ile  Lys
124      Ser Gly Thr Lys Leu Glu Ile  Lys
63       Gly Gly Thr Lys Leu Glu Ile  Lys
41       Gly Gly Thr Lys Leu Glu Ile  Lys
21       Gly Gly Thr Lys Leu Glu Ile  Lys
```

FIGURE 5: The V_κ regions of six light chains from the BALB/c mouse. M70 and M41 are taken from Gray *et al.* (1967); M21 from Milstein and Svasti (1971).

MV_κ regions and the ancestoral sequences for each of the three major human V_κ genealogic branches (V region subgroups). The ancestoral sequence in each case can be approximated by selecting the major residue expressed at each position. For example, the ancestoral sequence in the M70, M321, T124, M63 group for residues 27–30 (Figure 5) is

```
27  27a 27b 27c 27d 28  29  30
-Gln-Ser-Val-Asn-  ? -Tyr-Gly-Asn-
```

The residue at position 27d cannot be determined as each of the four MV_κ regions differs. The ancestoral sequences for the three human V_κ region subgroups are given in Milstein and Pink (1970). The mouse ancestoral V_κ sequence differs from each of the three human ancestoral sequences at 15 or more positions. If this particular branch of the mouse genealogic tree is encoded by multiple germ line genes, as seems to be suggested by considerations discussed above, then one must explain how multiple MV_κ genes (even 2) can evolve in parallel such that 10 or more residues distinguish them from most human V_κ regions. We feel that the MV_κ genes must have diverged from a common ancestor subsequent to the divergence of man and mouse. Thus it would appear that multiple gene systems such as immunoglobulins can undergo rapid gene expansion and contraction (for a more complete discussion of this point, see Hood and Prahl, 1971; Gally and Edelman, 1970). Such an explanation is consistent with the suggestion that increased diversity in the mouse κ chains (presumably reflecting an increased number of germ line MV_κ genes) may compensate for the apparent loss of mouse V_λ genes (as reflected in low serum expression and limited numbers of myeloma λ chains with restricted heterogeneity).

The extent of amino acid sequence diversity found in the amino-terminal portion of 44 light chains suggests that the V_κ immunoglobulin family of the BALB/c mouse is multigenic in nature. This supposition is reinforced by the observation that the extent of sequence diversity appears as great in the second 20 V_κ regions examined as in the first. The multigene nature of this immunoglobulin family is also consistent with our observations on V region sequences from four κ chains with nearly identical amino-terminal sequences. The question as to whether all of the information for immunoglobulin diversity is encoded in the germ line or whether somatic modification increases this diversity in a manner that is useful to the organism remains unanswered. However, the immunoglobulins are clearly a fascinating model for the study of evolutionary mechanisms in multigenic systems.

References

Appella, E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 590.

Benzer, S. (1961), *Harvey Lectures 56*, 211.

Cohn, M. (1971), *Ann. N. Y. Acad. Sci. 190*, 529.

Dayhoff, M. (1969), Atlas of Protein Sequence and Structure, Silver Spring, Md., National Biomedical Research Foundation.

Eisen, H. N. (1971), *Progr. Immunol. 1*, 243.

Gally, J., and Edelman, G. M. (1970), *Nature (London) 227*, 341.

Gray, W. R., Dreyer, W. J., and Hood, L. (1967), *Science 155*, 465.
Jerne, N. (1971), *Eur. J. Immunol. 1*, 1.
Hood, L., McKean, D., Farnsworth, V., and Potter, M. (1973), *Biochemistry 12*, 741.
Hood, L., and Prahl, J. (1971), *Advan. Immunol. 14*, 291.
McKean, D., Potter, M., and Hood, L. (1973), *Biochemistry 12*, 749.
Milstein, C., and Pink, J. R. L. (1970), *Progr. Biophys. Mol. Biol. 21*, 211.
Milstein, C., and Svasti, J. (1971), *Progr. Immunol. 1*, 33.
Okada, Y., Streisinger, G., Owen, J., Newton, J., Tsugita, A., and Inouye, M. (1972), *Nature (London) 236*, 338.
Smith, G. P., Hood, L., and Fitch, W. M. (1971), *Annu. Rev. Biochem. 40*, 969.
Weigert, M. G., Cesari, I. M., Yonkovich, S. J., and Cohn, M. (1970), *Nature (London) 228*, 1045.
Wu, T. T., and Kabat, E. (1970), *J. Exp. Med. 132*, 211.

Kinetics of the Reaction of Chymotrypsin A_α with Peptide Chloromethyl Ketones in Relation to its Subsite Specificity†

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ABSTRACT: The kinetics of the reaction of a series of peptide chloromethyl ketones with chymotrypsin A_α were investigated in order to relate rates of reaction in solution with the number of interactions which are observed in the crystallographic model of the inhibited enzyme (Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10*, 3728). The second-order rate constant (k_{obsd}/[I]) obtained at pH 5.02 and 5.80 in either 9 or 30% 1,2-dimethoxyethane varied by a factor of 27 from the slowest (CHO-PheCH_2Cl) to the fastest inhibitors (Z-Gly-Leu-PheCH_2Cl and Boc-Gly-Leu-PheCH_2Cl). The variation in the rates of inhibition with changing inhibitor structure could be a function of strength of binding of the inhibitor to the enzyme, stereoelectronic effects on the bond-forming step between enzyme and enzyme-bound inhibitor, or both. The increased reactivity of inhibitors containing a leucyl residue as the P_2 residue (Ac-Leu-PheCH_2Cl/Ac-Ala-PheCH_2Cl = 2.9, Boc-Gly-Leu-PheCH_2Cl/Boc-Ala-Gly-PheCH_2Cl = 3.5–3.9) is the result of a hydrophobic interaction between the Leu residue of the inhibitor and Ile-99 of the enzyme. This result correlated nicely with the previous observed "secondary specificity" of chymotrypsin for substrates with bulky aliphatic side chains as the P_2 residue. Inhibitors containing three amino acid residues reacted faster than Ac-PheCH_2Cl or dipeptide chloromethyl ketones. This again agrees with the crystallographic model since tripeptide inhibitors could form a β-sheet structure involving three hydrogen bonds with the enzyme while the others would form fewer hydrogen bonds. These results strengthen the view that the solution reactivity of peptide chloromethyl ketones and substrates can be explained on the basis of the crystal structures of chloromethyl ketone inhibited chymotrypsin derivatives.

The crystallographic determinations of the binding modes of peptide chloromethyl ketones to chymotrypsin A_γ (Segal *et al.*, 1971a,b) and to subtilisin BPN′ (Kraut *et al.*, 1971; Robertus *et al.*, 1972) have provided revealing insights into the interactions of inhibitors with these serine proteases. The inhibitors used in these studies are related to Tos-PheCH_2Cl, the stereospecific, active site-directed inhibitor of chymotrypsin which was designed by Schoellmann and Shaw (1963) and which is known to react irreversibly with His-57 in the enzyme (Ong *et al.*, 1964, 1965). For the crystallographic studies, the tosyl group of Tos-PheCH_2Cl was replaced by peptide chains of varying length so that the inhibitors would more closely resemble natural peptide substrates and would interact with subsites of the enzyme on the N-terminal side of the scissile bond of a peptide substrate. It was found that both enzymes presented an extended binding site composed of at least three subsites in the case of chymotrypsin and four in the case of subtilisin. Furthermore, the extended binding sites in the two proteases were found to be very similar in three-dimensional structure (Kraut *et al.*, 1971).

Although the crystallographic results are very convincing, great care must be exercised in extrapolating the structural features of crystalline enzyme–inhibitor complexes to models of the intermediates which take part in the dynamic processes of enzyme catalysis in solution. Relevant data from various types of physical chemical experiments in solution are required before conclusions drawn from the crystalline state can be satisfactorily evaluated.

One direct approach is to examine the crystallographic model for possible interactions between subsites and various amino acid residues substituted into the inhibitor chain, predict how these interactions would affect the binding of analogous substrates, and test the prediction by kinetic measurements of the hydrolysis rates of the corresponding synthetic peptide substrates. The results of some studies of this kind have been reported (Segal *et al.*, 1971b; Segal, 1972) and they

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‡ Dr. Philip E. Wilcox died on Nov 2, 1971. This is a great loss to those who have known him personally, or followed his work in protein chemistry.