Amino Acid Sequence of the Heavy-Chain Variable Region of the Crystallizable Human Myeloma Protein Dob†

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ABSTRACT: The amino acid sequence of the heavy-chain variable region of the crystallizable human myeloma protein Dob has been determined. This protein has previously been shown to have a deletion in the hinge region [Lopes, A. D., & Steiner, L. A. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1003; Steiner, L. A., & Lopes, A. D. (1979) *Biochemistry* (preceding paper in this issue)]. The complete sequence was established by analysis, in the automated sequenator, of the intact Fd′ piece and of three large overlapping fragments prepared from Fd′ by digestion with cyanogen bromide, by tryptic digestion of the citraconylated Fd′, and by cleavage with hydroxylamine. Portions of the sequence were confirmed by examination of the amino acid composition and the partial sequence of a variety of small peptides obtained by enzymatic degradation. The Dob heavy-chain variable region appears to belong to the V_HIII subgroup, but there are several unusual substitutions. Residue 45 in the Dob sequence is proline, although all other known heavy-chain sequences in man, mouse, rabbit, and guinea pig have leucine at this position. Positions 10 (aspartic acid), 68 (alanine), and 82 (leucine) in the Dob sequence are also atypical. There is no deleted segment in the variable region of the Dob heavy chain nor any abnormality in the variable–constant joining region. The hinge-region deletion appears to be the only gross structural anomaly in the Dob heavy chain.

The crystallizable human IgG1 myeloma protein Dob, which has been studied by X-ray diffraction (Sarma et al., 1971; Silverton et al., 1977), has been found to have a deletion in the hinge region[1] of the heavy chain (Lopes & Steiner, 1973; Steiner & Lopes, 1979). Fifteen amino acid residues (positions 216–230),[2] including the three half-cystines that ordinarily participate in interchain bonding in this subclass, are missing. Instead of four labile interchain disulfide bridges, the Dob protein has only one such bridge, which connects the two light chains; the heavy chains are monomeric. To determine whether any additional unusual structural features occur in the Dob molecule, we have determined the complete amino acid sequence of the variable region of its heavy chain. In other pathological immunoglobulins, deletions of varying extent have been found in this domain (Franklin & Frangione, 1975). We report here that there are no gross structural anomalies in the variable region of the Dob γ1 chain. Although there are some unusual amino acid substitutions, the Dob V_H sequence can be assigned to the V_HIII subgroup.

Experimental Procedures

Materials. Trypsin (treated with (L-tosylamido-2-phenyl)ethyl chloromethyl ketone), chymotrypsin, pepsin, and carboxypeptidase A (treated with diisopropyl fluorophosphate) were purchased from Worthington Biochemical Corp. Carboxypeptidase B (chromatographically purified) and soybean trypsin inhibitor were obtained from Sigma Chemical Co. Iodoacetic acid was recrystallized from ether–petroleum ether; [1-^{14}C]iodoacetic acid was obtained from New England Nuclear. Spectrapor No. 1 dialysis tubing with nominal molecular weight cutoff (6000–8000) and Spectrapor No. 3 dialysis tubing (cutoff 3500) were obtained from Spectrum Medical Industries, guanidine hydrochloride and urea (Ultra Pure) were from Schwarz/Mann, constant-boiling HCl, citraconic anhydride, ninhydrin, hydrindantin (dihydrate), and thiodiglycol were from Pierce, dimethyl sulfoxide was from Crown Zellerbach, and hydroxylamine hydrochloride was from Baker.

Dob Fd′ and Heavy Chains. The preparation of Fd′ from the Dob myeloma protein has been described (Steiner & Lopes, 1979). Heavy chains were isolated after partial reduction of Dob IgG (to break the interchain bond between the light chains) and alkylation with iodoacetamide, followed by separation of heavy and light chains by gel filtration with Sephadex G-100 in 8 M urea and 1 M acetic acid.

Cleavage of Fd′ with Cyanogen Bromide. Fd′ (600 nmol) in 1.2 mL of 70% formic acid was reacted with 108 mg of CNBr at room temperature for 24 h. Distilled water (30 mL) was added and the solution was lyophilized.

Reduction and Alkylation of Fd′. Fd′ (1.16 μmol) was reduced under N_2 in 2.0 mL of 0.015 M dithiothreitol, 7 M guanidine hydrochloride, 0.5 M Tris-HCl, and 0.002 M EDTA, pH 8.0, for 4 h at 37 °C. Alkylation was with 78 μmol of iodoacetic acid containing 50 μCi of [1-^{14}C]iodoacetic acid. After 1 h at room temperature in the dark, 0.1 mL of 0.3 M dithiothreitol was added, and incubation was continued at room temperature for 30 min. The protein was dialyzed in Spectrapor No. 1 tubing against 1 M and then 0.1 M acetic acid; it was analyzed in the sequenator and used for tryptic cleavage after citraconylation and for hydroxylamine cleavage, as

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[1] γ1 is the heavy chain of IgG1; Fd′ is the N-terminal half of the heavy chain, obtained after peptic digestion of the IgG1; V_H and V_L are the variable domains of the heavy and light chains, respectively; C_H1, C_H2, and C_H3 are the constant domains of the heavy chain; the hinge region is the segment of heavy chain between the C_H1 and C_H2 domains; V_HI, V_HII, and V_HIII are variable-region subgroups of human heavy chains.

[2] Residue positions in the constant domains of the Dob heavy chain are numbered according to the Eu γ1 sequence (Edelman, 1970); residue positions in the variable region are numbered according to the Dob sequence determined here, except for Figures 8 and 9, and associated discussion (including Abstract), in which the numbering system of Kabat et al. (1976) is used. For the variable regions of light chains, the numbering is that of Kabat et al.

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described below. The CNBr digest of Fd' was reduced in guanidine hydrochloride and alkylated with [1-^{14}C]iodoacetic acid by a similar procedure (see legend to Figure 2). For direct digestion with trypsin, Fd' was reduced and alkylated with iodoacetamide, as described in the preceding paper (Steiner & Lopes, 1979).

Reaction of Fd' with Citraconic Anhydride and Digestion with Trypsin. Reduced, alkylated Fd' (440 nmol; 2.9×10^6 cpm) was lyophilized, dissolved in 2.0 mL of 7 M guanidine hydrochloride and 0.05 M sodium carbonate buffer (pH 9.0), and reacted with citraconic anhydride (Dixon & Perham, 1968) at room temperature; 30 μL of reagent was added in 5-μL aliquots at 10-min intervals with constant stirring. The pH was maintained between 8.5 and 9.5 by the addition of 5 N NaOH. Fifteen minutes after the last addition of reagent, the solution was desalted on a column (1.1 × 20 cm) of Sephadex G-25 (fine) equilibrated in 0.1 M NH_4HCO_3 adjusted to pH 9.0 with NH_4OH. The effluent was monitored by measurement of the optical density, radioactivity, and spot test for guanidine (precipitation with $NaDodSO_4$).[3] The major protein-containing fractions (5.7 mL containing 97% of the total protein) were pooled, and 0.1 mg of trypsin was added. After 2 h at 37 °C another aliquot of 0.1 mg of trypsin was added, followed after another 2 h by 0.3 mg of soybean trypsin inhibitor. The digest remained clear. To determine the C-terminal residues of the tryptic peptides, aliquots were removed for amino acid analysis and for digestion with carboxypeptidases A and B. The rest of the digest was fractionated by gel filtration with Sephadex G-50 as described in the legend to Figure 4. The optical density and radioactivity of the fractions were determined immediately, and the fractions were frozen at −18 °C. Subsequently, citraconyl groups were removed by incubation in 5% formic acid for 24 h at room temperature.

Cleavage of Fd' with Hydroxylamine, Followed by Reaction with Citraconic Anhydride and Trypsin. Reduced, alkylated Fd' (88 nmol; 5.8×10^5 cpm) was lyophilized and dissolved in 1.5 mL of 2 M hydroxylamine and 6 M guanidine hydrochloride adjusted to pH 9.2 with LiOH, as described by Bornstein & Balian (1977). During the 4-h incubation at 45 °C, the pH dropped to 8.7. Formic acid (88%, 0.3 mL) was added to terminate the reaction, bringing the pH to 2.5, and the solution was applied to a 1.1 × 20 cm column of Sephadex G-25 in 9% formic acid. The material eluting in the void volume was lyophilized and taken up in 1.4 mL of 5 M guanidine hydrochloride and 0.07 M sodium carbonate buffer, pH 8.2. The solution was placed in an ice bath, and citraconic anhydride was added in six 2-μL aliquots at 10-min intervals with constant stirring. The pH was maintained near 8.0 (7.8–8.4) with 2 M NaOH in 5 M guanidine. Thirty minutes after the last addition, the solution was applied to the same Sephadex G-25 column, but in 0.1 M NH_4HCO_3 adjusted to pH 8.6. The material eluting in the void volume (now estimated to be 50 nmol) was pooled, and 27 μg of trypsin was added. After 1 h at 37 °C, 40 μg of trypsin inhibitor was added, and the sample was incubated for 15 min at room temperature. Aliquots were removed for amino acid analysis and for digestion with carboxypeptidase B, and the remainder of the sample was applied to a column of Sephadex G-50, as described in the legend to Figure 7.

Other Enzymatic Digestons. The digestion of reduced, alkylated Fd' with trypsin has been described (Steiner & Lopes, 1979). Fragment CB1 (154 nmol) was digested with 25 μg of trypsin in 100 μL of 0.2 M NH_4HCO_3 for 8 h. Tryptic peptides were digested with 7–10 μg of chymotrypsin or pepsin per 40 nmol of peptide in 50 μL of 0.1 M NH_4HCO_3 or 5% formic acid, respectively. For digestion with carboxypeptidase A or B, 1–5 μg of enzyme was added to 0.5–5 nmol of peptide in 50 μL of 0.1 M NH_4HCO_3; after 1 h at 37 °C, the sample was dried and taken up in 50 μL of pH 2.2 citrate buffer, and 40 μL was applied directly to the amino acid analyzer. Corrections were made for appropriate enzyme and substrate blanks.

Isolation of Peptides. Mixtures of peptides were separated by gel filtration, followed by chromatography and/or high-voltage electrophoresis on Whatman No. 1 paper. Two chromatographic systems were used: 1-butanol–acetic acid–pyridine–H_2O (15:3:10:12) and the upper (organic) phase of 1-butanol–acetic acid–H_2O (4:1:5). Chromatograms were developed in glass containers by the descending technique, usually for about 18 h. Electrophoresis was carried out at 50–70 V/cm for 40–60 min in Savant tanks with Varsol as the coolant. Three buffer systems were used: pH 6.5, pyridine–acetic acid–H_2O (33:1:300); pH 3.5, pyridine–acetic acid–H_2O (1:10:190); pH 1.9, formic acid–acetic acid–H_2O (1:4:45). Peptides were spotted along a strip (10–20 nmol/cm). When radiolabeled peptides were analyzed, autoradiography was carried out with Kodak XR-5 film for about 18 h. Guide strips (0.5 cm) were then cut and stained with cadmium–ninhydrin (Heilmann et al., 1957). If a peptide appeared to be well separated, it was eluted with dilute NH_4OH or acetic acid. If additional purification was necessary, the main strip was sewn into another sheet of Whatman No. 1 paper as described by Naughton & Hagopian (1962), and electrophoresis was carried out again, usually at another pH. For analytical purposes, two-dimensional analyses were carried out by chromatography, followed by electrophoresis, or by two steps of electrophoresis at different pH values. After electrophoresis at pH 6.5, the mobility of peptides was expressed relative to the distance between aspartic acid and the neutral amino acid spot. The net charge of peptides was calculated from data provided by Offord (1966) and was correlated with results of amino acid analysis to determine the number of amide groups.

Manual Sequence Analysis. The micro dansyl–Edman procedure (Hartley, 1970), as modified by Fleischman (1973), was used; approximately 0.5 nmol of peptide was taken for dansylation after each cycle of degradation. Sometimes, the reaction was carried out with a larger amount of peptide, and aliquots (1–2 nmol) were also removed after each cycle for amino acid analysis (subtractive method). The position of the amide group in peptide T6 was determined as follows: after zero, one, and two cycles of degradation, aliquots containing ~8 nmol of residual peptide were removed, and the mobility of these three peptides was determined by high-voltage electrophoresis.

Automated Sequence Analysis. Automated Edman degradation was performed in a Beckman 890B Sequencer modified with nitrogen blow-down and an undercut cup adaptation (Beckman Kit No. 335481), using a 0.1 M Quadrol program previously described (Brauer et al., 1975). Aliquots of PTH-norleucine were added to each tube in the fraction collector to serve as an internal standard.

PTH amino acids obtained after conversion at each cycle were identified by gas–liquid chromatography, thin-layer chromatography, and in certain instances by high-pressure liquid chromatography and amino acid analysis. Gas–liquid

[3] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

chromatography on 10% SP-400 was used to detect and quantitate the PTH derivatives of alanine, serine, cysteine (as *S*-(carboxymethyl)cysteine), glycine, valine, proline, threonine, leucine, isoleucine, methionine, and phenylalanine (Brauer et al., 1975). The temperature program was terminated before the elution of PTH-tyrosine and PTH-tryptophan. Three pairs of residues (the PTH derivatives of leucine and isoleucine, serine and *S*-(carboxymethyl)cysteine, and proline and threonine) coeluted in the gas–liquid chromatographic system; these were distinguished by another method. In one experiment the PTH derivatives of aspartic acid, glutamic acid, tyrosine, and tryptophan were identified by on-column silylation with bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.).

Two-dimensional thin-layer chromatography on polyamide sheets was carried out as described by Summers et al. (1973). The PTH derivatives of leucine and isoleucine, which could not be identified by thin-layer chromatography because they migrate with the internal standard, PTH-norleucine, were distinguished by high-pressure liquid chromatography. This was performed on a 0.4 × 30 cm μBondapak C_{18} column (Waters Associates) in a Waters Model ALC/6PC204 liquid chromatograph. Programs for the separation of 20 PTH amino acids using acetonitrile gradients have been described (Margolies & Brauer, 1978). The PTH amino acids recovered were quantitated with a Hewlett-Packard Model 3385 integrator. When no new PTH amino acid was found in the organic phase, PTH-histidine and PTH-arginine were sought in the aqueous phase. In certain instances PTH amino acids were hydrolyzed to the free amino acid with HI as described by Smithies et al. (1971) except that the hydrolysis was carried out for 6 h at 150 °C. Aliquots (10%) from selected cycles of Edman degradation were counted in Bray's solution in a Packard scintillation counter to confirm radiolabeled *S*-(carboxymethyl)cysteine.

At all cycles, two or more methods of PTH amino acid identification were used; at least one of these was quantitative. Yields of PTH amino acids were corrected for background, and repetitive yields were calculated as described previously (Brauer et al., 1975). Fragments Fd′ and HA-CT were analyzed in a single run, fragment CT6 was analyzed in two runs, and fragment CB2 was analyzed in three separate runs in the sequenator.

Amino Acid Analysis. Hydrolysis was carried out under vacuum in 10 × 70 mm ignition tubes containing 100 μL of constant-boiling HCl and maintained at 110 ± 1 °C in a heating block. A Durrum D-500 amino acid analyzer was used. To convert homoserine lactone to homoserine, hydrolyzed samples were treated as described by Ambler & Brown (1967). Values obtained for peptides eluted from paper were corrected by subtraction of appropriate blanks. Recovery of *S*-(carboxymethyl)cysteine was poor when peptides were hydrolyzed after elution from paper, and the presence of [1-^{14}C]-*S*-(carboxymethyl)cysteine was evaluated by autoradiography of the paper strip or by counting an aliquot of the peptide.

Electrophoresis in Polyacrylamide Gels Containing Sodium Dodecyl Sulfate. Analysis of proteins and large fragments was carried out as described by Steiner & Blumberg (1971). Samples in 0.1 M Tris-HCl, pH 8.0, were pretreated by adjusting them to 0.05 M dithiothreitol and 1% $NaDodSO_4$ and heating to 56 °C for 1 h, or by adjusting them to 0.05 M iodoacetamide and 1% $NaDodSO_4$ and boiling for 1 min. For the analysis of small fragments (molecular weight less than 15000), the urea–$NaDodSO_4$ system described by Swank & Munkres (1971) was also used.

Nomenclature. Peptides are numbered beginning at the N terminus. The prefixes CB, CT, HA, T, P, and Ch denote fragments or peptides produced by digestion with CNBr, trypsin (after citraconylation), hydroxylamine, trypsin, pepsin, and chymotrypsin, respectively.

Table I: Amino Acid Composition of Fd′ and CNBr Fragments[a]

amino acid	Fd′[b]		CB1[c]		CB2[d]	
CmCys[e]	4.0	(4)	0.7	(1)	2.8	(3)
Asp	17.7	(18)	3.1	(3)	15.0	(15)
Thr	16.1	(16)	0.1	(0)	15.2	(16)
Ser	29.2	(30)	3.6	(4)	25.7	(26)
Hse			1.0	(1)		
Glu	15.4	(15)	4.8	(5)	10.7	(10)
Pro	14.0	(14)	1.0	(1)	12.5	(13)
Gly	24.0	(24)	4.3	(4)	20.6	(20)
Ala	14.4	(14)	2.1	(2)	12.4	(12)
Val	20.9	(21)	3.0	(3)	17.9	(18)
Met	1.0	(1)	–	(0)	0.1	(0)
Ile	5.0	(5)	–	(0)	4.7	(5)
Leu	21.1	(21)	4.1	(4)	17.5	(17)
Tyr	8.9	(9)	1.0	(1)	7.7	(8)
Phe	8.1	(8)	2.0	(2)	6.2	(6)
His	4.1	(4)	1.0	(1)	2.9	(3)
Lys	11.1	(11)	0.1	(0)	10.7	(11)
Arg	6.1	(6)	2.0	(2)	4.2	(4)
Trp	nd[f]	(7)		(0)	nd	(7)
total		(228)		(34)		(194)
residue position	1–228		1–34		35–228	
yield (%)[g]			46		28[h]	

[a] Experimental values were normalized to the expected number of residues in each fragment, less tryptophan and *S*-(carboxymethyl)cysteine. Values in parentheses are the expected values based on the variable region sequence (determined here) and the known sequence of the N-terminal half of the γ1 chain (Cunningham et al., 1970), taking into account the deletion of 15 residues in the Dob hinge region (Steiner & Lopes, 1979). Where a blank appears the expected value is zero. [b] Values taken from Table I of Steiner & Lopes (1979). [c] Experimental values are averages of two 24-h hydrolysates of pool B, Figure 2, purified further as described in the text; (–) denotes less than 0.1 residue. [d] Experimental values were obtained from single 24-, 48-, and 72-h hydrolysates of pool A1, Figure 2, each analyzed in duplicate. Values shown are averages, except that those for threonine and serine are logarithmic extrapolations to zero time, that for valine is the 72-h value, and that for isoleucine is the average of the 48- and 72-h values. [e] *S*-(Carboxymethyl)cysteine. [f] Not determined. [g] Based on the amount of Fd′ digested with CNBr. [h] This was the recovery of CB2 in pool A1 (Figure 2); pool A2 contained an additional 46%. Thus, the total recovery of CB2 was 74%.

Results

Amino Acid Composition and N-Terminal Sequence of Fd′. The isolated Fd′ fragment moved as a single component in $NaDodSO_4$–polyacrylamide gel electrophoresis, either before reduction or after it had been extensively reduced and alkylated (Figure 1). The amino acid composition of Fd′ is shown in Table I; the results of automated sequence analysis of the fragment are presented in Table II. The sequence of the first 59 residues was established except for cycles 51, 56, and 57. The single methionine residue in Fd′ is located at residue position 34. The sequence of the N-terminal 12 residues of the Dob heavy chain was determined (data not shown); it was identical with the Fd′ sequence, establishing that the Fd′ fragment extends to the N terminus of the γ1 chain.

Preparation and Characterization of Cyanogen Bromide Fragments from Fd′. After reaction of Fd′ with CNBr, there was no change in the mobility of the unreduced digested protein in $NaDodSO_4$–polyacrylamide gel electrophoresis. Following reduction, however, two major and three minor components were detected. Presumably, the two major

Table II: Automated Edman Degradation of Fd′[a]

cycle no.	GLC[b]		TLC[c]	LC[d]		cpm[e]	cycle no.	GLC[b]		TLC[c]	LC[d]	
1	–[f]		E				31	–		E	E	(16.7)
2	V	(74)	V				32	Y[h]		Y		
3	–		Q				33	–		N	N	(8.9)
4	L/I[g]	(71)	–	L	(74)		34	M	(26)	M		
5	V	(72)	V				35	–		–	H	(3.2)
6	E[h]	(63)	E				36	W[h]		W		
7	S/C[g]	(38)	S				37	L/I	(19)	–	L	(21)
8	G	(49)	G				38	–		–	R	(3.6)
9	G	(54)	G				39	–		Q	Q[i]	(8.5)
10			D				40	G	(7.5)	G	G	
11	L/I	(44)	–	L	(52)		41	P/T	(9.3)	P	P	(7.1)
12	V	(43)	V				42	G	(8.9)	G	G	
13	–		Q				43	–		K	K	
14	P/T[g]	(8)	P				44	G	(5.4)	G	G	
15	G	(39)	G			45	45	P/T	(6.5)	P	P	(6.3)
16	–		–	R	(8.2)	37	46	–		E	E	(4.6)
17	S/C	(22)	S			42	47	–		W	W	(3.9)
18	L/I	(39)	–	L	(30)	34	48	V	(4.5)	V	V/M[j]	
19	–		–	R	(7.3)	41	49	S/C	(3.0)	S	S	
20	L/I	(39)	–	L	(30)	43	50	–		–	T	(1.4)
21	S/C	(18)	S			57	51	L/I	(3.5)	–		
22	S/C	(25)	C			554	52	P/T		T	T	(0.7)
23	A	(30)	A			185	53	–		W	W	(2.1)
24	A	(26)	A			83	54	–		N	N	(0.8)
25	S/C	(11)	S			44	55	G		G	G	(2.3)
26	G	(6.4)	G			35	56	–		–	–	
27	F	(26)	F			40	57	–		–		
28	–		N	N[i]	(13)	41	58	V	(2.8)		V/M	
29	F	(24)	F				59	L/I	(3.3)		L	
30	–		–	H	(5.0)							

[a] The sample was 154 nmol of reduced, alkylated Fd′. The repetitive yield was 95.6%. PTH amino acids are indicated by the one-letter code as defined in the legend to Figure 8, except that C denotes *S*-(carboxymethyl)cysteine. Values in parentheses are yields in nanomoles. [b] Gas–liquid chromatography. [c] Thin-layer chromatography. [d] High-pressure liquid chromatography. [e] The radioactivity in an aliquot (10%) from each cycle was determined to confirm the presence of PTH-*S*-(carboxymethyl)cysteine. [f] A horizontal line indicates that no new PTH amino acid was seen by this identification method at this cycle. [g] These two residues coeluted on gas–liquid chromatography and were distinguished by thin-layer chromatography and/or high-pressure liquid chromatography. [h] At certain cycles, the PTH derivatives of glutamic acid, tyrosine, and tryptophan were examined by gas chromatography following on-column silylation using bis(trimethylsilyl)trifluoroacetamide. [i] PTH-glutamine was recovered as a mixture of PTH-glutamine and PTH-glutamic acid. The extent of deamidation varied between 18 and 39%. PTH-asparagine was recovered as a mixture of PTH-asparagine and PTH-aspartic acid. The extent of deamidation varied between 10 and 25%. The yields given are the sums of the amide and acid forms. [j] PTH-valine and PTH-methionine were not separated in the high-pressure liquid chromatography elution program used in this experiment; they were distinguished by gas–liquid chromatography or thin-layer chromatography.

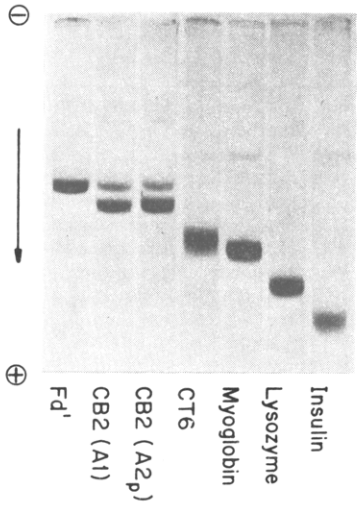

FIGURE 1: Major fragments used to determine the amino acid sequence of Fd′ analyzed by electrophoresis in 10% polyacrylamide–$NaDodSO_4$ gels. CB2 is the larger of the two fragments produced by cyanogen bromide cleavage of Fd′. A1 refers to the pool so designated in Figure 2; $A2_p$ is pool A2 (Figure 2) purified further as described in the text. Both samples of CB2 are contaminated with undigested Fd′, as shown. CT6 is the largest fragment produced after tryptic cleavage of citraconylated Fd′ (Figure 4, pool A).

fragments resulted from the cleavage of Fd′ at its single methionine residue at position 34. The most strongly staining component migrated slightly ahead of Fd′ and was probably the large fragment (designated CB2), extending from position 35 to the C-terminal end of Fd′. The second prominent component migrated just behind the dye front. This proved to be the N-terminal fragment, CB1, comprising residues 1–34 (see below). One of the minor components was identical in mobility with Fd′; the other two minor components, of unknown origin, moved between CB1 and CB2.

The two major fragments, CB1 and CB2, were separated as follows: the CNBr digest of Fd′ was reduced with dithiothreitol in guanidine hydrochloride, alkylated with [1-^{14}C]iodoacetic acid, and subjected to gel filtration with Sephadex G-75 in 5 M guanidine hydrochloride (Figure 2). A slightly asymmetric peak (A) appeared near the void volume, and a shallow broad peak (B) was barely evident just ahead of the "salt peak" (C). Peak A was divided into two pools (A1 and A2), as shown, and each pool was dialyzed in Spectrapor No. 3 tubing against 9% formic acid and then 0.1 M acetic acid. Analysis of these pools by $NaDodSO_4$–polyacrylamide gel electrophoresis indicated that pool A1 contained the large fragment, CB2, and undigested Fd′ but not the two fast-migrating contaminants (Figure 1). The amino acid composition of pool A1 was determined (Table I, CB2) and it was also analyzed in the sequenator. Two sequences were obtained (Table III). The major sequence (75–80%) corresponded to the Fd′ sequence beginning at position 35 and is therefore the N-terminal sequence of the

Table III: Automated Edman Degradation of CB2[a]

cycle no.	major sequence (CB2) GLC[b]	TLC[c]	LC[d]	minor sequence (Fd') GLC[b]	TLC[c]	LC[d]	cpm[e]
1	–[f]	–	H (5.2)	–	E	E (3.5)	
2	–	W	W (38)	V (12)	V		
3	L/I[g] (43)	–	L	–	Q	Q[h] (6.0)	
4	–	–	R (8.6)	L/I (13)	–		
5	–	Q	Q (24)	V (11)	V		
6	G (25)	G		–	E	E (6.4)	
7	P/T[g] (15)	P		S/C (6.1)	S		
8[i]	G (32)	G		(G)	–		
9	–	K		G (11)	G		
10	G (28)	G		–	(D)[j]	D (2.2)	
11	P/T (14)	P		L/I (8.2)	–		
12	–	E	E (21)	V (5.8)	V		
13	–	W	W (17)	–	(Q)	Q (1.5)	
14	V (19)	V		P/T (1.6)	(P)		
15	S/C[g] (6.2)	S		–	–		
16	P/T (6.9)	T		–	–	R (1.0)	
17	L/I (16)	–	F/I[k]	–	–		
18	P/T (4.9)	T		L/I (5)	–		
19	–	W	W (9.9)	–	–	R (0.8)	
20	–	N	N[h] (6.1)	L/I (2.4)	–		117
21	G (9.1)	G	G[l]	S/C (1.8)	–		163
22	G (11)	G	G	S/C (2.9)	C		403
23	S/C (7.3)	S	S	A (2.6)	(A)		182
24	V (8.5)	V	V/M[k]	A (2.4)	–		162
25	L/I (9.0)	–	L	–	–		
26	–	Y	Y (9.2)	G (0.5)	–		
27	A (8.8)	A	A	F (1.4)	–		
28	–	D	D (2.8)	–	–	N (0.4)	
29	S/C (4.4)	S		F (1.0)	–		
30	V (7.9)	V	V/M	–	–		
31	–	K	K	–	(E)		
32	G (1.5)	G	G	–	(Y)	Y (1.1)	
33	–	–	R (0.4)	–	–		
34	F (5.5)	F	F/I	–	–		
35	A (4.5)	A	A	–	–		
36	L/I (5.0)	–	I	–	–		
37	S (1.6)	S		–	–		
38	–	–	R (0.4)	–	–		
39	–	D	D (1.0)	–	–		
40	–	N	N (1.1)	–	–		
41	A (2.2)	A		–	–		
42		Q	Q (1.2)	–	–		
43	–	K		–	–		
44	P/T (0.9)	–		–	–		
45	L/I (1.8)	–	L	–	–		

[a] The sample was 93 nmol of pool A1 (Figure 2). The repetitive yield was 93.6%. PTH amino acids are indicated by the one-letter code as defined in the legend to Figure 8, except that C denotes *S*-(carboxymethyl)cysteine. Values in parentheses are yields in nanomoles. [b] Gas–liquid chromatography. [c] Thin-layer chromatography. [d] High-pressure liquid chromatography. [e] The radioactivity in an aliquot (10%) from each cycle was counted to confirm the presence of PTH-*S*-(carboxymethyl)cysteine. [f] A horizontal line indicates that no new PTH amino acid was seen by this identification method at this cycle. [g] These two residues coeluted on gas–liquid chromatography and were distinguished by thin-layer chromatography and/or high-pressure liquid chromatography. [h] PTH-glutamine was recovered as a mixture of PTH-glutamine and PTH-glutamic acid. The extent of deamidation varied between 18 and 39%. PTH-asparagine was recovered as a mixture of PTH-asparagine and PTH-aspartic acid. The extent of deamidation varied between 10 and 25%. The yields given are the sums of the amide and acid forms. [i] At cycle 8, the only PTH amino acid found was PTH-glycine. From the identity of the minor sequence with that of intact Fd', PTH-glycine would be expected at cycle 8. However, the yield (32 nmol) is much greater than expected from the minor sequence; therefore PTH-glycine is assigned at position 8 in the major sequence. This identification appears to be correct since position 42 of the intact Fd' (which corresponds to position 8 of CB2) is PTH-glycine. [j] Parentheses for identifications by thin-layer chromatography indicate that the spot was faint. [k] PTH-valine/methionine and PTH-phenylalanine/isoleucine were not separated in the high-pressure liquid chromatography elution programs used in this experiment; they were distinguished by gas–liquid chromatography or thin-layer chromatography. [l] Identifications by high-pressure liquid chromatography for cycles 21–24, 27, 30–32, 34, and 35 are from a duplicate sequenator run.

CB2 fragment; the minor sequence (20–25%) was identical with that of Fd'. Since the sequence of Fd' had been determined previously (Table II) and the CB2 fragment was present in a greater amount, the residues could be assigned to each of the two sequences.

Pool A2 contained CB2 and Fd' and also the two smaller minor components. This pool was purified further in order to remove the latter two contaminants. After gel filtraton in 9% formic acid, repeated gel filtration in 5 M guanidine hydrochloride, and gel filtration again in 9% formic acid (all with Sephadex G-75), CB2 and the contaminating Fd' were separated from the other minor components. The pattern obtained in $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1) and the amino acid composition of the purified pool A2 were indistinguishable from the corresponding analyses of pool A1. In the sequenator, two sequences were again obtained, corresponding to CB2 (75–80%) and Fd' (20–25%).

Pool B was prepared from fractions in the region of the shallow broad peak, as shown in Figure 2. It was desalted on a 2.0 × 89 cm column of Sephadex G-25 in 9% formic acid. Very little material absorbing at 278 nm was eluted, and the peptide material was located by its radioactivity. Its amino

Table IV: Amino Acid Composition of Peptides Derived from Cyanogen Bromide Fragment CB1[a]

amino acid	CB1	CB1-T1	CB1-T2	CB1-T3	CB1-T1-P1	CB1-T1-P2	CB1-T1-P3	CB1-T1-P1a	CB1-T1-P2a	CB1-T3-Ch1	CB1-T3-Ch2	CB1-T3-Ch3
CmCys[b]	(1)	–	–	0.7 (1)						0.7 (1)	–	–
Asp	(3)	1.0 (1)	–	2.0 (2)	–	1.0 (1)	–	–	1.0 (1)	–	1.0 (1)	1.0 (1)
Ser	(4)	1.0 (1)	1.0 (1)	1.7 (2)	–	0.9 (1)	–	–	0.9 (1)	1.8 (2)	–	–
Hse	(1)	–	–	1.0 (1)						–	–	1.0 (1)
Glu	(5)	4.0 (4)	–	1.1 (1)	2.0 (2)	1.1 (1)	1.1 (1)	2.0 (2)	1.2 (1)	–	1.0 (1)	–
Pro	(1)	1.1 (1)	–	–	–	–	1.0 (1)	–	–	–	–	–
Gly	(4)	3.0 (3)	–	1.3 (1)	–	2.1 (2)	1.0 (1)	–	2.0 (2)	1.1 (1)	–	–
Ala	(2)	–	–	2.1 (2)	–	–	–	–	–	2.1 (2)	–	–
Val	(3)	2.9 (3)	–	–	1.0 (1)	0.9 (1)	1.0 (1)	1.0 (1)	0.9 (1)	–	–	–
Leu	(4)	1.9 (2)	1.0 (1)	1.0 (1)	–	1.0 (1)	0.9 (1)	1.0 (1)	–	1.0 (1)	–	–
Tyr	(1)	–	–	0.9 (1)	–	–	–	–	–	–	0.8 (1)	–
Phe	(2)	–	–	2.0 (2)	–	–	–	–	–	1.0 (1)	1.1 (1)	–
His	(1)	–	–	0.9 (1)	–	–	–	–	–	–	1.1 (1)	–
Arg	(2)	1.0 (1)	1.0 (1)	–	–	–	1.0 (1)	–	–	–	–	–
position	1-34	1-16	17-19	20-34	1-3	4-10	11-16	1-4	5-10	20-27	28-32	33-34
yield (%)[c]		59	67	58	18	9	30	6	4	20	9	12
mobility[d]		−0.34	+0.45	−0.20	−0.44	−0.53	+0.34	−0.35	−0.64	−0.27	~N	~N

[a] Experimental values are based on one or two 24-h hydrolysates and are expressed as residues per peptide; (–) denotes <0.1 residue per peptide. Values in parentheses are those expected from the sequence. Where a blank appears the expected value is zero. [b] *S*-(Carboxymethyl)cysteine; values were determined from the specific activities of the hydrolyzed peptides. [c] Expressed relative to amount of CB1 digested with trypsin. [d] Mobility in high-voltage electrophoresis at pH 6.5, expressed relative to aspartic acid. Positively charged peptides are designated (+); negatively charged peptides are designated (–); N denotes neutral peptides.

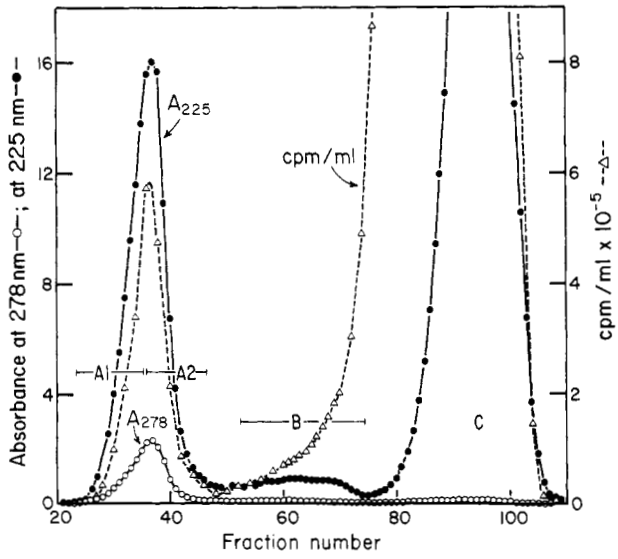

FIGURE 2: Gel filtration of cyanogen bromide digest of Fd′. After lyophilization, the digest (600 nmol) was taken up in 2 mL of 7 M guanidine hydrochloride, 0.2 M Tris-HCl, and 0.002 M EDTA (pH 8.0), reduced with 20 μmol of dithiothreitol, and alkylated with 50 μmol of iodoacetic acid containing 40 μCi of [1-^{14}C]iodoacetic acid. The digest was applied to a 1.5 × 81 cm column of Sephadex G-75 equilibrated in 5 M guanidine. The fraction size was 1.3 mL. Pool A1 contained fragment CB2 as well as a small amount of undigested Fd′ (see Figure 1). Pool A2 contained these components, as well as additional contaminants, and was purified as described in the text. Pool B contained fragment CB1 and was purified as described. Pool C contained low molecular weight material including non-protein-bound [1-^{14}C]iodoacetic acid.

acid composition resembled that expected for fragment CB1 (residues 1–34) of Fd′, but some contaminating residues were also present. Moreover, analysis by $NaDodSO_4$–urea–polyacrylamide gel electrophoresis indicated that two contaminants were present. Accordingly, the material was purified further by gel filtration on a 1.5 × 120 cm column of Sephadex G-50 in 0.05 M NH_4OH. A single symmetrical peak, eluting at 1.3× the void volume and free of the contaminants, was obtained. The amino acid composition of this purified fragment (CB1) is shown in Table I.

Isolation and Characterization of Peptides Derived from CB1. When fragment CB1 was digested with trypsin, the expected three peptides, comprising residues 1–16 (CB1-T1), residues 17–19 (CB1-T2), and residues 20–34 (CB1-T3), were obtained. Peptides CB1-T1 and CB1-T3 were digested with pepsin and chymotrypsin, respectively, and the resulting peptides were purified by high-voltage electrophoresis. The amino acid compositions, yields, and mobilities of all of these purified peptides are summarized in Table IV, and their location within CB1 is shown in Figure 3. The data are consistent with the results obtained by automated sequence analysis.

Isolation and Characterization of Tryptic Peptides Derived from Citraconylated Fd′. The first constant domain of the γ1 chain lacks arginine, except for chains of allotype G1m(4), which have arginine at position 214 (Cunningham et al., 1970). In the Dob γ1 chain, residue 214 is lysine (Steiner & Lopes, 1979). Therefore, if tryptic cleavage is confined to arginine, a large fragment, beginning in the variable region and extending to the end of Fd′, should be produced. Accordingly, Fd′ was reacted with citraconic anhydride to block lysines and then digested with trypsin. After adding trypsin inhibitor, aliquots of the digest were removed and analyzed for amino acid composition and C-terminal end groups. Digestion with carboxypeptidase B released 3.8 mol of arginine per mol of

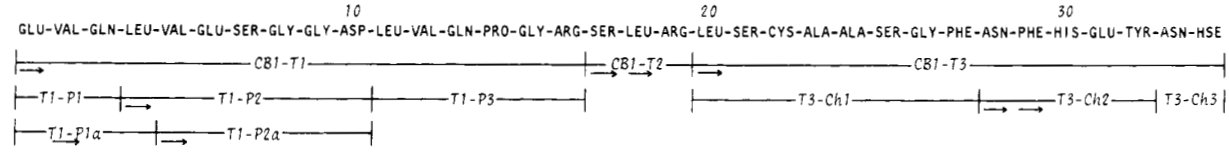

FIGURE 3: The arrangement of peptides derived from cyanogen bromide fragment CB1. When CB1 was digested with trypsin, three peptides were obtained, as shown. CB1-T1 was digested with pepsin and CB1-T3 was digested with chymotrypsin, producing five and three peptides, respectively. The properties of these peptides are described in Table IV. → denotes sequence determined by the manual dansyl–Edman method.

Table V: Amino Acid Composition of Tryptic Peptides of Citraconylated Fd'[a]

amino acid	CT1[b]	CT2	CT3	CT4[b]	CT5	CT6
CmCys[c]			0.3 (1)			2.9 (3)
Asp	1.2 (1)	–	2.2 (2)	2.0 (2)	–	13.0 (13)
Thr	0.4	–	–	1.8 (2)	–	13.7 (14)
Ser	1.3 (1)	0.9 (1)	1.9 (2)	2.6 (3)	0.9 (1)	19.6 (22)
Glu	3.6 (4)	–	1.0 (1)	2.4 (2)	–	8.3 (8)
Pro	0.7 (1)	–	0.1	2.2 (2)	–	11.5 (11)
Gly	3.7 (3)	–	1.4 (1)	6.0 (6)	–	14.7 (14)
Ala	0.3	–	2.1 (2)	1.1 (1)	1.1 (1)	10.4 (10)
Val	2.9 (3)	–	0.2	3.0 (3)	–	15.2 (15)
Met	–	–	0.7 (1)	–	–	–
Ile	0.3	–	–	1.0 (1)	1.0 (1)	3.0 (3)
Leu	1.6 (2)	1.1 (1)	2.0 (2)	1.2 (1)	–	15.2 (15)
Tyr	0.2	–	0.8 (1)	1.0 (1)	–	6.9 (7)
Phe	0.1	–	2.1 (2)	–	1.0 (1)	5.2 (5)
His	–	–	1.8 (2)	–	–	2.1 (2)
Lys	0.4	–	–	1.7 (2)	–	9.1 (9)
Arg	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)
Trp	nd[d]		nd (1)	nd (2)	nd	nd (4)
position	1-16	17-19	20-38	39-67	68-72	73-228
yield (%)[e]		25	1[f]		22	59
mobility[g]	−0.33	+0.49	N, +0.05[f]		+0.35	

[a] Experimental values are based on one or two 24-h hydrolysates and are expressed as residues per peptide; (–) denotes <0.1 residue per peptide. Values in parentheses are those expected from the sequence. Where a blank appears the expected value is zero. [b] These peptides were only partially purified, as described in the text. [c] *S*-(Carboxymethyl)cysteine. The value for CT3 was determined from the specific activity of the hydrolyzed peptide and that for CT6 was determined by amino acid analysis. [d] Not determined. [e] Expressed relative to the amount of citraconylated Fd' digested with trypsin. [f] Two closely spaced peptides. [g] Mobility in high-voltage electrophoresis at pH 6.5, expressed relative to aspartic acid. Positively charged peptides are designated (+); negatively charged peptides are designated (–); N denotes neutral peptides.

Fd' (76% of the theoretical yield, since one of the six arginine residues in Fd' is N terminal to a proline residue). In contrast, the amount of lysine released was only 0.2 mol/mol of Fd' (2% yield). Treatment of the tryptic digest with carboxypeptidase A released 0.8 mol of valine per mol of Fd', consistent with the previous finding that the C-terminal residue in Fd' is valine (Steiner & Lopes, 1979).

The peptides in the tryptic digest were fractionated by gel filtration with Sephadex G-50. As shown in Figure 4, three peaks of peptide material were eluted. Of the total radioactive material eluted from the column, 64% was in the region of peak A and 27% was in the region of peak C. This distribution suggested that the cleavage with trypsin had occurred within the first disulfide loop of the Fd' fragment, with three of the four *S*-(carboxymethyl)cysteine residues contained within a large peptide that extends from the variable region to the C-terminal end of Fd'. The homogeneity of the material in pool A was evaluated by polyacrylamide gel electrophoresis. Both in the standard $NaDodSO_4$ gel system (shown in Figure 1, CT6) and in $NaDodSO_4$–urea gels, a single component, slightly retarded relative to myoglobin, was seen. The amino acid composition of this material is shown in Table V (CT6). Digestion with carboxypeptidase A released valine, and digestion with carboxypeptidase B released neither arginine nor lysine, indicating that CT6 is the C-terminal peptide of Fd'. Analysis of this component in the sequenator revealed a single sequence. Details of one of the two sequenator runs carried out with this fragment are provided in Figure 5. It is apparent that a marked drop in yield occurred between steps 29 and 35. The possibility that this is the result of a rearrangement involving the asparaginyl-glycyl sequence at positions 31–32 of this fragment will be discussed later.

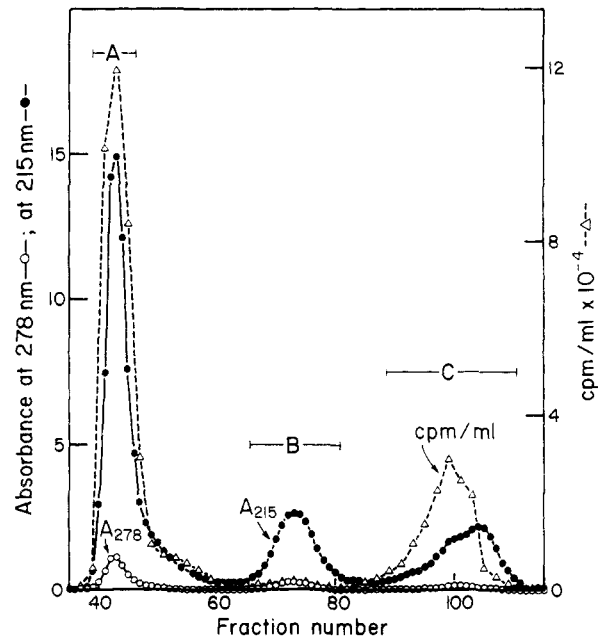

FIGURE 4: Gel filtration of a tryptic digest of Fd' that had been reduced, alkylated with [1-^{14}C]iodoacetic acid, and reacted with citraconic anhydride. The digest (440 nmol) was applied to a 1.5 × 122 cm column of Sephadex G-50 equilibrated at room temperature in 0.05 M NH_4HCO_3 adjusted to pH 9.3 with NH_4OH; the fraction size was 2.1 mL. Pool A contained a single large peptide (CT6), as demonstrated by electrophoresis in $NaDodSO_4$–polyacrylamide gels (Figure 1); data concerning the determination of its N-terminal sequence are provided in Figure 5. Pools B and C contained the other five peptides that, together with CT6, account for the entire Fd' piece.

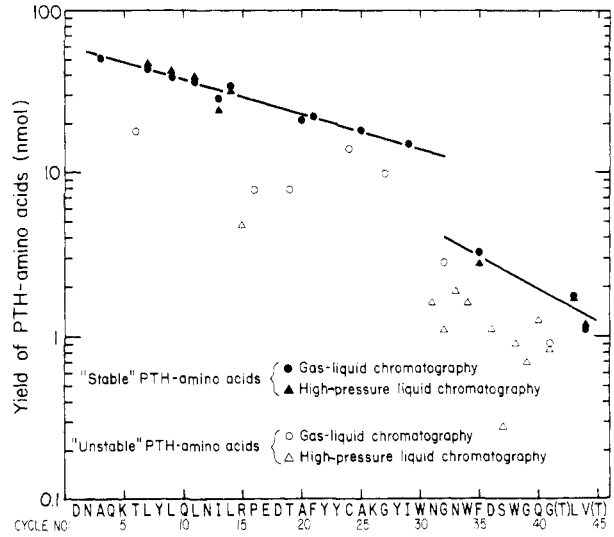

FIGURE 5: Yields of PTH amino acids obtained by automated Edman degradation of 100 nmol of fragment CT6 (Figure 4, pool A). "Stable" PTH amino acids are those recovered consistently in high yield; "unstable" PTH amino acids are those recovered in lower and variable yield. The sequence for 45 steps is indicated at the bottom of the graph in the one-letter code (see legend to Figure 8) and also includes the results of identifications by thin-layer chromatography; the latter data were not plotted since they are not quantitative. Cys was identified as *S*-(carboxymethyl)cysteine. Note the apparent falloff in yield between the isoleucyl residue at position 29 and the phenylalanyl residue at position 35. This may be the result of cyclization of the asparaginyl side chain at position 31 with subsequent formation of a β-aspartyl–glycyl bond (see the discussion in the text). The average repetitive yield (based on linear regression analysis of yields of the stable PTH amino acids) was 95.2% for cycles 1–29 and 91.1% for cycles 35–44.

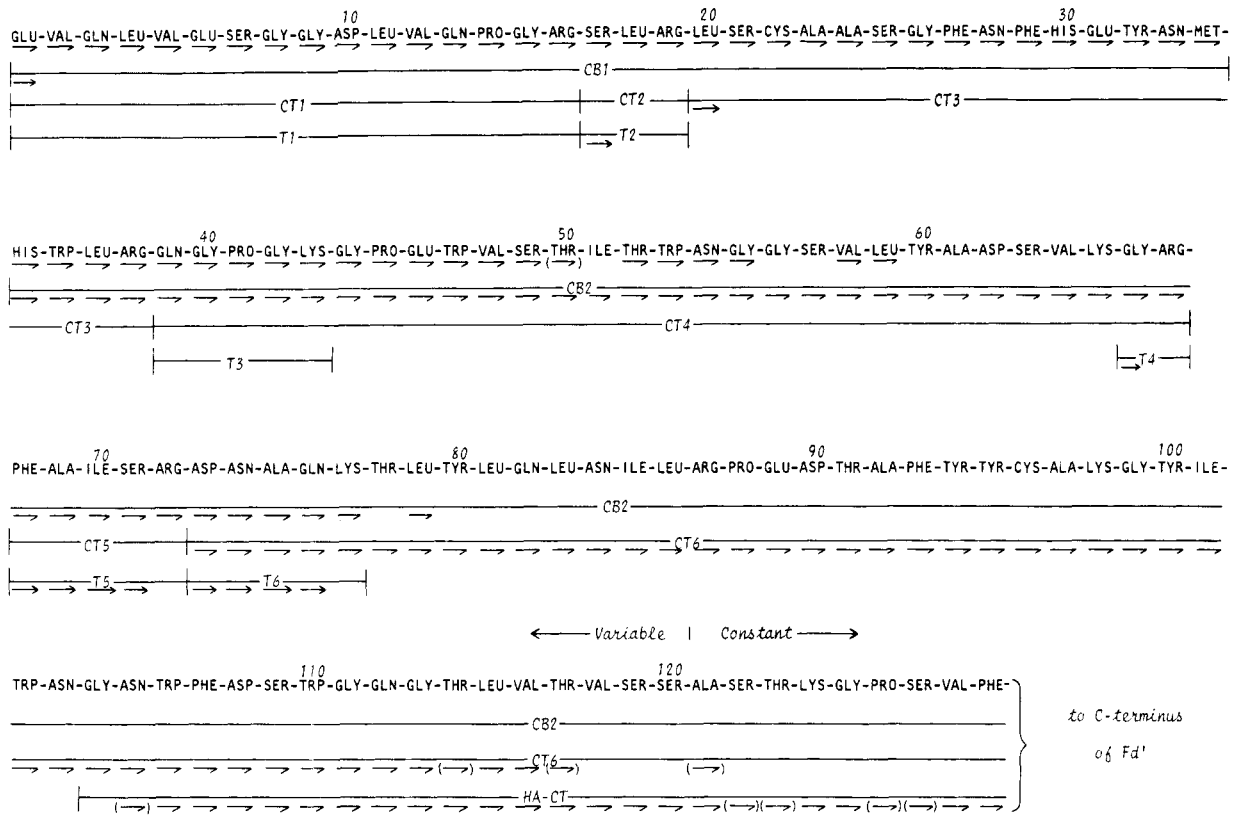

FIGURE 6: The amino acid sequence of the variable region of the Dob $\gamma 1$ chain, including the overlap into the constant region. The major fragments and peptides used to establish the sequence are indicated; CB, CT, HA, and T denote cleavage by cyanogen bromide, trypsin (following citraconylation), hydroxylamine, and trypsin, respectively. Results obtained by analyzing the intact Fd′ fragment are indicated by arrows below the printed sequence; results obtained by analyzing the individual fragments are indicated by arrows below the designated fragments. → indicates residues identified after manual Edman degradaton. — indicates residues identified after automated Edman degradation by at least two identification methods and/or in two separate sequenator runs. (—) indicates identification by only one method.

Digestion with carboxypeptidases A and B indicated that, as expected, the only C-terminal residue of the peptides in pools B and C was arginine. Three peptides (Table V: CT2, CT3, and CT5) were isolated from pool C by gel filtration with Sephadex G-25 and high-voltage electrophoresis (after removal of citraconyl blocking groups). Analytical high-voltage electrophoresis demonstrated that pool B contained two peptides; these were partially separated from each other, after deblocking, by gel filtration with Sephadex G-25 but were not purified further. Their amino acid compositions are also presented in Table V (CT1 and CT4). Peptides CT1–CT5 comprise the N-terminal 72 residues of Fd′, as determined by the analysis of Fd′ and CB2 in the sequenator. Together with peptide CT6, these peptides account for the entire Fd′.

Hydroxylamine Cleavage of Fd′ and Purification of a Fragment Containing the C-Terminal Portion of the Variable Region. The falloff in yield that occurred when CT6 was analyzed in the sequenator prevented completion of the variable-region sequence with data obtained from this fragment. However, it seemed possible that the ability of hydroxylamine to cleave asparaginyl–glycyl bonds (Bornstein & Balian, 1977) could be utilized to generate a fragment beginning at Gly-104 and extending to the end of Fd′ (see Figure 6). Analysis of such a fragment should provide the remaining sequence of the variable region. It was noted that the sequence Asn-Gly also occurs at positions 54–55 of Fd′. Since hydroxylamine cleavage might not be complete, the piece beginning at Gly-104 would probably have to be isolated from a mixture of fragments, which might prove to be difficult. Accordingly, the cleavage of reduced, alkylated Fd′ with hydroxylamine was followed by citraconylation and tryptic digestion of the mixture of fragments. From the earlier results of digestion of citraconylated Fd′ with trypsin, it was known that all arginines are N-terminal to residue 73. Therefore, the fragment beginning at Gly-104 should not be affected by this treatment, whereas other fragments (e.g., 1–54 and 1–103) would presumably be cleaved into smaller peptides that should be easy to separate from the desired large piece.

The extent of cleavage by trypsin at arginine and lysine residues of the hydroxylamine-treated, citraconylated Fd′ was evaluated by amino acid analysis and carboxypeptidase B treatment of the digest. Cleavage at arginine was essentially complete, whereas cleavage at lysine was negligible. The tryptic digest was fractionated by gel filtraton with Sephadex G-50. Comparison of the elution pattern (Figure 7) with that obtained earlier after citraconylation and tryptic digestion of Fd′ (Figure 4) indicated that hydroxylamine cleavage had probably occurred to a significant extent at both positions 54–55 and 103–104. Fractions 35–45 were pooled and analyzed by electrophoresis in $NaDodSO_4$–urea–polyacrylamide gels (Figure 7). The major component migrated between myoglobin and cytochrome *c*. A faint band was present in the position of fragment CT6. When pool 35–45 was analyzed in the automated sequencer, two distinct sequences were obtained in approximately equal yield (Table VI). The data were consistent with the interpretation that one of these sequences (B) is that of CT6 (Figure 5) and the other sequence (A) is that of the fragment (designated HA-CT) beginning at position 104. Since fragment CT6 was only a minor component when pool 35–45 was examined by electrophoresis in $NaDodSO_4$–urea gels (Figure 7), the relative yield of sequence B was higher than expected. Nevertheless, the data obtained from the mixed sequence were sufficient to complete the variable-region sequence and to extend nine residues into

Table VI: Automated Edman Degradation of HA-CT[a]

	sequence A (HA-CT)				sequence B (CT6)			
cycle no.	GLC[b]	TLC[c]	LC[d]	BH[e]	GLC[b]	TLC[c]	LC[d]	BH[e]
1[f]								
2[g]	–[h]	(N)	–			(N)		
3	–	W	W (4.7)		A (1.5)	–		
4	F (6.8)	F	F/I (6.7)		–	Q	Q[i] (1.1)	
5	–	D	D (3.6)		–	K	K	
6	S/C[j] (3.3)	S	S (0.9)		P/T	T	T (1.0)	
7	–	W	W (3.5)		L (2.9)	–	L (3.0)	
8	G (3.7)	G	G (3.6)		–	Y	Y (2.5)	
9	–	Q	Q (2.7)		L/I	–	L (2.3)	
10	G	G	G (1.8)		(Q)[k]	–	–	
11	P/T[j] (1.0)	T	T (2.5)		L/I (2.6)	–	L (2.1)	
12	L/I[j] (2.8)	–	L (3.5)		–	N	N[i] (1.3)	
13	V	–	V/M (3.3)	V (1.6)	L/I	–	I (3.5)	I[l] (1.3)
14	P/T	–	T (1.4)	T[m] (1.9)	L/I	–	L (2.9)	L (1.6)
15	V	–	V (2.8)	V (1.3)	–	–	–	R (0.6)
16	S/C (2.4)	–	S (0.1)	A[n] (1.4)	P/T (0.4)	–	P (0.9)	P (0.7)
17	S/C (2.4)	–		A[n] (1.5)	–	–	E (0.6)	E (1.2)
18	A		A (1.0)	A (1.5)	–	–	D (1.0)	D (0.6)
19	S/C (1.3)	–	–		P/T (0.2)	–	–	
20	–	–	T (0.2)		A (0.6)	–	A	
21	–	K	K		F	–	F/I (1.0)	
22	G (0.7)	–	G (0.8)		–	Y	Y (0.7)	
23	–	–	P (0.3)		–	–	Y (0.5)	
24	–	–	S (0.1)		–	–		
25	V (0.7)	–	V (0.7)		A (0.4)	–	A (0.5)	
26	F (0.5)	–	F/I		–	–	–	

[a] The sample (~34 nmol) was a pool of fractions 35–45, Figure 6. Before beginning the Edman degradation the sample in the sequenator cup was exposed to cleavage acid (heptafluorobutyric acid) for 6 min to remove the citraconyl blocking groups. The average repetitive yield was 92.3%. PTH amino acids are indicated by the one-letter code as defined in the legend to Figure 8, except that C denotes *S*-(carboxymethyl)cysteine. Values in parentheses are yields in nanomoles. [b] Gas–liquid chromatography. [c] Thin-layer chromatography. [d] High-pressure liquid chromatography. [e] Back-hydrolysis. [f] Fraction 1 was lost. [g] At cycle 2, PTH-asparagine was the major spot detected by thin-layer chromatography. This residue is expected in both sequences and is therefore indicated in parentheses. In addition, at cycle 2, 3.2 nmol of PTH-phenylalanine was detected; this could not be assigned to either sequence. At cycles 5 and 6, small amounts of contaminating PTH amino acids were seen. After cycle 6, contaminating sequences were not detected. [h] A horizontal line indicates that no new PTH amino acid was seen by this identification method at this cycle. [i] PTH-glutamine was recovered as a mixture of PTH-glutamine and PTH-glutamic acid. The extent of deamidation varied between 18 and 39%. PTH-asparagine was recovered as a mixture of PTH-asparagine and PTH-aspartic acid. The extent of deamidation varied between 10 and 25%. The yields given are the sums of the amide and acid forms. [j] These two residues coeluted on gas–liquid chromatography and were distinguished by another identification method. [k] The PTH-glutamine in sequence B at cycle 10 could not be established unequivocally because of the presence of PTH-glutamine in cycle 9 of sequence A and the somewhat higher than average overlap. This was not essential because both sequences through this region had been determined from previous runs. [l] PTH-isoleucine was determined as a mixture of isoleucine and alloisoleucine. [m] PTH-threonine was determined as α-aminobutyric acid. [n] After hydrolysis, PTH-serine was detected as alanine but distinguished from PTH-alanine by the results obtained in the other identification procedures.

the constant region.

Isolation and Characterization of Tryptic Peptides Derived from Fd′. Fd′ was reduced in guanidine, alkylated with [1-^{14}C]iodoacetamide, and digested with trypsin, and the soluble peptides were divided into two pools (A and B) by gel filtration with Sephadex G-25 in 0.05 M acetic acid. Details of these procedures, as well as the characterization of peptides derived from the constant region of Fd′, are provided in the preceding paper (Steiner & Lopes, 1979). Except for peptide T1, the remaining five peptides from the variable region were recovered from pool B, which contained the smaller peptides. Amino acid compositions of these peptides, after purification by high-voltage electrophoresis and chromatography, are summarized in Table VII. Four of the peptides (T2, T4, T5, and T6) were partially sequenced by the manual dansyl–Edman method, as shown in Figure 6. In the case of peptides T5 and T6, the sequence was confirmed by the subtractive method. The position of the amide group in T6, established in the sequenator run of CT6, was confirmed by determining the mobilities of peptide T6 and the peptides obtained after the first and second cycles of the Edman degradation. Peptide T1 was identical in composition with CB1-T1 and was not studied further. No dansyl derivative of the amino terminus was obtained from peptide T3 nor was any obtained after one cycle of Edman degradation. This suggested that the NH_2 terminus may be blocked. Since analysis in the sequenator indicated that the N-terminal residue (position 39) of the peptide was glutamine, the failure to obtain a dansyl derivative is consistent with the possibility that cyclization of this residue to pyrrolidonecarboxylic acid had occurred. Moreover, this peptide was neutral in electrophoretic mobility at pH 6.5, also consistent with cyclization of the N-terminal residue. A trace amount of a peptide with similar composition was also obtained from a basic region, but there was not enough for further analysis. The composition and sequence of the peptides listed in Table VII are consistent with the results obtained by automated sequence analysis.

Discussion

The amino acid sequence of the variable region of the Dob heavy chain and the data on which this sequence is based are summarized in Figure 6. The complete sequence was established by analysis in the automated sequenator of the intact Fd′ piece and of three large overlapping fragments prepared from Fd′: the cyanogen bromide fragment (CB2), the largest peptide obtained after citraconylation and digestion with trypsin (CT6), and a peptide obtained after hydroxylamine cleavage, citraconylation, and tryptic digestion (HA-CT). Data obtained by determining the amino acid composition and

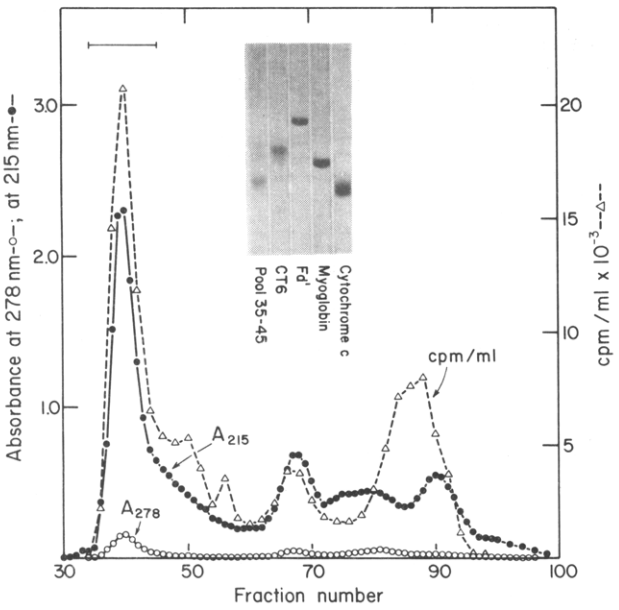

FIGURE 7: Gel filtration of reduced, [^{14}C]carboxymethylated Fd′ (88 nmol) that had been treated with hydroxylamine and, following citraconylation, digested with trypsin (see Experimental Procedures). The tryptic digest was applied to a 1.1 × 120 cm column of Sephadex G-50 in 0.05 M NH_4HCO_3 adjusted to pH 8.6 with NH_4OH. The fraction size was 1.2 mL. Fractions 35–45 were pooled, as indicated by the horizontal line. The photograph shows the results of electrophoresis of this pool in $NaDodSO_4$–urea–polyacrylamide (10%) gels. For comparison, CT6, Fd′, myoglobin, and cytochrome *c* were also analyzed on the same gels. Analysis of pool 35–45 in the automated sequencer indicated that there were two sequences, one corresponding to that of CT6 and the other corresponding to a fragment (HA-CT) beginning at Gly-104 in the Dob Fd′ sequence (Table VI).

partial sequence of a variety of smaller peptides were consistent with the results of automated Edman degradation and are also summarized in Figure 6, as well as in Figure 3.

A technical problem was encountered in the analysis of fragment CT6. As shown in Figure 5, a significant falloff in yield of the PTH amino acids occurred after cycle 29. Asparaginyl peptides are prone to form cyclic imides that may hydrolyze to a mixture of α- and β-aspartyl peptides. Such rearrangements, which are favored if glycine is the adjacent C-terminal residue, would impede the Edman degradation [reviewed by Bornstein & Balian (1977)]. Accordingly, the unusual decrease in yield in the sequence analysis of CT6 could be related to the asparaginyl-glycyl sequence at positions 31–32 of this fragment. To circumvent this problem in the determination of the sequence of fragment CT6, hydroxylamine was used to cleave the asparaginyl–glycyl bond, yielding a fragment (HA-CT) beginning at glycine-104. It has been proposed that this cleavage occurs after nucleophilic addition of hydroxylamine, under alkaline conditions, to the cyclic imide, anhydroaspartylglycine (Bornstein & Balian, 1977). The fragment HA-CT was obtained after citraconylation and tryptic digestion of the hydroxylamine digest of Fd′ and was found to be contaminated with fragment CT6. However, the sequence of CT6 had been determined previously, and analysis of the mixture sufficed to obtain a sufficiently long N-terminal sequence of HA-CT to complete the sequence of the variable region and to provide an overlap into the constant region. In addition to the Asn-Gly sequence in CT6 (positions 103–104 of Fd′), this pair of residues also occurs in cyanogen bromide fragment CB2 (positions 54–55 of Fd′). However, the problem of an unusual decrease in yield was not encountered when fragment CB2 was analyzed in the sequenator.

Table VII: Amino Acid Composition of Soluble Tryptic Peptides from the Variable Region of Fd′[a]

amino acid	T1	T2	T3	T4	T5	T6
Asp	1.0 (1)	–	–	–	0.1	2.0 (2)
Thr	–	–	–	–	–	–
Ser	1.1 (1)	0.9 (1)	–	–	1.0 (1)	0.1
Glu	4.0 (4)	0.1	1.0 (1)	–	0.1	1.0 (1)
Pro	0.9 (1)	–	1.0 (1)	–	–	–
Gly	3.1 (3)	–	2.0 (2)	1.0 (1)	0.1	–
Ala	–	–	–	–	1.1 (1)	1.0 (1)
Val	3.0 (3)	–	–	–	0.2	–
Met	–	–	–	–	–	–
Ile	–	–	–	–	1.0 (1)	–
Leu	1.9 (2)	1.1 (1)	–	–	–	–
Tyr	–	–	–	–	–	–
Phe	–	–	–	–	0.9 (1)	–
His	–	–	–	–	–	–
Lys	0.1	–	1.0 (1)	0.1	0.1	1.0 (1)
Arg	1.0 (1)	1.0 (1)	–	1.0 (1)	1.1 (1)	–
position	1–16	17–19	39–43	66–67	68–72	73–77
yield (%)[b]	18	7	8	34	22	51
mobility[c]	−0.36	+0.51	N[d]	+0.72	+0.34	N

[a] Experimental values are based on a single 24-h hydrolysate and are expressed as residues per peptide. (–) denotes <0.1 residue per peptide. Values in parentheses are those expected from the sequence. Where a blank appears the expected value is zero. [b] Expressed relative to amount of Fd′ digested with trypsin. [c] Mobility in high-voltage electrophoresis at pH 6.5, expressed relative to aspartic acid. Positively charged peptides are designated (+); negatively charged peptides are designated (–); N denotes neutral peptides. [d] The N-terminal residue of the peptide has probably cyclized to pyrrolidonecarboxylic acid, as discussed in the text.

The complete, or nearly complete, amino acid sequences of 23 other variable regions of human heavy chains have been reported. These are compared with the sequence of the Dob heavy chain in Figure 8. Variable regions have been subdivided into segments of greater and lesser variability known as hypervariable and framework regions, respectively (Wu & Kabat, 1970; Capra & Kehoe, 1974a). Some residues in the hypervariable segments participate in the antibody-combining region, and, hence, these segments are also known as complementarity-determining regions. In addition, as indicated in Figure 8, the variable regions of human heavy chains have been divided into three subgroups based largely upon homology in amino acid sequence among their amino terminal or first framework regions; this division is independent of the class of the constant region (Kohler et al., 1970; Wang et al., 1970; Capra, 1971). The sequences in the more C-terminal segments of the variable regions lend themselves less readily, if at all, to stratification into these subgroups (Kehoe & Capra, 1971; Florent et al., 1974). As the number of known variable-region sequences has increased, it has become more difficult to establish unambiguous criteria for the classification, i.e., to demonstrate residues that are invariably present in a particular subgroup.

The variable region of the Dob heavy chain appears to belong to the V_HIII subgroup. However, there are several unusual substitutions. The residue at position 45 (according to the numbering used in Figure 8) is proline. Regardless of subgroup, all other human heavy-chain variable regions shown in Figure 8, and three additional ones that have been partially sequenced, have leucine at this position (Kabat et al., 1976).[4] Moreover, leucine occurs at this position in all heavy chains

[4] Residues 45 and 46 in protein Lay and Pom are listed by Kabat et al. (1976) as Glu-Leu; the correct sequence is Leu-Glu (Capra & Kehoe, 1974b).

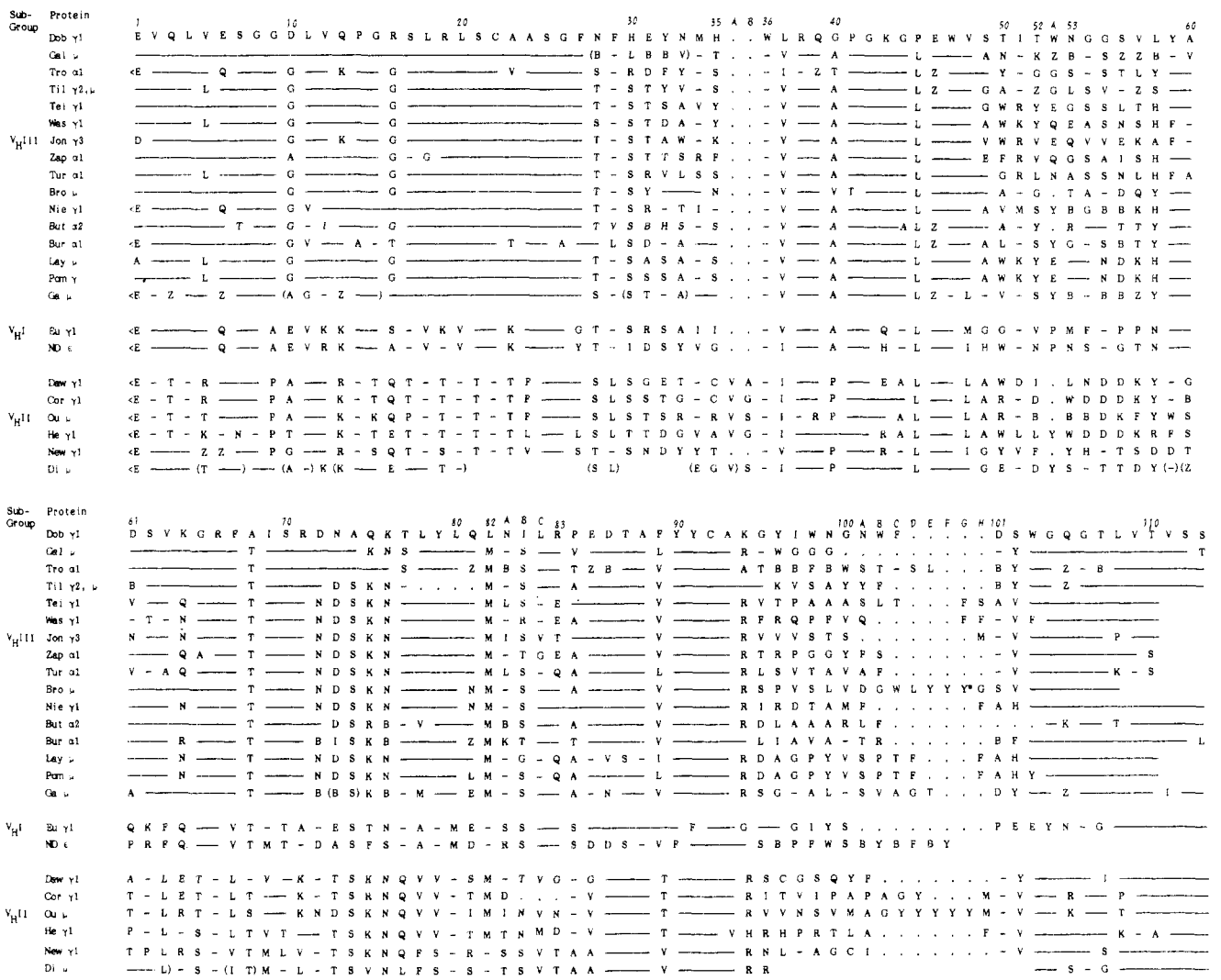

FIGURE 8: Comparison of variable-region sequences of human heavy chains. The class of the constant region is indicated next to the name of the protein. The three variable region subgroups are designated V_HI, V_HII, and V_HIII. The one-letter amino acid code is used: A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Z, Glx. <E denotes pyrrolidonecarboxylic acid. Solid lines indicate identity in amino acid sequence to that of the Dob γ1 chain in the top line. Residues in parentheses have not been ordered; periods indicate gaps introduced to improve alignment with longer sequences. In protein Bro, * means that an additional tyrosyl residue occurs between 100 G and 100 H. Blank spaces denote no reported sequence. The numbering system is that used by Kabat et al. (1976). The following are the references for the sequences: Dob (this paper); Gal (Watanabe et al., 1973); Tro (Kratzin et al., 1975); Til (Wang et al., 1977); Tei, Was, Jon, Zap, and Tur (Capra & Kehoe, 1974a; Capra & Hopper, 1976); Bro (Capra & Hopper, 1976); Nie (Ponstingl et al., 1970); But (Torano & Putnam, 1978); Bur (Liu et al., 1976); Lay and Pom (Capra & Kehoe, 1974b; Capra & Hopper, 1976); Ga and Di (Florent et al., 1974); Eu (Cunningham et al., 1970); ND (Bennich & Von Bahr-Lindström, 1974; Dorrington & Bennich, 1978); Daw and Cor (Press & Hogg, 1970); Ou (Putnam et al., 1973); He (Cunningham et al., 1971); New (Poljak et al., 1977). The subgroup assignments used here are those proposed in these references.

of mouse myeloma proteins (10 examples), as well as in all rabbit (5) and guinea pig (4) heavy-chain sequences listed by Kabat et al. (1976). This replacement could result from a single base change in a codon. Position 45 is a contact residue between the variable domains of the light and heavy chains (Padlan, 1977; Saul et al., 1978), which makes the finding of a physicochemically nonconservative substitution surprising. The possibility of an error in the sequence at position 45 is unlikely since PTH-proline was identified by three methods at position 45 of Fd′ (Table II). Moreover, *both* PTH-proline and PTH-leucine/isoleucine were found at cycle 11 of three separate analyses of CB2 (e.g., Table III). Since Fd′ is a contaminant of the CB2 preparation, the PTH-leucine/isoleucine is presumably derived from the leucine at position 11 in Fd′ and the PTH-proline from position 11 in CB2, which is position 45 in Fd′. Although the amount of PTH-proline recovered in the degradation of CB2 was not much greater than the amount of PTH-leucine (Table III), the yield of the former is ordinarily much lower than that of the latter; thus, PTH-proline and PTH-leucine are probably derived from the major and minor sequences, respectively.

Examination of the detailed three-dimensional structure of Fab′ New (Saul et al., 1978) and other immunoglobulin molecules (E. Padlan, personal communication) reveals, in fact, that a proline probably could be accommodated without difficulty at position 45 of V_H. Residues 37 and 45 in V_H contact the nearly invariant phenylalanine at position 98 in V_L (Saul et al., 1978). In Dob, V_H-37 is leucine, but in New (V_HII) and in most V_HIII proteins, it is valine. The larger side chain of leucine might compensate for the smaller proline that occurs uniquely at V_H-45 in Dob. (As shown in Figure 8, most V_HI and V_HII chains have isoleucine at position 37, but all of these have leucine at position 45. The terminal methyl group in the isoleucyl side chain, which replaces a hydrogen in one of the methyl groups of the valyl side chain, must be accommodated in the three-dimensional structure of

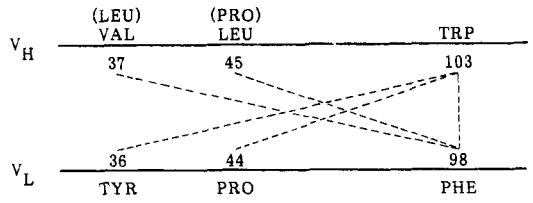

FIGURE 9: Structural relations among certain residues in V_H and V_L. The two chains are aligned by comparison of their three-dimensional structures. Contacts between V_H and V_L are indicated by the dashed lines. [These data are abstracted from Saul et al. (1978).] Leu-45 and Trp-103 are nearly invariant residues in V_H. Position 37 is Val in most V_HIII proteins but is frequently Ile in V_HI or V_HII. The substitutions found at V_H-37 and V_H-45 in Dob are indicated in parentheses. Tyr-36, Pro-44, and Phe-98 are invariant or nearly invariant in most subgroups of V_L (Kabat et al., 1976).

these proteins.) Positions 37 and 45 in V_H are homologous in structure to positions 36 and 44 in V_L. The latter two residues both contact the nearly invariant trytophan at V_H-103, a position that is homologous to V_L-98. This tryptophan (V_H-103) also forms a contact with the phenylalanine at V_L-98. The structural relations among these residues are indicated in Figure 9. Evidently, the unusual proline at V_H-45 in Dob has a structural role similar to that of the invariant (in human κ and λ) proline at V_L-44. We have recently determined that position 44 in the Dob light (κ) chain is, indeed, proline (Steiner and Margolies, unpublished experiments).

Position 68 of the Dob variable region is alanine; all of the other proteins listed in Figure 8 have threonine at this position except for Daw (V_HII), which also has alanine and Ou (V_HII), which has serine (both single base changes). At position 82, Dob has leucine instead of methionine (a single base change), which has previously been found in all V_HIII proteins. However, both V_HI and two V_HII proteins also have leucine at this position. At positions 72–76, nine of the sixteen V_HIII proteins listed in Figure 8, as well as protein Ou (V_HII), have the sequence Asn-Asp-Ser-Lys-Asn. The sequence of the Dob and Tro V_HIII proteins at these positions is Asp-Asn-Ala-Gln-Lys (all single base substitutions). Interestingly, the sequence of the V_HIII protein Gal is a "recombinant": Asp-Asn-Ala-Lys-Asn [see discussion in Kabat et al. (1978)]. Another atypical residue in the variable region of the Dob heavy chain is the aspartic acid at position 10. Of the 62 V_HIII sequences listed in Figure 8 and by Kabat et al. (1976) that include position 10, 53 have glycine at this position, 3 (Dob, Gal, and Ski) have aspartic acid (or Asx), and the others have alanine or valine (single base changes). Of the 12 V_HI or V_HII proteins, only New (V_HII) has glycine at this position. The N-terminal sequences of eight feline and canine myeloma proteins have been reported and all have aspartic acid at position 10; all of these proteins have been classified as V_HIII (Kehoe & Capra, 1972).

It is evident from the sequence data reported here that the gross structural abnormality in the Dob heavy chain does not involve the variable region. Indeed, the anomaly appears to be confined to a deletion of 15 residues in the hinge region, between C_H1 and C_H2. Possible explanations for this deletion are discussed in the preceding paper (Steiner & Lopes, 1979).

Acknowledgments

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Magnesium Ion Dependent Adenosine Triphosphatase Activity of Heavy Meromyosin as a Function of Temperature between +20 and −15 °C†

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ABSTRACT: The hydrolysis of Mg^{2+}–adenosine 5′-triphosphate (ATP) by heavy meromyosin has been studied between +20 and −15 °C, especially in the low-temperature range, in a medium containing 30% (v/v) ethylene glycol by fluorometric, spectrophotometric, and potentiometric measurements. The time course of the fluorescence changes of the enzyme during the reaction depends markedly on the temperature in consequence of large differences between the activation energies of the various steps. The observed kinetics have been analyzed according to the simplified scheme of Bagshaw & Trentham [Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331–349]. The following results have been obtained. (1) The rate-limiting step of the reaction changes in this temperature range; at 20 °C M**·ADP·P_i is the predominant steady-state complex, and M*·ADP predominates at −15 °C, with a half-life of ~10 min. (2) As expected, on the basis that it is the dissociation of the M*·ADP complex which becomes rate limiting at low temperature, one observes, in the pre-steady-state below 0 °C, both a proton burst and a lag phase in ADP release. (3) At low temperature, the equilibrium M*·ATP ⇌ M**·ADP·P_i is displaced to the left All the kinetic data obtained in this study are compatible with a simple pathway for the Mg^{2+}–ATP hydrolysis by myosin and with sequential release of the reaction products.

$$M + ATP \rightarrow M^*\cdot ATP \rightleftharpoons M^{**}\cdot ADP\cdot P_i \rightarrow M^*\cdot ADP \rightleftharpoons M + ADP + P_i + H^+$$

Myosin is one of the main protein components of muscle, and its interaction with actin in a cyclic process is responsible for muscular contraction. The hydrolysis of ATP[1] supplies the energy required to drive this cycle, and an active site for this reaction resides on each globular head of the myosin molecule.

The mechanism of ATP hydrolysis by myosin is still the object of debate (Taylor, 1977a; Tonomura & Inoue, 1977). Bagshaw & Trentham (1974), in particular, have proposed a seven-step mechanism for myosin Mg^{2+}-dependent ATP hydrolysis and have drawn attention to the strong temperature dependence of some of the steps. Thus, the rate-limiting step was found to change with temperature between +20 and 0 °C; the magnitude of this change, which is still a matter of uncertainty (Inoue et al., 1977), was small, however, and investigations over a wider temperature range are required to establish it with more certainty and also to better separate the different steps of the reaction.

Douzou (1973, 1977) and Fink (1976) have developed kinetic techniques applicable to studies at subzero temperatures and have emphasized the interest of such studies for the temporal resolution of complex enzymatic reactions into their elementary steps. We have accordingly embarked on a study of the hydrolysis of Mg^{2+}–ATP by heavy meromyosin, a proteolytic subfragment of myosin, between +20 and −15 °C, in a medium containing 30% (v/v) ethylene glycol. In the first place, the effect of ethylene glycol on the conformation and the activity of heavy meromyosin was examined. The fluorescence changes of the enzyme during the hydrolysis reaction were then measured under different experimental

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMPPNP, β,γ-imidoadenosine 5′-triphosphate; AMPCPP, α,β-methyleneadenosine 5′-triphosphate; ATPase, adenosine triphosphatase; HMM, heavy meromyosin; β-NADH, β-nicotinamide adenine dinucleotide; P_i, inorganic phosphate.

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