Preparation of a Semisynthetic Antibody†

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ABSTRACT: The antibody combining site of the mouse myeloma protein 315 is contained in the Fv fragment, composed of the variable region of light chain (V_L) and heavy chain (V_H) [Hochman, J., et al. (1973) *Biochemistry 12,* 1130]. It was also shown that the binding site for the dinitrophenyl (Dnp) ring is located in V_L [Gavish, M., et al. (1977) *Biochemistry 16,* 3154]. We now describe the solid phase synthesis of a linear polypeptide of 115 amino acid residues with the sequence of V_L of protein 315. The synthetic product was removed from the resin and the single disulfide bond was formed after reduction in 8 M urea. The synthetic material showed common antigenic determinants with native V_L and 40% of it was adsorbed on anti-V_L-Sepharose. Fractionation on Dnp-lysyl-Sepharose showed that 24% of the synthetic V_L can be adsorbed specifically on this column. Equilibrium dialysis with [^{3}H]Dnp-lysine demonstrated that the affinity-purified synthetic V_L binds 1 mol of hapten per mol of assumed dimer with an association constant of 2.9×10^3 mM^{-1}. Under these conditions native V_L dimer binds 2 mol of hapten per mol of protein with a similar affinity [Gavish, M., et al. (1977) *Biochemistry 16,* 3154]. Affinity-purified synthetic V_L and native V_H were associated by dilution from 8 M urea or 6 M guanidine hydrochloride into buffer and the solution was purified by affinity chromatography on Dnp-lysyl-Sepharose followed by elution with Dnp-glycine. The binding analysis of this material demonstrated 0.2 site with high affinity ($K_A = 7.8 \times 10^5$ M^{-1}), for Dnp-lysine, similar to the affinity of Fv for this ligand and the yield of synthetic V_L participating in this fraction was 7.1% of the affinity purified V_L. It was therefore concluded that 1.7% (0.71 × 0.24) of the synthetic V_L is able to associate with V_H yielding a semisynthetic combining site which resembles native Fv.

Antibodies are a family of closely related proteins with diverse specificity. The sequence variability of antibodies of the same class is confined to the N-terminal 110–120 residues (V_L or V_H)[1] of the light and heavy chains. The principal variability is localized in three hypervariable regions in each V domain (Wu & Kabat, 1970) which are clustered together to form the antibody combining site (Amzel et al., 1974). The rest of the sequence in the V domains may be regarded as constructing a rigid framework whose three-dimensional structure is very similar in different antibodies, and to which are attached the hypervariable loops forming the specific site (Padlan et al., 1977). It is not yet possible to correlate precisely sequence and specificity, or to predict which combining site will be generated by a particular sequence. Also, it is not always clear what the relationship is between replacements in hypervariable regions and in framework residues. It is conceivable that synthetic homologues of antibodies will enable a systematic step-by-step replacement of amino acids in the V region and will help to elucidate the contribution of each segment in the V domain to the overall structure and to the specificity of the combining site. It is hoped that, if the synthetic methods will be found suitable for the synthesis of V domains, they can also be utilized to prepare synthetic antibodies with known specificity.

The experience with the present methods of solid phase synthesis of proteins was predominantly with polypeptides of sizes no longer than 120–150 residues, such as RNase (Gutte & Merrifield, 1971), lysozyme (Sharp et al., 1973), or growth hormone (Li & Yamashiro, 1970). Previously we reported the preparation of Fv fragment from protein 315, a mouse myeloma IgA, with high affinity to nitrophenyl ligands (Inbar et al., 1972; Hochman et al., 1973). This fragment has a molecular weight of 25 000 and retains full binding activity. It is composed of V_L and V_H, each 110–120 residues long and, therefore, provides the suitable material to attempt chemical synthesis of V domains. The conditions for association of V_L and V_H, after reduction of disulfide bonds and unfolding, to form an active Fv were described (Hochman et al., 1976). It was also shown recently that V_L by itself contains the binding subsite for the Dnp moiety of Dnp ligands with a measurable affinity ($K_A = 2.3 \times 10^3$ M^{-1}) and that it is possible to adsorb V_L onto Dnp-lysyl-Sepharose (Gavish et al., 1977). Hence our approach to prepare a synthetic antibody began with an attempt to synthesize the V_L fragment of protein 315. This provides several advantages in the purification and analysis of the binding activity of the synthetic product: (a) it is possible to isolate the binding molecule from the synthetic mixture by affinity chromatography on Dnp-lysyl-Sepharose; (b) it is possible to measure the binding activity of V_L by itself; (c) the association between V_L and V_H is a very efficient process (Hochman et al., 1976) and the active material can again be purified on Dnp-lysyl-Sepharose; (d) the affinity for Dnp-lysine of the V_L–V_H recombinant should be higher than that of V_L by itself.

It should also be realized that the fact that the antibody site is formed by two peptide chains puts an additional constraint on the synthetic product (V_L), namely, the recognition of another peptide chain (V_H). Hence the synthetic V_L should have a binding site for Dnp ligands and affinity for V_H and should contain the correct sequence to contribute contact residues at the combining site of Fv.

We describe here the synthesis and analysis of the V_L portion of protein 315 and its use for the association with natural V_H to form a semi-synthetic Fv.

Experimental Section

The preparation and characterization of protein 315, its Fv,

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[1] Abbreviations used: V_L and V_H, variable portion of light and heavy chain, respectively; V_L(s), synthetic V_L; Fv, variable fragment of antibody composed of V_L and V_H; Dnp, 2,4-dinitrophenyl; PBS, 0.01 M sodium phosphate-0.14 M NaCl, pH 7.4; Gdn·HCl, guanidine hydrochloride; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; Tos, tosyl (*p*-tolylsulfonyl); im-, imidazole; DMF, dimethylformamide; TFA, CF_3COOH; DCC, dicyclohexylcarbodiimide.

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TABLE I: Assignment of Amides in V_L.

Residue no.	Result	Peptide[a] analyzed	Residues[b] spanned	Mobility,[c] pH 6.5	Net charge,[d] pH 6.5	R_f[e]	Assignment by[f]
39	Gln	Th 3	38–45	+0.1	0	0.57	Edman degradation
40	Glu	Th 3	38–45	+0.1	0	0.57	Edman degradation
43	Asp	Th 3	38–45	+0.1	0	0.57	Net charge
81	Gln	Th 1	77–88	−0.53	−3		Edman degradation
83	Glu	Th 1	77–88	−0.53	−3		Net charge
96	Asn	Th 4	96–98	+0.43	+1	0.34	Net charge
96	Asn	Th 5	96–97	+0.58	+1	0.14	Net charge

[a] Th 3 = Ile-Gln-Glu-Lys-Pro-Asp-His-Leu (Asp, 0.9; Glu, 1.5; Pro, 0.6; Ile, 1.0; Leu, 0.9; Lys, 1.0; His, 0.9); Th 1 = Ile-Thr-Gly-Ala-Gln-Thr-Glu-Asp-Asp-Ala-Met-Tyr (Asp, 2.0; Thr, 1.9; Glu, 2.0; Gly, 1.3; Ala, 1.9; Met, 0.8; Ile, 0.9; Tyr, 0.8); Th 4 = Asn-His-Phe (Asp, 1.0; His, 0.9; Phe, 0.9); Th 5 = Asn-His (Asp, 1.0; His, 0.8). [b] According to the sequence published by Dugan et al. (1973). [c] Mobility of aspartic acid was taken as −1. [d] Calculated according to Offord (1966). [e] Chromatography was in butanol:acetic acid:water:pyridine (15:3:12:10). [f] Peptide Th 3 has one His and the assignment of Gln and Glu in the second and third positions, respectively, was by the identification of the Pth-amino acids after Edman degradation. Peptide Th 1 has three charges and two known Asp. Since residue 5 was Gln, the other unknown residue was assigned as Glu. Peptide Th 4 is positively charged and therefore must have Asn.

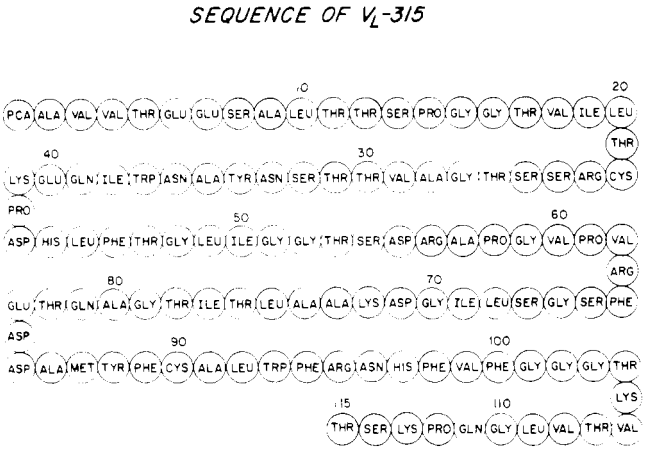

FIGURE 1: Sequence of V_L according to Dugan et al. (1973) and the assignment of amides reported here.

V_L and V_H fragments, were previously described (Hochman et al., 1973; Gavish et al., 1977). The sequence of protein 315 light chain was determined by Dugan et al. (1973), and for the purpose of our synthesis of V_L the sequence of the N-terminal 115 residues was used. However, in six positions of this sequence the amides were not determined (Glx at 39, 40, 81, and 83 and Asx at 43 and 96). In order to determine the residues at these positions V_L was reduced with 0.1 M β-mercaptoethanol in 8 M urea, alkylated with iodoacetamide, and dialyzed against 0.1 M NH_4HCO_3 to remove reagents. The reduced and alkylated V_L was digested with thermolysin and the peptides were separated by high voltage paper electrophoresis and chromatography. Since residues 34 and 88 are tyrosine and residues 44 and 97 are histidine (Dugan et al., 1973), we anticipated that the peptides containing the unknown residues will also contain either His or Tyr. Hence, Pauli positive peptides were isolated and analyzed for their mobility, composition, and partial sequence. Four Pauli positive peptides were obtained. Their analysis is given in Table I. The data indicate that residues 39 and 81 are Gln, residues 40 and 83 are Glu, residue 43 is Asp and residue 96 is Asn.

The complete sequence, including these assignments, is given in Figure 1.

Synthetic Procedure. Cross-linked chloromethylated polystyrene beads (1%) (200–400 mesh) were obtained from Bio-Rad (SX-1 Bio-Beads No. 13668) and contained 0.89 mequiv of chloride/g of resin. The chloromethylated resin (4 g) was esterified by refluxing in 100 mL of ethanol with 616 mg (2 mmol) of Boc-L-Thr(Bzl) and 0.28 mL of triethylamine (1.8 mmol) for 48 h. The resin was filtered and washed with ethanol, water, and CH_2Cl_2 and dried in vacuo at 25 °C. A sample of the Boc-L-Thr(Bzl)-resin was hydrolyzed at 110 °C for 4 h in concentrated hydrochloric acid:propionic acid (1:1) in evacuated sealed tube, and 24 h in 6 N hydrochloric acid. The threonine content was determined on the amino acid analyzer to be 0.15 mmol per g. All amino acids were protected at the α-amino position with Boc group (Schnabel, 1967), and the following side chain blocking groups were used: Arg(Tos), Asp(-OBzl), Cys(3,4-Me_2-Bzl), Glu(-OBzl), His(im-Tos), Lys(2,6-Cl_2Z), Ser(Bzl), Thr(Bzl), Trp(CHO-), and Tyr(2,6-Cl_2Bzl). The Boc-amino acid derivatives were either synthesized in our laboratory or purchased from Bachem (Marina Del Ray). The purity of each derivative was checked by paper chromatography in butanol:acetic acid:water:pyridine (15:3:12:10).

The synthesis was performed manually with a small (40 mL volume) reaction vessel which contained 4 g of the Boc-L-Thr(Bzl)-resin (containing 0.6 mmol of threonine). Boc-amino acid derivatives (1.2 mmol in 8 mL of CH_2Cl_2) were coupled three successive times: twice for 2 h and once for 18 h. The coupling was mediated with *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2 (Sheeham et al., 1955) except for Boc-Asn and Boc-Gln which were coupled as *O*-nitrophenyl esters in DMF (Bodansky & Sheeham, 1964). Due to the low solubility of Boc-L-Arg(Tos) and Boc-L-His(im-Tos), they were first dissolved in DMF and then diluted with CH_2Cl_2 before adding to the peptide resin. One cycle of synthesis consisted of: CH_2Cl_2, twice for 2 min; CH_2Cl_2:TFA:anisole (50:48:2), 2 min; CH_2Cl_2:TFA:anisole (50:48:2), 20 min; CH_2Cl_2, five times for 2 min; $CHCl_3$, three times for 3 min; CH_2Cl_2, three times for 2 min; Boc-amino acid in 10 mL of CH_2Cl_2, for 5 min; and then DCC in CH_2Cl_2 was added for further 2 h; $CHCl_3$:MeOH (1:1), twice for 5 min; CH_2Cl_2, twice for 2 min. When *O*-nitrophenyl esters were used, the CH_2Cl_2 wash before addition of the amino acid derivative was replaced with DMF and the DCC step was omitted. During all coupling steps, 100 mg of 1-hydroxybenzotriazole was added (Konig & Geiger, 1973).

The coupling and deblocking of Boc-amino acids were monitored by ninhydrin test according to Kaiser et al. (1970). The above procedures for stepwise addition of protected amino acids were repeated 114 times according to the sequence of V_L starting from $Ser^{114}L$. The final product was removed from

the resin with HF in the presence of anisole (Sakakibara et al., 1967). After removal of the HF at 0 °C under vacuum, the resin and cleaved product were precipitated with ether, washed several times, dried, and suspended in 10% acetic acid. The suspension was filtered and the solution was lyophilized. Formyl groups were removed by incubation in 1 M NH_4HCO_3 (pH 9) for 24 h followed by dialysis and lyophilization. The final product was obtained in 66% yield (5 g) assuming molecular weight of 12 000 for V_L.

The Effect of HF on Fv. The conditions for removal of synthetic V_L from the resin were checked on native Fv as follows: 10 mg of Fv was placed in a plastic tube and 2 mL of anisole and 8 mL of HF were added at 0 °C. After 30-min incubation at 0 °C the solvent was evaporated in vacuum for 15 min and in an oil pump for 2 h. The residue was suspended in 5 mL of 0.1 M acetic acid and extracted four times with 5 mL of ether to remove the anisole. The water phase was centrifuged for 10 min at 10 000 rpm and the precipitate containing more than 90% of Fv was dissolved in 4 mL of 8 M urea, 0.1 M Tris-HCl (pH 9.0). It was then diluted 50-fold with PBS and incubated at 4 °C for 24 h, after which time the material was passed through Dnp-lysine-Sepharose. The adsorbed material was eluted with 0.1 M NH_4OH and dialyzed against PBS. Analysis of the binding activity of this Fv by fluorescence quenching with Dnp-lysine indicated full recovery of activity in the Fv eluted from Dnp-lysine-Sepharose. Hence 42% of Fv can be recovered after HF treatment under these conditions, which are similar to those employed for removal of synthetic V_L from the resin.

Reduction and Reoxidation of Disulfide Bonds. This was performed according to the procedure of Hochman et al. (1976). Briefly V_L (4 mg/mL) in 8 M urea, 0.1 M Tris-Cl (pH 8.0) was reduced with 0.1 M β-mercaptoethanol. The reduced material was dialyzed against 8 M urea to remove mercaptoethanol until SH content was below 10^{-4} M, followed by dialysis against several changes of 0.1 M NH_4HCO_3 and lyophilization.

Physical and Chemical Measurements. Equilibrium dialysis was performed as described (Sharon & Givol, 1976) at 4 °C. Protein concentrations were evaluated from absorbance measurements at 280 nm in Zeiss PMQII spectrophotometer. Fluorescence spectra were measured with Perkin-Elmer fluorescence spectrophotometer Model MPF-31.

Amino acid analyses were performed on a Beckman Model 120B amino acid analyzer. Resin peptide samples were hydrolyzed at 110 °C for 4 h in HCl:propionic acid (1:1, v/v) in evacuated sealed tubes followed by hydrolysis in 6 N HCl for 20 h at 110 °C. For analysis of tryptophans, the hydrolysis was performed in 6 N HCl in the presence of 0.04% thioglycolic acid for 24 h at 110 °C in evacuated sealed tubes (Matsubara & Sasaki, 1969).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in slabs of 15% gel in Tris-Cl buffer according to Laemmli (1970). Cellulose-acetate electrophoresis was performed in a microzone electrophoresis cell (Model R-101 Beckman-Spinco) using 8 M urea, 0.1 M Tris-Cl (pH 9.0).

The extinction coefficients, $E_{280}^{0.1\%}$, of 1.5, 2.0, and 1.0 were used for Fv, V_H, and V_L, respectively (Hochman et al., 1973). The extinction coefficient E_{360}^{M} of Dnp-lysine is 17 400.

Pure organic solvents commercially available were used. Ether was washed with double-distilled water for removing traces of alcohol and then it was dried over $CaCl_2$, for 1 week, filtered, and dried on metallic sodium. Dimethylformamide was purchased from Fluka. It was distilled in high vacuum and dried over molecular sieves. HF was prepared according to Stewart & Young (1969). Dicyclohexylcarbodiimide and anisole were purchased from Fluka. [^{3}H]Dnp-lysine (1.1 mCi/mmol) was purchased from New England Nuclear (Boston). Urea (analar) was purchased from British Drug House. Antiserum to V_L was prepared in rabbits by injecting 1 mg of V_L in complete Freund's adjuvant. The antibodies were purified on V_L-Sepharose column.

Results

Solid Phase Synthesis. During the synthetic work the individual coupling steps and deprotecting steps were monitored by the ninhydrin test (Kaiser et al., 1970). Repeated coupling (three times) was used to ensure complete reaction. The couplings of the following amino acids were not satisfactory according to the ninhydrin test and therefore they were coupled 4–5 times (3–4 times in CH_2Cl_2 and once in DMF:CH_2Cl_2, 1:1): Val-108, Thr-107, Lys-105, His-97, His-44. The couplings in positions 27–33 were also less satisfactory than the other positions. Deprotection after Val-60, Pro-61, Phe-62, Arg-63, Phe 64 was performed twice according to the results of the ninhydrin test.

The synthesis started with 4 g of Boc-Thr-resin (0.15 mmol/g) and ended with 9.6 g of dry resin. After cleavage with HF, 5 g of polypeptide was obtained; hence the yield of crude peptide obtained in this work was 66%, based on the starting Boc-Thr content. This suggests that the average yields of individual steps in the synthesis were very high and that only few peptides have been lost from the resin during the course of the synthesis.

The final product was removed from the resin with HF in the presence of anisole. After removal of the HF at 0 °C under vacuum the resin and product were precipitated with ether, washed several times, dried, and suspended in 10% acetic acid. The suspension was filtered and the solution was lyophilized. To remove formyl groups from tryptophans, the material was dissolved (5 mg/mL) in 1 M NH_4HCO_3 (pH 9.0) for 24 h at room temperature followed by dialysis and lyophilization.

Reduction and Reoxidation of Disulfide Bonds. Nine hundred milligrams of synthetic V_L was dissolved in 200 mL of 8 M urea, 0.1 M Tris-Cl and was reduced with 0.1 M 2-mercaptoethanol for 30 min at 37 °C. The protein solution was oxidized by dialysis against several changes of 8 M urea–0.1 M Tris (pH 8.0) until the SH concentration was lower than 10^{-4} M (Hochman et al., 1976). The oxidized synthetic material was then dialyzed several times against 0.1 M NH_4HCO_3 to remove urea, and the material was kept in solution at −20 °C.

Characterization of the Synthetic Material. Amino Acid Analysis. Amino acid analysis of the synthetic material during several stages of the synthesis and of the final product shows that the synthetic material is very similar in comparison to the native V_L in its amino acid composition (Table II).

Size of the Synthetic Material. Sephadex G-75 chromatography in 8 M urea, 0.1 M Tris-Cl (pH 8.0), 0.05 M 2-mercaptoethanol (Figure 2) shows that a large portion of the material (~70%) has higher molecular weight than the native V_L. This high molecular weight material of the synthetic product is apparently an aggregate of synthetic V_L, even in 8 M urea. In contrast, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2) shows that a relatively small proportion of the synthetic material migrates like the native V_L while the rest migrates as a broad band having a lower molecular weight than that of the native V_L (mol wt = 12 500).

Electrophoretic Mobility. The electrophoresis in 8 M urea (pH 9.0) of native V_L and synthetic V_L (Figure 3) shows that the synthetic material is very similar to V_L in its electrophoretic

TABLE II: Amino Acid Analysis of Synthetic V_L.[a]

Amino acid	Cleaved product V_L(s)
Lys	5.1 (4)
His	2.2 (2)
Arg	3.5 (4)
Asp	8.0 (8)
Thr	14.3 (16)
Ser	8.5 (9)
Glu	8.4 (8)
Pro	6.2 (5)
Gly	16.3 (14)
Ala	10.0 (10)
½-Cys	1.6 (2)
Val	8.4 (9)
Met	1.0 (1)
Ile	4.3 (5)
Leu	8.6 (8)
Tyr	2.0 (2)
Phe	7.8 (6)
Trp	2.0 (2)
Total residues	115

[a] Amino acid analysis was performed as described in the Experimental Section. ½-Cys was determined as Cys-O_3H after performic acid oxidation. Trp was analyzed according to Matsubara & Sasaki (1969). Numbers in parentheses give the residue content in natural V_L. Data on amino acid composition at various stages of the synthesis can be found in the microfiche version of the journal (see Supplementary Material Available paragraph at end of paper) and will be provided by the authors on request.

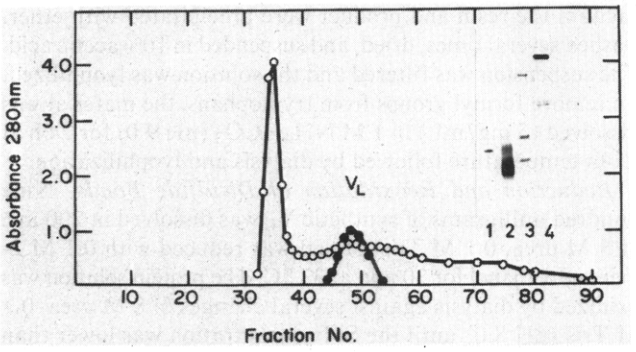

FIGURE 2: Gel filtration of crude synthetic V_L on Sephadex G-75. The column (5 × 93 cm) was equilibrated with 8 M urea, 0.1 M Tris-Cl (pH 8.0), and 0.05 M β-mercaptoethanol. (○) V_L(s); (●) V_L. (Inset) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of V_L(s) and markers. (1) V_L; (2) V_L(s); (3) markers of (from top to bottom) ovalbumin (43 000), RNase (13 800), and *Naja naja* toxin (7800); (4) heavy and light chains of mouse IgM (MOPC 104E). Polyacrylamide concentration was 15%. Electrophoresis was from top to bottom.

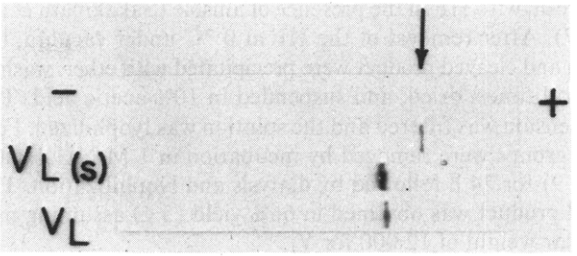

FIGURE 3: Cellulose-acetate electrophoresis of V_L(s) and V_L. Electrophoresis was in 8 M urea, 0.1 M Tris-Cl (pH 9.0). Arrow indicates the point of application.

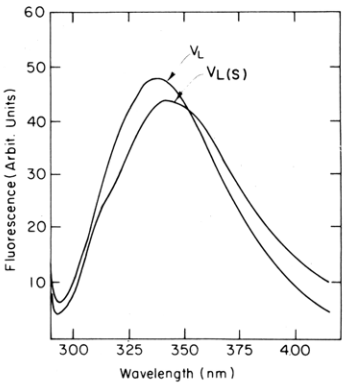

FIGURE 4: Emission fluorescence spectrum of V_L and V_L(s). Excitation was at 280 nm. Protein concentration was 0.1 mg/mL.

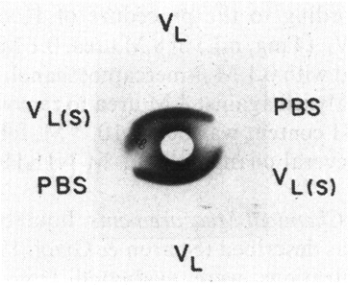

FIGURE 5: Agar gel immunodiffusion between rabbit anti-V_L and V_L or V_L(s). Center well contained anti-V_L.

mobility under these conditions.

The emission spectrum of synthetic V_L as compared with that of native V_L in PBS (Figure 4) shows that the native V_L has a maximum fluorescence at 337 nm whereas the synthetic V_L has a maximum fluorescence at 343 nm but the two spectra overlap considerably. The fluorescence yield of the two proteins is quite similar in agreement with the analysis of tryptophan which demonstrated that synthetic V_L has 2 tryptophans as expected (Table II).

Solubility. The synthetic material is soluble (10 mg/mL) in 0.1 M NH_4HCO_3 or in 0.1 M acetic acid; higher concentrations were not tested.

$E_{0.1\%}^{280}$ of synthetic V_L was found to be 1.1 whereas that of native V_L is 1.0.

Antigenic Activity of Synthetic V_L. Immunodiffusion reaction between synthetic V_L, native V_L, and anti-V_L (Figure 5) shows that the synthetic material has antigenic determinants in common with the native V_L. Forty percent of the synthetic material is adsorbed on a column of anti-V_L-Sepharose.

Binding Activity of Synthetic V_L. As was shown (Gavish et al., 1977), native V_L has a binding site for Dnp ligands and is specifically adsorbed on a column of Dnp-lysine-Sepharose. In order to check if the synthetic V_L has a similar affinity, 9.3 mL of V_L(s) in PBS (6 mg/mL) was passed on a column of Dnp-lysine-Sepharose (2.2 × 5.0 cm) and it was found that 30% of the synthetic material was adsorbed on the column. The adsorbed material was eluted with 0.05 M Dnp-glycine and dialyzed extensively against PBS. A second passage of the eluent (7 mg/mL) on such Dnp-lysine-Sepharose column showed that 80% of the material was adsorbed. The eluted material from the second run was used for binding analysis using [^{3}H]Dnp-lysine. Equilibrium dialysis of V_L and synthetic V_L (eluent of Dnp-lysine-Sepharose) in PBS against [^{3}H]-Dnp-lysine (Figure 6) shows that the synthetic V_L binds one ligand with $K_A = 2.9 \times 10^3$ whereas the native V_L binds two ligands with $K_A = 1.7 \times 10^3$. The binding data were calculated per V_L dimer which has a molecular weight of 25 000 (Gavish et al., 1977), although V_L(s) is predominantly an aggregate

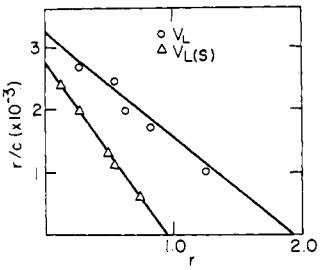

FIGURE 6: Scatchard's plot of the binding data of $V_L(s)$ and [^{3}H]Dnp-lysine. Concentration of $V_L(s)$ was 1.5 mg/mL and initial hapten concentration ranged between 10^{-3} and 10^{-4} M.

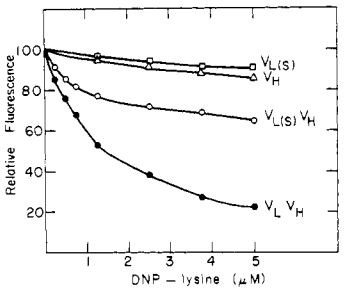

FIGURE 7: Fluorescence quenching titration of the recombinant V_L – V_H, $V_L(s)$ – V_H and of V_H and $V_L(s)$. Titration was with 50 μM N^{ϵ}-Dnp-lysine at room temperature. Protein solutions were of 0.1 OD_{280nm}.

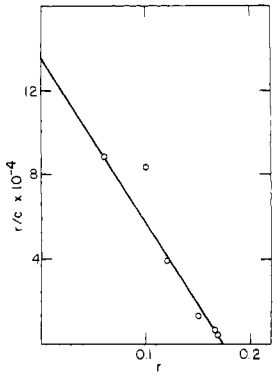

FIGURE 8: Scatchard's plot of the binding data of $V_L(s)$ – V_H recombinant after purification on Dnp-lysyl-Sepharose. Protein concentration was 1.5 mg/mL and initial hapten concentration ranged between 9×10^{-5} and 2.5×10^{-6} M.

under these conditions. The results suggest either that only 50% of the molecules bind two ligands or that one ligand is bound per dimer equivalent of $V_L(s)$. We have shown recently that V_L dimer can bind either one or two ligands per dimer depending on the pH. At pH below 7 when only one ligand is bound the affinity of V_L for Dnp-lysine is three- to four fold higher than at pH 7.4 (Gavish et al., 1977, and unpublished results). Analysis of the $V_L(s)$ fraction which binds to Dnp-Sepharose demonstrated that this material has a similar size heterogeneity to that of the initial $V_L(s)$.

Association of $V_L(s)$ with V_H. In order to test the possibility that a fraction of synthetic V_L may associate with V_H to form an active Fv, recombination experiments between synthetic V_L, previously purified on Dnp-lysine-Sepharose, and V_H were performed according to the procedure previously described (Hochman et al., 1976). Synthetic V_L in 6 M Gdn·HCl (6 mg/mL) and V_H in 8 M urea, 0.1 M Tris-Cl (pH 9) (5 mg/mL) were mixed in a 2.5:1 ($V_L(s)$:V_H) weight ratio and diluted 40-fold into PBS. After 24 h at 4 °C, the solution was passed

TABLE III: Binding Properties of Synthetic V_L ($V_L(s)$).

	$V_L(s)$	V_L[a]
Binding to Dnp-lysyl-Sepharose	30%	96%
Rechromatography on Dnp-lysyl-Sepharose	80%	
Sites	1[b]	2
K_a (M^{-1})	2.9×10^3	2.3×10^3
Yield	24%	

[a] Data from Gavish et al. (1977). [b] Calculated per dimer equivalent.

TABLE IV: Binding Properties of Recombination Product of $V_L(s)$ with V_H.

	$V_L(s)$	V_H	$V_L(s) - V_H$
Mixture (mg)[a]	12.0	5.0	17.0
Bound to Dnp-Sepharose (mg)			8.5
Yield (bound)			50%
Sites[b]			0.2
K_A (M^{-1})			8×10^5
Active material (mg)	0.85	0.85	1.7
Yield (active)[c]	7.1%		
Total yield (0.24×0.071)[d]	1.7%		

[a] $V_L(s)$ previously purified on Dnp-Sepharose was in 6 M Gdn·HCl and V_H was in 8 M urea, 0.1 M Tris-Cl (pH 9.0). [b] By equilibrium dialysis (Figure 9). [c] Yield of $V_L(s)$ from affinity purified $V_L(s)$. [d] Yield of recombined $V_L(s)$ in active Fv. See Table III for the estimate of 24%.

through a Dnp-lysyl-Sepharose column and the adsorbed material was eluted with 0.05 M Dnp-glycine and dialyzed against PBS. The eluted material contained 50% of the OD_{280} applied to the column and, on the basis of a control solution which contained $V_L(s)$ alone that was run separatively, this material contained 30% V_H and 70% synthetic V_L by weight. If indeed recombination between synthetic V_L and V_H took place, the indication is that the eluted material contained at least 40% of unassociated synthetic V_L. Analysis of the binding properties of this material by the fluorescence quenching method is shown in Figure 7. It is shown that the fluorescence of the recombinant between synthetic V_L and V_H is partially quenched by Dnp-lysine; whereas control V_H or synthetic V_L alone does not show measurable fluorescence quenching.

The material eluted from the Dnp-Sepharose column was also analyzed by equilibrium dialysis for binding of [^{3}H]-Dnp-lysine. The condition of the analysis was such that only material having high affinity for Dnp-lysine would be detected (protein concentration, 1.5 mg/mL; initial ligand concentration ranged between 9×10^{-5} and 2.5×10^{-6} M). Figure 8 demonstrates that the recombinant between $V_L(s)$ and V_H that was purified on Dnp-lysyl-Sepharose has 0.2 site for Dnp-lysine with an association constant of 7.8×10^5 M^{-1}. This indicates that 20% of the $V_L(s)$–V_H recombinant is similar in its binding affinity to native Fv ($K_A = 2 \times 10^6$ M^{-1}). The results of these experiments are summarized in Tables III and IV and indicated that 1.7% of the synthetic V_L may have, like native V_L, the capability to combine with V_H and to form an active Fv. This is in contrast to 24% of synthetic V_L which shows binding of Dnp-lysine, similar to that of native V_L. The results also indicate that the association between V_L and V_H to form an Fv imposes an additional requirement on the synthetic molecule which may be more stringent than the mere binding of the ligand. The electrophoresis of the affinity-purified recombinant between $V_L(s)$ and V_H in sodium dodecyl sulfate-polyacryl-

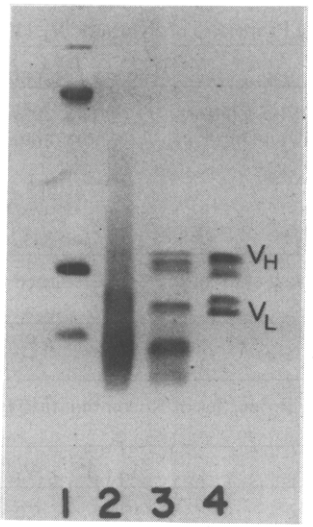

FIGURE 9: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the $V_H - V_L(s)$ recombinant purified on Dnp-lysyl-Sepharose. (1) Markers of ovalbumin (43 000), RNase (13 800), and *Naja naja* toxin (7800); (2) $V_L(s)$; (3) $V_H - V_L(s)$ purified on Dnp-lysyl-Sepharose; (4) Fv from which V_H was obtained. In this preparation of Fv size heterogeneity of V_L or V_H was observed. The presence of discrete bands in the pattern of $V_H - V_L(s)$, one of them of V_L size, indicates that V_H binds selectively to some populations of $V_L(s)$. Conditions are as in Figure 2.

amide gel is shown in Figure 9, in comparison with Fv and $V_L(s)$. The results indicate that V_H binds a selected population of $V_L(s)$ and one of the bands in the gel is similar in size to that of native V_L.

Discussion

Using the Merrifield solid phase peptide synthesis we synthesized a 115-residue long polypeptide with the sequence of V_L of protein 315. The choice of mouse myeloma protein 315 for the attempt of chemical synthesis of an antibody has several advantages: (a) This protein exhibits high affinity for nitrophenyl ligands (Eisen et al., 1968) relative to other myeloma proteins. (b) The preparation of Fv fragment comprised only of V_L and V_H was established for this protein (Hochman et al., 1973) and there is no doubt that this fragment by itself contains the combining site. (c) The conditions for efficient separation and reassociation of V_L and V_H were analyzed (Hochman et al., 1976). (d) The V_L chain by itself binds Dnp ligands and can be purified by affinity chromatography on Dnp-lysyl-Sepharose (Gavish et al., 1977). Hence there are several possible steps of affinity purification of the active polypeptide from the synthetic mixture which include: binding of $V_L(s)$ to Dnp-lysyl-Sepharose, association of $V_L(s)$ with V_H, and again binding to Dnp-lysyl-Sepharose.

A preliminary report of this synthesis was previously reported (Givol et al., 1977; Gavish et al., 1976) and in the synthesis reported here several improvements in the protecting groups were introduced (Erickson and Merrifield, 1976). The side chain blocking group for Cys was changed from MeOBzl to 3,4-diMeBzl; for His, im-Boc was replaced by Tos; for Tyr, Bzl was replaced by 2,6-Cl_2Bzl; for Arg, NO_2 was replaced by Tos; for Lys, Z was replaced by 2,6-Cl_2Z and Trp was protected by formyl group. The results indeed demonstrate improvement in the final yield of active material from 0.1% (Gavish et al., 1976) to 1.7%. In the product of this synthesis the tryptophan fluorescence yield was similar to that of V_L and the content of tryptophans (two per mole) was as expected (Dugan et al., 1973).

The most disturbing aspect of the result was the aggregation tendency of the final product even in 8 M urea (Figure 2). However, on sodium dodecyl sulfate–polyacrylamide gel most of the material is of molecular weight lower than 8000 with a distinct band at the position of V_L (Figure 2). The high tendency for aggregation may be due to incomplete removal of blocking groups during the HF treatment. This was also supported by the absorbance spectrum of $V_L(s)$ which showed a broad shoulder in the region of 260–280 nm. The low molecular weight chains in sodium dodecyl sulfate–polyacrylamide gel are due to incomplete synthesis, probably because of unavailability of the amino terminal groups at later stages of the synthesis. This may be due either to incomplete unblocking of Boc groups or to inavailability of terminal amino groups because of hydrophobic interaction of the growing chain with the resin. This suggests that for long polypeptide synthesis there is a need for improvement in the general strategy of synthesis such as fragment condensation or other methods which will overcome the difficulty in obtaining high yields of long (above 80 residues) peptide chains.

The analysis of activity in synthetic V_L involved several criteria. $V_L(s)$ shares antigenic determinants with V_L as detected by rabbit anti-V_L (Figure 5) and 40% of $V_L(s)$ can be specifically adsorbed on anti-V_L-Sepharose column. As far as binding activity is concerned there are two different criteria for the synthetic product: the binding of hapten by $V_L(s)$ and the association of $V_L(s)$ with V_H to form an active Fv. We found that $V_L(s)$ can be purified by affinity chromatography on Dnp-lysyl-Sepharose. Thirty percent of $V_L(s)$ was bound to the column and 80% of the adsorbed material was readsorbed in a second passage through this column. This material (24% of the initial $V_L(s)$) exhibits an apparent one binding site, calculated on the basis of equivalent weight of V_L. Under the conditions of this analysis V_L binds 2 mol of Dnp-lysine per dimer (molecular weight 25 000), whereas $V_L(s)$ showed only 1 mol of ligand bound per 25 000 g. This may suggest that the mode of ligand binding by $V_L(s)$ is different from that of V_L. However, the similarity in the affinity of $V_L(s)$ and V_L for Dnp-lysine (Figure 6) indicates that this binding is as specific as that of V_L. It is not clear, however, if this ligand binding is due only to the population of molecules which are identical in size to V_L. The high yield (24%) of binding molecules in $V_L(s)$ seems to exceed the proportion of material migrating like V_L in SDS polyacrylamide gel (Figure 2). Indeed the affinity purified $V_L(s)$ migrates on sodium dodecyl sulfate–polyacrylamide gel similarly to unpurified $V_L(s)$. It is conceivable that even molecules smaller than V_L may bind Dnp-lysine due to the interaction with Trp-93 which was suggested to be in the combining site and to bind Dnp by stacking interaction (Padlan et al., 1977; Dwek et al., 1977).

The conditions for ligand binding by V_L may be less stringent than the conditions required for association of V_L and V_H and the formation of the Fv combining site with higher affinity for the hapten. Indeed as is shown in Table IV only 7.1% of the material purified as active $V_L(s)$ can associate with V_H to form molecules similar in binding properties to Fv. This is deduced from the fact that the recombinant between $V_L(s)$ and V_H which was purified on Dnp-lysyl-Sepharose contains only 20% of material that binds Dnp-lysine with an association constant similar to that of Fv. When equilibrium dialysis was performed with higher initial hapten concentrations, a higher number of sites with lower affinity can be measured. This may be due to unassociated V_L which is present in the mixture. If, however, we are interested in the $V_L(s)$–V_H recombinant which is similar to native Fv the yield is around 1.7% (24% × 7.1%). These molecules represent a semisynthetic antibody composed of native V_H and synthetic V_L.

In conclusion the general approach should be reconsidered. The antibody family provides a very important system where a synthetic approach is desirable. Due to the similarity in structure and to the confinement of combining site residues to limited hypervariable regions (Wu & Kabat, 1970), the synthesis of homologues can help understanding of the chemical basis of antibody specificity. The system of protein 315 chosen in our study is probably the best available system to attempt such a synthesis. However, the low yield of authentic product obtained here does not permit yet the use of chemical synthesis to solve the aims of this study. This is because the final yield of active product is around 2% and activity is measured as a binding activity and not as an enzymatic reaction. It will therefore be very difficult to detect variations from this low activity as a result of synthetic homologues. Hence an effort should be made to improve the methodology of synthesis of long polypeptides. It is likely that improvements in the synthetic procedure in the future will allow further study of the problem of antibody diversity by the approach of chemical synthesis.

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Supplementary Material Available

Data on amino acid composition at various stages of synthesis (1 page). Ordering information is available on any current masthead page.

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