A. (1974), *Science 185,* 435.
Ladner, J. E., Jack, A., Robertus, J. D., Brown, R. S., Rhodes, D., Clark, B. F. C., and Klug, A. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4414.
Ladner, J. E., Jack, A., Robertus, J. D., Brown, R. S., Rhodes, D., Clark, B. F. C., and Klug, A. (1975b), *Nucleic Acids Res. 2,* 1629.
Levy, J., Rialdi, G., and Biltonen, R. (1972), *Biochemistry 11,* 4138.
Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High-resolution Nuclear Magnetic Resonance, New York, N.Y., McGraw-Hill, Chapter 10.
Quigley, G. J., Seeman, N. C., Wang, A. H.-J., Suddath, F. L., and Rich, A. (1975), *Nucleic Acids Res. 2,* 2329.
Reid, B. R., and Robillard, G. (1975), *Nature (London) 257,* 287.
Rich, A., and RajBhandary, U. L. (1976), *Annu. Rev. Biochem. 45,* 805.
Römer, D., Riesner, D., and Mauss, G. (1970), *FEBS Lett. 10,* 352.
Schneider, D., Solfert, R., and v. d. Haar, F. (1972), *Hoppe-Seyler's Z. Physiol. Chem. 353,* 1330.
Sprinzl, M., and Cramer, F. (1973), *Nature (London), New Biol., 245,* 3.
Sprinzl, M., Wagner, T., Lorenz, S., and Erdmann, V. E. (1976), *Biochemistry 15,* 3031.
Stout, C. D., Mizuno, H., Rubin, J., Brennan, T., Rao, S. T., and Sundaralingam, M. (1976), *Nucleic Acids Res. 3,* 1111.
Sussman, J. L., and Kim, S.-H. (1976), *Biochem. Biophys. Res. Commun. 68,* 89.
Ts'o, P. O. P., Ed. (1974), Basic Principles in Nucleic Acid Chemistry, Vol. 2, New York, N.Y., Academic Press, Chapter 5.

Comparison of the Fine Specificity of Anti-Dinitrophenyl-Combining Site Composed of Either V_L Dimer or V_L and V_H of Protein 315†

Moshe Gavish, Raymond A. Dwek,‡ and David Givol*

ABSTRACT: The Fv fragment (Hochman, J., Inbar, D., and Givol, D. (1973), *Biochemistry 12,* 1130) derived from mouse myeloma protein 315, possessing anti-dinitrophenyl (Dnp) activity, is composed of the variable portions of light chain (V_L) and heavy chain (V_H) of the intact immunoglobulin. After dissociation of Fv in 8 M urea, V_L and V_H were isolated and analyzed for their hapten binding properties. V_H was found to be an aggregate without anti-Dnp binding activity, whereas V_L was a dimer with a molecular weight of 24 000 and possessed two binding sites for N^{ϵ}-2,4-dinitrophenyllysine with an association constant of 2.3×10^3 M^{-1}. The binding properties of V_L dimer were found to be identical with those of the light chain dimer of protein 315 previously reported (Schechter, I., Ziv, E., and Licht, A. (1976), *Biochemistry 15,* 2785) and exclude the constant part of light chain from participating in the combining site of L dimer. The fine specificity of the anti-Dnp binding site of V_L dimer (V_L-V_L) or L dimer (L-L) was compared with that of Fv (V_L-V_H), or of the intact protein (L-H) by analyzing the binding of a homologous series of Dnp ligands. The affinity of protein 315 for Dnp-lysine is approximately 1000-fold greater than that of V_L dimer, whereas for DnpOH the affinity of the intact protein is only 6.5-fold greater than that of V_L dimer. Thus the subsite for binding the Dnp ring per se can be localized within V_L. On the other hand, the interaction with the side chain of Dnp ligands is negligible in V_L dimer or L dimer, whereas it is very pronounced in the intact protein, suggesting that V_H contributes most of these interactions. The binding of "strange" cross-reacting haptens (Michaelides and Eisen, 1974) like dinitronaphthol and menadione was also localized within V_L dimer. Moreover the affinity of V_L dimer toward Dnp-lysine, dinitronaphthol, and menadione is similar, whereas the intact protein, or Fv, binds Dnp-lysine much better than they bind dinitronaphthol and menadione. Upon binding of dinitronaphthol or Dnp-caproate to V_L dimer, a red shift is observed in their absorbance spectrum. This red shift is similar, although not identical, to that observed with Fv and indicates that in either V_L dimer or Fv the ligands interact with a tryptophan residue. V_L dimer has only two tryptophan residues one of which (Trp-35L) is a constant residue present in the domain interior, whereas the other tryptophan is localized in the third hypervariable region of V_L (Trp-93L) and must therefore be the one interacting with the aromatic haptens. This assignment is in agreement with a model building study of protein 315 combining site (Padlan et al., 1977). The problems of antibody multispecificity and of subsites localized on different chains are discussed.

The Dnp binding site of the mouse myeloma protein 315 (Eisen et al., 1968) has been the subject of many investigations. Several parameters of the fine structure of this site were analyzed by affinity labeling (Goetzl and Metzger, 1970; Givol et al., 1971; Haimovich et al., 1972), binding of "strange" cross-reacting ligands (Michaelides and Eisen, 1974) by kinetic mapping with various ligands (Haselkorn et al., 1974), circular dichroism (Hochman et al., 1973; Freed et al., 1976), and magnetic resonance (Dwek et al., 1975a,b). These studies elucidated the dimensions of the site and the contribution of various side chains to the binding site. They also helped to divide the site into several subsites with hydrophobic or positively

† From the Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel (M.G. and D.G.), and the Department of Biochemistry, University of Oxford, Oxford, United Kingdom (R.A.D.). *Received January 18, 1977.* Supported in part by a grant from the Israel Commission for basic science.

charged components according to the contribution of various side chains of Dnp ligands to the binding energy (Haselkorn et al., 1974). A recent model building study of protein 315 binding site supports most of these analyses (Padlan et al., 1977). Moreover the model and NMR studies indicated that the predominant binding of the Dnp ring is via a stacking interaction with Trp-93 of the light chain (Padlan et al., 1977; Dwek et al., 1977). This suggests the possibility that the light chain by itself may exhibit a binding site for Dnp ligands. Indeed it was shown recently (Schechter et al., 1976) that light chain dimer (L_2)[1] of protein 315 has two homogeneous binding sites which bind Dnp-lysine with approximately 1000-fold lower affinity than that of the intact protein. It is important to determine if the binding site is constructed by the variable region (V_L) alone and if there is one binding site which accommodates two ligands. It is also necessary to establish which part of the hapten interacts with the combining site in L_2.

To analyze these points and also to compare the fine specificity of the L-L combining site with that of the L-H (intact protein) combining site, we investigated the binding properties of the variable portion (V_L) of the L chain of protein 315. Our results demonstrate that V_L is present as a dimer (molecular weight of 24 000) which binds two Dnp ligands with the same affinity as the intact light chain dimer. On the basis of crystallographic analysis of another V_L dimer (Epp et al., 1974), we favor the interpretation that the dimer has one binding site which accommodates two ligands. Cross-reacting ligands like dinitronaphthol and menadione also bind to the $(V_L)_2$ site. The affinity for DnpOH is similar for both $(V_L)_2$ and protein 315, whereas for Dnp ligands with a longer side chain, the affinity of protein 315 is much greater than that of $(V_L)_2$. In addition, the "positive subsite" in protein 315 combining site which interacts with a negative charge on the side chain of Dnp ligands (Haselkorn et al., 1974) is absent in $(V_L)_2$. This study indicates that Trp-93L binds the Dnp ring in either protein 315 or $(V_L)_2$ and suggests that it may be possible to localize subsites on different isolated chains (H or L) of the antibody molecule.

Experimental Section

Materials. Plasmacytoma MOPC 315 was generously provided by Dr. M. Potter (National Cancer Institute, Bethesda, Md.) and variant O.P. by Dr. H. N. Eisen (M.I.T., Cambridge, Mass.). The tumor was maintained in Balb/c mice and the production of large amounts of ascites fluid containing protein 315 was achieved by injecting the tumor intraperitoneally into (Balb/c × DBA/2)F_1 mice which were injected 3-12 weeks previously with 0.5 mL of pristane (Potter et al., 1972). After 12-14 days ascites fluid was collected every other day (total approximately 30 mL/mouse) and contained 6-8 mg/mL of protein 315. The preparation of protein 315, its Fv fragment, and V_L and V_H were described earlier (Inbar et al., 1971; Hochman et al., 1973). H and L chain were separated on Sephadex G-100 equilibrated with 4 M urea-1 M propionic acid. The proteins were dialyzed against water and lyophilized.

The following haptens were used in binding experiments: [^{3}H]-N^{ϵ}-Dnp-L-lysine (1.1 Ci/mmol) was purchased from New England Nuclear (Boston); menadione (2-methyl-1,4-naphthoquinone) was from Serva (Heidelberg); 2,4-dinitro-1-naphthol-7-sulfonic acid was from Eastman (Rochester); and dinitrophenol was obtained from Fluka. Dnp-glycine, Dnp-aminobutyric acid, Dnp-ornithine, Dnp-aminocaproic acid, and Dnp-L-lysine were prepared as described by Porter (1959). Dnp-aminopropylamine was prepared by a slight modification of previous procedure (Haselkorn et al., 1974): 1,3-propylenediamine (20 mmol) in 10 mL of 0.05 M $NaHCO_3$ was brought to pH 9.0 with 6 N HCl and was reacted with FDNB (1 mmol) with stirring for 30 min. The reaction mixture was adjusted to 1 N HCl and extracted four times with ether to remove all bis-Dnp product and DnpOH. The solution was then extracted with 1-butanol, and the extract was evaporated to dryness, dissolved in ethanol, and precipitated by ether. The product was found to be pure by paper electrophoresis at pH 3.5 and contained one yellow spot with a positive electrophoretic mobility, which was also ninhydrin positive.

Methods. Equilibrium dialysis was performed as previously described (Sharon and Givol, 1976). Protein concentrations were 10^{-4} and 1.4×10^{-4} M for V_L and L, respectively, and the initial hapten ([^{3}H]Dnp-L-lysine) concentration ranged from 3×10^{-3} to 3×10^{-4} M. For protein 315 or Fv, protein concentrations were around 5×10^{-6} M. The binding of various ligands was evaluated from their inhibition of the binding of [^{3}H]Dnp-L-lysine by protein 315, L, or V_L in equilibrium dialysis. The inhibition experiments were performed with that concentration of [^{3}H]Dnp-L-lysine which gave 40% occupancy of the combining sites when determined by equilibrium dialysis with [^{3}H]Dnp-L-lysine as the sole ligand. K_I, the intrinsic association constant for the inhibiting ligand, was determined from Karush's equation (Karush, 1956; Michaelides and Eisen, 1974), $K_I = (r/r' - 1)(1 + K_c)/I$, where r and r' refer to moles of [^{3}H]Dnp-L-lysine bound per mole of protein in the absence (r) and presence (r') of the inhibitor. The free concentrations of the reference ligand [^{3}H]Dnp-L-lysine and the inhibitor are C and I, respectively, and K is the association constant for ϵ-Dnp-L-lysine.

Measurements of sedimentation velocity were made in a Spinco Model E ultracentrifuge at 56 000 rpm at a constant temperature of 20 °C. Diffusion measurements were made in the same centrifuge with a synthetic boundary cell at 20 000 rpm. Partial specific volume ($\bar{v}$) for V_L and L was calculated from amino acid composition to be 0.71 and 0.73, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in slabs of 15% acrylamide in Tris-Cl buffer according to Laemmli (1970). Cellulose acetate electrophoresis was performed in a microzone electrophoresis cell (Model R-101 Beckman-Spinco) using 0.05 M potassium phosphate buffer, pH 7.0.

Protein concentrations were evaluated from absorbance measurements at 280 nm in a Zeiss PMQII spectrophotometer and difference spectra were determined using a Cary spectrophotometer Model 118. The extinction coefficients $E_{280}^{0.1\%}$ of 1.0, 1.1, 1.5, and 1.4 were used for V_L, L, Fv, and protein 315 respectively (Underdown et al., 1971; Hochman et al., 1973). The following molar extinction coefficients at pH 8.2 were used for the ligands: DnpOH, $E_{360}^{M} = 14\,900$; DnpGly, $E_{360}^{M} = 15\,890$; Dnp-aminobutyric acid, Dnp-aminopropylamine, Dnp-lysine, and Dnp-ornithine, $E_{360}^{M} = 17\,400$; Dnp-aminocaproic acid, $E_{365}^{M} = 17\,800$; menadione, $E_{335}^{M} = 2200$; and dinitronaphthol-7-sulfonate, $E_{426}^{M} = 15\,700$.

Results

Preparation and Characterization of V_L. V_L and V_H were isolated from Fv on DEAE-cellulose in 8 M urea as previously

[1] Abbreviations used: H, heavy chain; L, light chain; V_H and V_L are the variable fragments of H and L, respectively; 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; Tris, tris(hydroxymethyl)-aminomethane; DEAE, diethylaminoethyl; NMR, nuclear magnetic resonace.

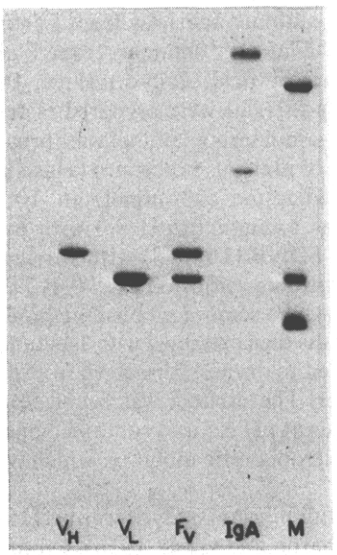

FIGURE 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein 315 IgA, its Fv fragment, and V_L and V_H isolated from Fv. M, standard markers which include *Naja naja* toxin (7800), lysozyme (14 500), and ovalbumin (43 000). IgA yields L chain (22 500) and H chain (55 000). The samples were reduced and run on a slab gel (15%) and the gel was stained with Coomassie brilliant blue. The anode was at the bottom of the gel.

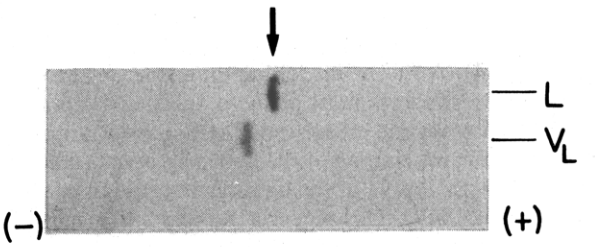

FIGURE 2: Cellulose acetate electrophoresis of V_L and L from protein 315. Electrophoresis was at pH 7.0. Arrow indicates the point of application.

TABLE I: Comparison of Some Properties of the Subunits of Protein 315.

	L	V_L	V_H
MW($NaDodSO_4$)	22 600	12 000	13 500
MW(PBS)	43 500	24 000	Aggregate
$s_{20,w}$	3.16	2.18	Aggregate
$D_{20,w} \times 10^{-7}$ ($cm^2\ s^{-1}$)	6.65	7.74	Aggregate
Dnp binding sites	2	2	0
$K_A \times 10^{-3}$	2.3	2.3	−
Binding to Dnp-Lys-Sepharose	+ (96%)	+	− (6%)

described (Hochman et al., 1973). The protein, recovered in the first (V_L) and second (V_H) fractions of this column, was dialyzed exhaustively against 0.1 M NH_4HCO_3 and either was lyophilized, or used directly for further experiments. V_L (10 mg/mL) was dissolved either in 0.01 M sodium acetate, pH 5.5 or above pH 8.5, and was dialyzed against PBS. The electrophoretic migration of V_L and V_H in sodium dodecyl sulfate-polyacrylamide gel is shown in Figure 1. Under the conditions used, the lysozyme marker deviates from the linear relationship between log molecular weight and mobility and migrates like V_L whereas V_H migrates like RNase. Calculation of the molecular weights in $NaDodSO_4$ shows that V_L and V_H have molecular weights of 12 000 and 13 500 respectively. The difference in electrophoretic mobility in neutral buffer between L and V_L is depicted in Figure 2. V_L is more positively charged than L and the two proteins are homogeneous by electrophoretic criteria. The sedimentation pattern of V_L dimer (5 mg/mL) indicated the size homogeneity of this fragment and the sedimentation coefficient was found to be 2.18 S (Table I). The molecular weights in PBS of V_L and L were calculated from sedimentation velocity and diffusion measurements in the ultracentrifuge (Table I). The results clearly show that both V_L and L are present as dimers. V_H was present as an aggregate under this condition and its solubility was not more than 1 mg/mL at neutral pH. On the other hand, V_L dissolves well (10 mg/mL) at pH 5.5 or above pH 8 and can then be brought by dialysis against PBS to neutrality. V_L can also be dissolved in 8 M urea and dialyzed against PBS with full recovery of activity.

Binding Properties of V_L and V_H. When V_L (1 mg/mL) or V_H (0.5 mg/mL) were passed on Dnp-Lys-Sepharose, only V_L showed significant binding whereas 94% of V_H was not adsorbed on this column (Table I). The bound V_L was eluted with 0.05 M Dnp-glycine (80% recovery). The results of equilibrium dialysis experiments of V_L and L are given in Figure 3. It is shown that both V_L and L bind two Dnp-lysines per dimer with the same affinity ($K_A = 2.3 \times 10^3\ M^{-1}$). The Scatchard plot in Figure 3 indicates that the binding properties of V_L and L are identical and implies that the constant portion (C_L) of L does not affect the binding and that the combining site of L is exclusively located with V_L. It is also indicated by the Scatchard plot that the two binding sites are homogeneous and independent. Equilibrium dialysis of V_H and [^{3}H]Dnp-lysine did not show binding in the range analyzed for V_L.

On the basis of x-ray crystallography of other L or V_L dimers, we assume that V_L (or L) has one combining site which binds two Dnp-lysines (Edmundson et al., 1974; Epp et al., 1974). This is compatible with each monomer having one Dnp ligand in stacking arrangement with its Trp-93. This assignment is supported by the difference spectrum of either Dnp aminocaproate or dinitronaphthol when bound to V_L or Fv as shown in Figure 4. The red shifts and hypochromic effect observed indicate that either the Dnp ligand or dinitronaphthol interact with a tryptophan residue (Little and Eisen, 1967). The difference spectrum of the same ligands with L was similar to that of V_L.

Fine Specificity of the Combining Site of $(V_L)_2$ as Compared with Fv or the Intact IgA. The combining site of protein 315 shows strong dependence of the association constant on the size and character of the side chain of various Dnp ligands (Haselkorn et al., 1974). If our interpretation that the Dnp ring binds to Trp-93 of V_L (Padlan et al., 1977; Dwek et al., 1977) is correct, it is anticipated that most of the binding of Dnp ligands to V_L is due to the Dnp ring, whereas very little is contributed by the side chain of the ligand. Hence a homologous series of Dnp ligands of increasing length and varied charge was used to map the subsites of the combining site of V_L (or L) and of the intact IgA (or Fv). In addition two other ligands, dinitronaphthol and menadione were included in this analysis since they represent some "strange" cross-reaction with Dnp ligands (Michaelides and Eisen, 1974). The results (Table II) include the binding data of these ligands as obtained by the method of inhibition of binding of [^{3}H]Dnp-L-lysine. Several features are obvious from this comparative mapping of the combining sites. It is clear that the fine specificity of the combining sites of V_L and L is very similar since the K_I of the

TABLE II: Binding of Various Ligands to Protein 315 IgA and Its L or V_L Subunits.

Ligand	K_I ($M^{-1} \times 10^{-3}$) L	V_L	IgA[a]	K_I(IgA)/K_I(V_L)	K_{rel} V_L	L	IgA
(1) Dnp-OH	0.38	0.46	3	6.5	5.2	6.6	567
(2) Dnp-$NHCH_2COOH$	1.1	1.60	50	31	1.5	2.3	34
(3) Dnp-$NHCH_2CH_2CH_2COOH$	1.2	1.40	5200	3714	1.7	2.1	0.33
(4) Dnp-$NHCH_2CH_2CH_2NH_2$	1.0	1.20	870	725	2.0	2.5	1.95
(5) Dnp-$NHCH_2CH_2CH_2CH(NH_2)COOH$	1.6	2.0	3400	1700	1.2	1.6	0.50
(6) Dnp-$NHCH_2CH_2CH_2CH_2CH_2COOH$	2.4	2.2	3600	1636	1.1	1.0	0.47
(7) Dnp-$NHCH_2CH_2CH_2CH_2CH(NH_2)COOH$	2.5[b]	2.4[b]	1700	708	1.0	1.0	1.0
(8) Dinitronaphthol-7-sulfonate	3.8	4.5	10	2.2	0.5	0.7	170
(9) Menadione (2-methyl-1,4-naphthoquinone)	2.3	1.5	70	47	1.6	1.1	24

[a] Similar results were obtained with Fv which is composed of V_L and V_H. [b] Direct binding with [^{3}H]Dnp-L-lysine yielded K_A of 2.3×10^3 M^{-1} for either L or V_L and 2000×10^3 M^{-1} for intact protein 315. [c] K_{rel} is the ratio between the association constants (K_I) of Dnp-lysine and the K_I of the respective inhibitor.

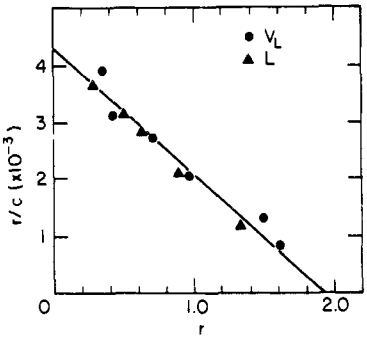

FIGURE 3: Scatchard plot of equilibrium dialysis results obtained with $(V_L)_2$ or L_2 and [^{3}H]Dnp-lysine.

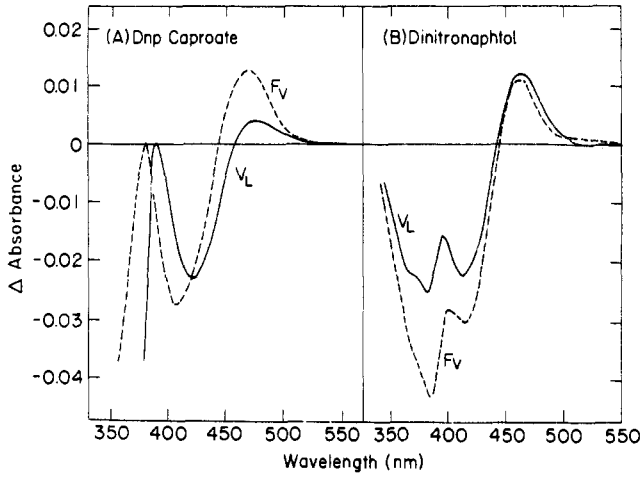

FIGURE 4: Difference spectra with Fv fragment and $(V_L)_2$ of protein 315. Each spectrum was obtained as the difference between protein and ligand in one cell and the same total ligand concentration in the reference cell. The following concentrations were employed: Fv (6×10^{-5} M) and dinitronaphthol (7.5×10^{-5} M) or Fv (1.1×10^{-5} M) and Dnp caproate (2.5×10^{-5} M); V_L (8×10^{-5} M) and dinitronaphthol (7.5×10^{-5} M) or Dnp caproate (1.5×10^{-4} M). Similar difference spectra were also measured for the light chain and were found to be similar to those of V_L.

various ligands agree very well. Some significant features of the combining site of protein 315 are absent in those of either L or V_L. The increase in affinity with the increase in length of the ligand side chain disappears almost completely. Thus, K_{rel} (the ratio of the association constants for Dnp-lysine and a particular inhibitor, Table II) for DnpOH is 5.2 and 6.6 in V_L and L, respectively, whereas it is 567 in the IgA. However, the association constant for DnpOH is quite similar (6.5-fold different) in V_L and in IgA (0.46×10^3 and 3.0×10^3 M^{-1}, respectively) in contrast to the large difference in K_I of other ligands (e.g., a 3714-fold difference for Dnp-aminobutyrate). This implies that most of the binding energy for the Dnp ring in the binding site of protein 315 (composed by heavy and light chain) is contributed by the light chain. It is also shown that increasing the length of the side chain of the ligands does not affect much the K_I of V_L or L for Dnp ligands. This is particularly pronounced with respect to the disappearance in V_L of the "positive subsite" (Haselkorn et al., 1974) which is expressed in protein 315 by the difference in K_I for Dnp-aminobutyric acid as compared with Dnp-aminopropylamine (ligands 3 and 4 in Table II).

Another remarkable feature is that the binding of the cross-reacting haptens dinitronaphthol and menadione to V_L or L are similar to that of Dnp-lysine in contrast to the situation in protein 315 (Table II and Michaelides and Eisen, 1974). This indicates that the site for binding these cross-reacting ligands is also localized in V_L.

Discussion

The Combining Site of V_L. The results reported here confirm the observation of Schechter et al. (1976) that L_2 of protein 315 combines with Dnp-lysine with K_A which is approximately 1000-fold lower than that of the intact protein. In addition it is shown that this binding activity resides within $(V_L)_2$ which has two binding sites with the same affinity toward Dnp-lysine (Figure 3) and the same fine specificity (Table II) as those of L_2, and therefore that the constant part of L is not involved in the combining site of L_2.

It is not easy to determine whether $(V_L)_2$ has two binding sites or one binding site which accommodates two Dnp ligands. On the basis of x-ray analysis of L_2 (McG) and $(V_L)_2$ (Rei or Au) (Schiffer et al., 1972; Fehlhammer et al., 1975; Epp et al., 1974), there is only one way of dimerization of V_L domains. The interaction between V_L domains is governed by very significant hydrophobic and other interactions (Edmundson et al., 1975; Fehlhammer et al., 1975) dictated by the "immunoglobulin fold" of the V_L domain. The domains are related by a pseudo-twofold symmetry axis, the structure of V_L dimer is identical in all cases analyzed, and the contacts between V regions do not depend on those present in the C region. The $(V_L)_2$ present in protein Rei or McG has a binding site composed of the hypervariable regions of both domains, and in McG L_2 (Edmundson et al., 1974) it binds more than one Dnp ligand, whereas in Rei $(V_L)_2$ only one Dnp ligand is bound (R.

Huber, personal communication). Assuming this structure holds also for $(V_L)_2$ of protein 315, we propose that it contains a similar binding site which must therefore accommodate two Dnp ligands. This means that the binding site is much broader than that of protein 315 and this is probably reflected also in its specificity (see below). As demonstrated by the Scatchard plot (Figure 3), the binding of the two ligands is independent and no cooperativity is observed in spite of their presumed proximity in the same binding site.

Fine Specificity and Subsites in Protein 315 and $(V_L)_2$. A remarkable feature of the results presented in Table II is the small difference in the association constant for DnpOH of $(V_L)_2$ and protein 315. For example, the affinity of protein 315 for Dnp-lysine or Dnp-aminocaproate is respectively 708 and 1636 greater than that of $(V_L)_2$, whereas the affinity of protein 315 for DnpOH is only 6.5-fold greater than that of $(V_L)_2$. This indicates that the predominant binding energy of the Dnp ring is provided by V_L, apparently, mainly by Trp-93. It is therefore a clear cut illustration of the subdivision of the antibody combining site into defined subsites which can in this particular case be identified with one of the chains. It is also noteworthy that in $(V_L)_2$ there is almost no increase in K_I between DnpOH and other ligands with longer side chains, whereas, in protein 315 combining site which is composed of both V_L and V_H, the affinity for Dnp-lysine is 567-fold greater than for DnpOH (Table II). Moreover, different side chains of the ligand can sense special features of protein 315 combining site. Thus Dnp-aminobutyric acid binds about 6-fold better than Dnp-aminopropylamine due to a "positive subsite" in protein 315 (Haselkorn et al., 1974). This feature is also absent in $(V_L)_2$. It is suggested that a significant part of the interactions with side chains of Dnp ligands are due to contributions from the heavy chain, whereas the Dnp ring is bound predominantly to the light chain.

Another interesting feature of the $(V_L)_2$ binding site is the relatively strong binding of dinitronaphthol and menadione to $(V_L)_2$ which is similar to that of Dnp-lysine. This is in contrast to the 24- or 170-fold lower affinity of menadione and dinitronaphthol, respectively, to the intact IgA when compared with Dnp-lysine (Table II). As discussed above with regard to DnpOH, this indicates that these "strange" cross-reacting ligands also bind to the subsite present in V_L.

Previous difference spectral analysis of menadione bound to protein 315 or present in a tryptophan solution demonstrated that menadione is associated with a Trp residue in the protein 315 combining site (Michaelides and Eisen, 1974). Figure 4 shows that the red shift and hypochromia observed in the absorbance of Dnp-aminocaproate or dinitronaphthol when bound to either $(V_L)_2$ or Fv are similar, indicating that the ligands occupy the same environment in both proteins. Similar results were obtained also with L_2. Since these difference spectra are due to interaction with tryptophan residues (Little and Eisen, 1967), it indicates that the Trp residue in protein 315 which is responsible for binding either Dnp ligands, menadione or dinitronaphthol, is localized within V_L. Since (as will be discussed below) there is only one Trp residue in V_L which is a candidate for this interaction, we suggest that dinitronaphthol is bound to the same site to which the Dnp ring is bound. This evidence when applied to $(V_L)_2$ rather than to V_L-V_H leaves less room for suggestions about multispecificities based on *different* sites in the V domains (Richards and Konigsberg, 1973). Rather it indicates that "strange" cross-reactions between ligands may take place in the antibody combining site with a significant overlapping of their location at the same site. It should be pointed out that, whereas the difference spectra of dinitronaphthol bound to V_L and Fv are very similar, the difference spectra of Dnp-caproate has some significant differences, mainly in the 470-nm region. The red shift in this wavelength region is much less pronounced with V_L and may indicate that the location of the Dnp ligand in $(V_L)_2$ is somewhat different from that of protein 315; in addition it suggests that, although the Dnp ring and the dinitronaphthol are near the same Trp, they are not occupying precisely the same space. These results provide a structural basis for suggestions concerning multispecificity of antibodies recently put forward by Johnston and Eisen (1976). In their article they provide evidence that the cross-reaction between Dnp ligands and menadione is not "strange" at all but represents a typical cross-reaction based on structural similarity of the ligands which is very obvious to the antibodies site, but sometimes looks "strange" to the investigator's eye. Our results indeed support their conclusion.

NMR analysis (Dwek et al., 1977) as well as CD analysis (Freed et al., 1976) clearly indicate the involvement of a Trp residue in the binding of the Dnp ring in protein 315 as well as in its light chain. A model building study by Padlan et al. (1977) placed the Dnp ring parallel to Trp-93L with a stacking interaction between them. This was not self evident since Trp-35 of the H chain is also close to the site. The results reported here verify the assignment made in our model building (Padlan et al., 1977) and NMR analysis (Dwek et al., 1977). V_L has only two Trp: Trp 35 which is a constant Trp and is buried in the domain interior near the disultide bond, and therefore cannot be available to the Dnp ligand, and Trp 93 which is in the third hypervariable region and must therefore be the one responsible for the binding. Hence it is also Trp-93L which causes the red shift of Dnp absorbance (Little and Eisen, 1967; Eisen et al., 1967), the induced CD spectrum near 300 nm (Freed et al., 1976), and some of the ring current shifts in the magnetic resonance of Dnp protons (Dwek et al., 1977). We therefore suggest that in $(V_L)_2$ each Dnp ligand stacks to Trp-93 of one of the domains and that the two ligands are situated side by side in the same combining site.

Finally it should be emphasized that the results reported here represent a particular case of the antibody site of protein 315, where the Dnp subsite of Dnp ligands can be localized in the V_L region. Other examples like that of the anti-phosphorylcholine myeloma proteins clearly show that the predominant contact residues are contributed by the V_H region (Segal et al., 1974). It is possible that more information on the distribution of such subsites and on their contribution to the antibody site formed by both H and L chains will increase our understanding of the nature of antibody diversity.

Added in Proof

We now find that $(V_L)_2$ exists in two pH-dependent conformations: at pH 7.4 it binds two ligands per dimer, whereas at pH 6.0 $(V_L)_2$ binds only one ligand per dimer with higher affinity. The transition between the two conformers is at pH 6.9 and indicates conformational flexibility of antibody combining site composed of identical chains.

Acknowledgments

We thank Sylvia Wilder and Rivka Azulai for excellent technical assistance.

References

Dwek, R. A., Jones, R., Marsh, D., McLaughlin, A. C., Press, E. M., Price, N. C., and White, A. K. (1975a), *Philos. Trans. R. Soc. London, Ser. B 272*, 53.

Dwek, R. A., Knott, J. C., Marsh, D., McLaughlin, A. C., Press, E. M., Price, N. C., and White, A. I. (1975b), *Eur. J. Biochem. 53,* 25.

Dwek, R. A., Wain-Hobson, S., Dower, S., Gettins, P., Sutton, B., Perkins, S. J., and ivol, D. (1977), *Nature (London) 266,* 31.

Edmundson, A. B., Ely, K. R., Abola, E. E., Schiffer, M., and Panagiotopoulos, N. (1975), *Biochemistry 14,* 3953.

Edmundson, A. B., Ely, K. R., Girling, R. L., Abola, E. E., Schiffer, M., Westholm, F. A., Fausch, M. D., and Deutsch, H. F. (1974), *Biochemistry 13,* 3816.

Eisen, H. N., Simms, E. S., and Potter, M. (1968), *Biochemistry 7,* 4126.

Epp, O., Colman, P., Fehlhammer, H., Bode, W., Schiffer, M., Huber, R., and Palm, W. (1974), *Eur. J. Biochem. 45,* 513.

Fehlhammer, H., Schiffer, M., Epp, O., Colmar, P. M., Lattman, E. E., Schwager, P., Steigeman, N., and Schram, H. J. (1975), *Biophys. Struct. Mech. 1,* 139.

Freed, R. M., Rockey, J. H., and David, R. C. (1976), *Immunochemistry 13,* 509.

Givol, D., Strausbauch, P. H., Hurwitz, E., Wilchek, M., Haimovich, J., and Eisen, H. N. (1971), *Biochemistry 10,* 3461.

Goetzl, E. J., and Metzger, H. (1970), *Biochemistry 9,* 3862.

Haimovich, J., Eisen, H. N., Hurwitz, E., and Givol, D. (1972), *Biochemistry 11,* 2389.

Haselkorn, D., Friedman, S., Givol, D., and Pecht, I. (1974), *Biochemistry 13,* 2210.

Hochman, J., Inbar, D., and Givol, D. (1973), *Biochemistry 12,* 1130.

Inbar, D., Hochman, J., and Givol, D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2659.

Inbar, D., Rotman, M., and Givol, D. (1971), *J. Biol. Chem. 246,* 6272.

Johnston, M. F. M., and Eisen, H. N. (1976), *J. Immunol. 117,* 1189.

Karush, F. (1956), *J. Am. Chem. Soc. 78,* 5519.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Little, R. J., and Eisen, H. N. (1967), *Biochemistry 6,* 3119.

Michaelides, M. C., and Eisen, H. N. (1974), *J. Exp. Med. 140,* 687.

Padlan, E. A., Davies, D. R., Pecht, I., Givol, D., and Wright, C. (1977), *Cold Spring Harbor Symp. Quant. Biol.* (in press).

Porter, R. R. (1959), Methods in Medical Research, Vol. 3, Gerard, R. W., Ed., Chicago, Ill., Yearbook Publishers, p 256.

Potter, M., Humphrey, J. G., and Walters, J. L. (1972), *J. Natl. Cancer Inst. 49,* 305.

Richards, F. F., and Konigsberg, W. H. (1973), *Immunochemistry 10,* 545.

Segal, D. M., Padlan, E. A., Cohen, G. H., Rudikoff, S., Potter, M., and Davies, D. R. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4298.

Schechter, I., Ziv, E., and Licht, A. (1976), *Biochemistry 15,* 2785.

Schiffer, M., Girling, R. M., Ely, K. R., and Edmundson, A. B. (1973), *Biochemistry 12,* 4620.

Sharon, J., and Givol, D. (1976), *Biochemistry 15,* 1591.

Underdown, B. J., Simms, E. S., and Eisen, H. N. (1971), *Biochemistry 10,* 4359.