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Preparation of Fv Fragment from the Mouse Myeloma XRPC-25 Immunoglobulin Possessing Anti-Dinitrophenyl Activity†

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ABSTRACT: The myeloma IgA protein, produced by plasmacytoma XRPC-25, was isolated by affinity chromatography on dinitrophenyllysine-Sepharose. The affinity constant of the intact protein or its Fab′ toward 2,4-dinitrophenyl-L-lysine (Dnp) was found to be 2.6×10^5 M^{-1}. In order to prepare an Fv fragment (Hochman, J., Inbar, D., and Givol, D. (1973), *Biochemistry 12,* 1130) from this protein, the heavy and light chains were separated and the light chain was digested with trypsin at pH 8.2 to yield half a light chain. This digest was reassociated with the heavy chain and the recombinant was digested with papain at pH 5.7. Fractionation of this digest on a Sephadex G-75 column and Dnp-lysine-Sepharose resulted in the isolation of an Fv fragment which possesses one binding site for Dnp-lysine ($K_a = 2.0 \times 10^5$ M^{-1}). The active Fv fragment has a molecular weight of 23 400 and is composed of two peptide chains, each having a molecular weight of approximately 12 000. The N-terminal residues of these chains are aspartic and glutamic acids, which are also N-terminal in the heavy and light chains, indicating that the Fv is composed of V_L and V_H.

The heavy (H)[1] and light (L) chains of immunoglobulins are comprised of a linear array of compact domains resulting from the folding of homology regions of approximately 110 residues each. The L and H chains have two and four such domains, respectively (Edelman, 1971; Poljak et al., 1972). It has been demonstrated that the antibody combining site is contained entirely in the Fv region which is composed of the N-terminal V_L and V_H domains (see Givol, 1975, for a review). An Fv fragment was indeed isolated from the mouse myeloma protein 315 and was shown to possess an intact combining site (Inbar et al., 1972; Hochman et al., 1973) and all the idiotypic determinants of protein 315 (Wells et al., 1973). Hence, Fv is the elementary unit of recognition and specificity in the immune system and it is desirable to develop methods for its preparation from other immunoglobulins.

The preparation of Fv from human myeloma IgM, using pepsin digestion, was reported by Kakimoto and Onou (1974). Various attempts in our laboratory to prepare Fv fragments by digesting the Fab′ fragment from mouse myeloma proteins other than protein 315 were unsuccessful. Similarly, digestion with papain, pepsin, or trypsin of the intact Ig, reduced and alkylated Ig, or molecules recombined from separated H and L chains, did not yield Fv fragment. A reasonable explanation for this may be that protein 315 has a unique type of L chain (λ2) which is not present in other mouse immunoglobulins (Schulenburg et al., 1971). This λ2 differs significantly from other L chains in its constant (C_L) portion and therefore its interaction with C_H1 may be less tight, thus rendering these domains susceptible to peptic digestion, leaving an intact Fv of protein 315.

We therefore used another approach for the preparation of Fv, based on the following considerations. Human L chain can be split into two halves (Solomon and McLaughlin, 1969; Karlsson et al., 1969; Poulsen et al., 1972) and conditions for the preferential recovery of either V_L or C_L were described (Seon et al., 1972). The preparation of V_H domain from rabbit heavy chain was also reported recently (Huston et al., 1972; Mole et al., 1975). It is conceivable that either V_L or V_H can recombine with the other chain to yield an active recombinant in which the Fv region will be protected from enzymic digestion.

We have tried this approach on the mouse myeloma IgA produced by plasmacytoma XRPC-25 which possesses anti-Dnp[2] activity. The L chain was split by trypsin and the digest was recombined with the H chain. Papain digestion of this recombinant yielded an active Fv fragment of this protein.

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[1] Nomenclature and abbreviations for immunoglobulins correspond to those recommended by the World Health Organization (1964). The proteins and their fragment are referred to as protein 25, Fv 25, or Fv 315, corresponding to the plasmacytomas that secrete these proteins.

Materials and Methods

Preparation and Purification of Protein 25. Plasmacytoma XRPC-25 was generously provided by Dr. M. Potter (National Cancer Institute, Bethesda, Md.). The tumor was maintained in Balb/c mice and the production of large amounts of ascites fluid containing protein 25 was achieved by injecting the tumor intraperitoneally into (Balb/c × DBA/2) F_1 mice which were injected 3 weeks previously with 0.5 ml of pristane (Potter et al., 1972). Ascites fluid from these mice was collected every 2–3 days (average 15–20 ml/mouse) and protein 25 was purified by affinity chromatography on Dnp-lysine-Sepharose as described for protein 315 (Inbar et al., 1971), except that elution of the adsorbed protein 25 was by 0.2 M NH_4OH. The eluted protein was brought to pH 8.0, dialyzed against water, and lyophilized. The yield was 3–4 mg/ml of ascites fluid. Protein 25 is an IgA containing κ type L chain (M. Potter, personal communication). The Fab′ fragment was prepared by pepsin digestion as described (Inbar et al., 1971).

Separation and Recombination of Light and Heavy Chains. Lyophilized protein 25 was dissolved in 0.2 M Tris-Cl (pH 8.2) and reduced with 0.1 M 2-mercaptoethanol for 1 h at room temperature, followed by alkylation with 0.15 M iodoacetamide. The alkylated protein was dialyzed against 0.1 M NaCl, brought to 1 M propionic acid, and chromatographed at room temperature on a Sephadex G-100 column, equilibrated and developed with 4 M urea–1 M propionic acid. The separated light and heavy chains were dialyzed exhaustively against water to remove urea and propionic acid, and lyophilized.

Lyophilized heavy or light chain was dissolved in water (10 mg/ml) by raising the pH to 11.0 with 1 M NaOH, followed by neutralization immediately after dissolution of the protein (approximately 1 min). Association of H and L chains to yield active molecules was followed by the adsorption of such mixtures on Dnp-lysine-Sepharose.

Enzymic Digestion. Tos-PheCH_2Cl-trypsin, soybean trypsin inhibitor, pepsin, and papain were obtained from Worthington Biochemical Corp., Freehold, N.J. Digestion of L chain with trypsin was performed at 37 °C in 0.1 M Tris-Cl (pH 8.2) and was terminated with soybean trypsin inhibitor (1:1 weight ratio with respect to trypsin). Papain digestion was performed in 0.01 M sodium acetate–0.01 M 2-mercaptoethanol (pH 5.7) at 37 °C, and terminated with iodoacetamide as described for rabbit Fd (Mole et al., 1975).

Physical and Chemical Measurements. Equilibrium dialysis was performed in small Lucite chambers. Protein (50 μl) (2×10^{-5} M) in PBS[1] and an equal volume of 2,4-[^{3}H]Dnp-L-lysine (Amersham) were added to opposite sides of the membrane. The cells were equilibrated at 4 °C for 20 h and 25 μl was withdrawn from each compartment and counted in 3 ml of Bray's solution (Bray, 1960) in a Packard liquid scintillation counter.

Measurements of sedimentation velocity were made in a Spinco Model E ultracentrifuge at 60 000 rpm at a constant temperature of 20.3 °C. Diffusion measurements were made in the same centrifuge with a synthetic boundary cell at 20 000 rpm. Samples were equilibrated by dialysis against 0.14 M NaCl–0.01 M sodium phosphate (pH 7.4). The molecular weight was calculated according to the Svedberg equation: $M = RTs_{20,w}/D_{20,w}(1 - \bar{v}\rho)$. The partial specific volume ($\bar{v}$) for Fv was assumed to be 0.730.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in slabs of 12.5 or 15% gels in Tris-Cl buffer, as described (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 was fixed and stained with Ponceau S stain solution. Protein concentrations were evaluated from absorbance measurements at 280 nm with a Zeiss PMQII spectrophotometer. The extinction coefficient of a 1% solution of protein 25 or its L and H chains was taken as 14.0. N-Terminal amino acids were determined by the dansyl method (Gray, 1967), and the dansylamino acids were identified on polyamide sheets (Chang Ching Trading Co. Ltd., Taiwan).

Results

Tryptic Digestion of L Chain. Tryptic digestion of L chain (10 mg/ml) for various time intervals and at different pH values was followed by sodium dodecyl sulfate disc gel electrophoresis. Optimal conditions for obtaining half L chains were found to be 90 min of digestion in 0.1 M Tris-Cl (pH 8.2) at 37 °C, using an enzyme to substrate weight ratio of 1:30 (Figure 1). It is seen that the digest contains half L chain fragments which are slightly smaller than ribonuclease and also undigested L chain.

Recombination of L Chain Digest with H Chain. Conditions for recombination between L and H chains were sought by mixing the chains at different pH values and binding to columns of Dnp-lysine-Sepharose (2 × 1 cm). When equimolar amounts of light and heavy chains at pH 7.8 and 1 mg/ml were mixed and allowed to stand for 4 hr at room temperature, 80% of the mixture was found to bind to Dnp-lysine-Sepharose, indicating good recovery of active IgA molecules. Similar conditions were used also for the recombination of the tryptic digest of L chain with H chain except that the molar ratio of L to H was 1.5:1, to compensate for loss of product by extensive digestion of L chain.

A tryptic digest of 36 mg of L chain was diluted with water to 1 mg/ml and mixed with 54 mg of heavy chain (1 mg/ml) at pH 7.8. After storing overnight at room temperature the solution was dialyzed against 0.01 M sodium acetate (pH 5.7). This solution was made 0.01 M mercaptoethanol and digested with papain (enzyme to protein weight ratio, 1:200) for 80 min at 37 °C, and the digestion was terminated by alkylation with iodoacetamide (0.015 M) at pH 8.2. The turbidity which was present during the digestion at pH 5.7 disappeared upon raising the pH. The clear solution was concentrated and fractionated on a Sephadex G-75 column in PBS and the protein that emerged in the fraction corresponding to Fv (Figure 2) was dialyzed against water and lyophilized. This fraction contained 15 mg which is 42% of the maximum yield expected on the basis of the starting light chain.

Characterization of the Fv Fragment of Protein 25. Preliminary analysis of the Fv fragment eluted from the Sephadex G-75 column showed that 85% of this protein was adsorbed specifically on Dnp-lysine-Sepharose. Figure 3 depicts sodium dodecyl sulfate disc electrophoresis of the Fv fragment before and after its purification on Dnp-lysine-Sepharose. It is seen that the purified Fv is composed of two major peptide chains having molecular weights of approximately 12 000. The sizes of these peptide chains are similar to those of Fv obtained from protein 315 (Figure 3).

[1] Abbreviations used are: Dnp, 2,4-dinitrophenyl; PBS, 0.15 M NaCl–0.01 M sodium phosphate (pH 7.4); Tos-PheCH_2Cl, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

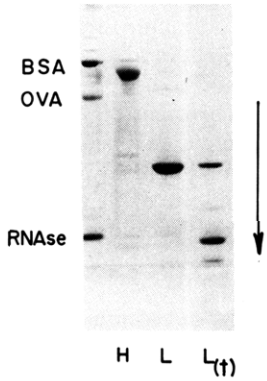


FIGURE 1: Sodium dodecyl sulfate disc gel electrophoresis of a trypic digest of L_{25}. Digestion conditions are specified in the text; $L_{(t)}$, tryptic digest of L chain.

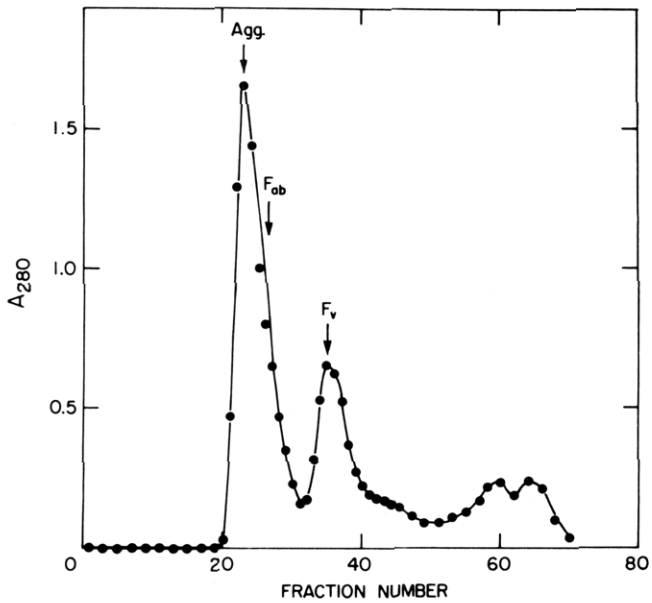


FIGURE 2: Fractionation of a papain digest of the recombinant between H chain and L chain digest, on a Sephadex G-75 column. The column (2.5 × 80 cm) was in PBS and fractions of 8 ml were collected. Agg, material that emerged at the front of the column, was found to be composed of small fragments on sodium dodecyl sulfate gel; Fab, position where marker Fab emerged from the column. These fractions contained Fab also in this fractionation.

From sedimentation velocity and diffusion data an s_{20} of 2.3 S and D_{20} of 9.03×10^{-7} cm^2 s^{-1} were determined for the Fv fragment. The molecular weight of Fv was calculated to be 23 400. This and the results of the sodium dodecyl sulfate disc gel electrophoresis indicate that Fv is composed of two peptide chains of similar size, each approximately 12 000 daltons.

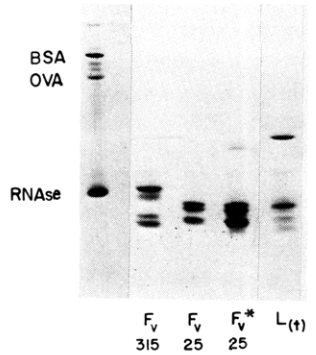


FIGURE 3: Sodium dodecyl sulfate disc gel electrophoresis of Fv 25 as compared with Fv 315: Fv^*_{25}, the fraction emerged from the Sephadex G-75 column; Fv 25, after affinity chromatography on Dnp-lysine-Sepharose.

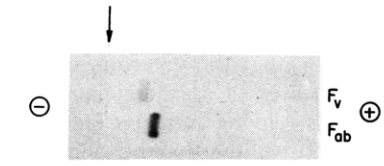


FIGURE 4: Cellulose acetate electrophoresis of Fv and Fab derived from protein 25. Electrophoresis was at pH 7.0. Arrow indicates the point of application.

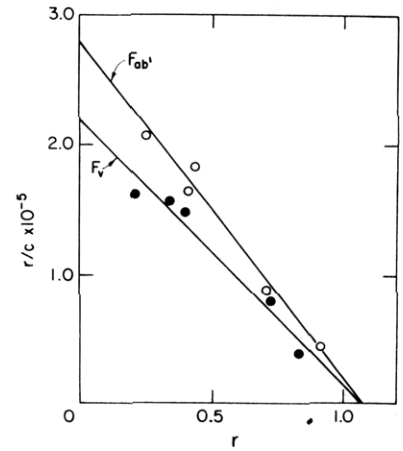


FIGURE 5: Scatchard plot of equilibrium dialysis results obtained with Fab or Fv and [^{3}H]Dnp-lysine.

N-Terminal analysis of Fv 25 determined Asp and Glu as the only N-terminal residues, similar to the N-terminal residues of heavy (Glu) and light (Asp) chains. Thus, the peptide chains of Fv are V_L and V_H. The difference in electrophoretic mobility between Fab′ and Fv fragments of protein 25 is shown in Figure 4.

The binding activity of Fv 25 and Fab′ 25 was measured by equilibrium dialysis with [^{3}H]Dnp-L-lysine. The Scatchard plot of the results shown in Figure 5 demonstrates that Fv like Fab′ has one binding site and the association constants of both are very similar ($K_a = 2 \times 10^5$ M^{-1} and 2.6×10^5 M^{-1} for Fv and Fab′, respectively).

Discussion

This paper describes the preparation of an active Fv fragment from mouse myeloma IgA produced by plasmacytoma XRPC 25, and possessing anti-Dnp activity. The molecular weight of this Fv is 23 400 and it contains one binding site with an association constant of 2×10^5 M^{-1} which is very similar to that of the Fab or the intact protein 25. Fv 25, like the previously obtained Fv 315 (Hochman et al., 1973), is composed of two peptide chains, V_L and V_H, and the sizes of these chains are indeed similar for the two Fv fragments, although the V_H of Fv 315 is slightly larger than that of Fv 25 (Figure 3). The heterogeneity of the peptide chains of Fv_{315} or Fv_{25} on sodium dodecyl sulfate gel is due to slight differences in size of V_H and V_L, probably due to heterogeneity in the positions of the enzymic split.

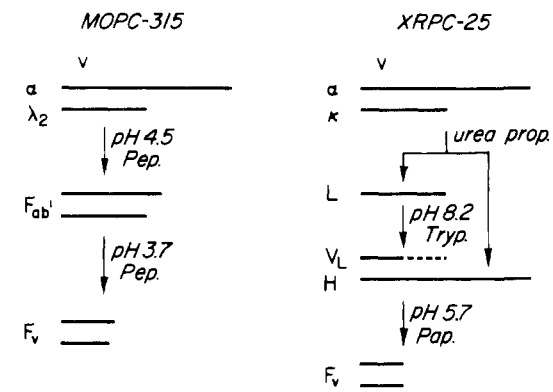
This study further substantiates the existence of Fv fragments due to noncovalent interaction between V_L and V_H. It was, however, obvious from the preliminary studies on protein 25, and other mouse myeloma proteins, that the conditions used for obtaining Fv 315, i.e., by peptic diges-

tion of Fab′ at pH 3.7 (Hochman et al., 1973), are not suitable for the digestion of Fab′ derived from other mouse Ig. It should be recalled that the digestion of Fab′ 315 with pepsin at pH 3.7 cleaves the constant parts, C_L and C_H1, into small fragments, leaving an intact Fv, rather than split the switch region between the V and C domains. Hence, the difference in susceptibility to peptic digestion of Fab 315 and other mouse Fab′ lies in the different stabilities of their constant domains. This may be correlated with the unique type of L chain (λ2) in protein 315 which is not present in any other mouse Ig (Dugan et al., 1973; Schulenburg et al., 1971).

To circumvent the difficulty encountered in digesting Fab′, other than that derived from protein 315, we tested the possibility of limited digestion of L chain followed by reassociation of the digest with H chain. The recombinant may contain molecules composed of V_L and H where the Fv region will be protected from enzymic digestion. Trypsin partially split L chain to yield fragments which are half the size of L (Figure 1). These fragments were not characterized further and they may include both V_L and C_L.

However, 85% of the fraction emerging from Sephadex G-75 at a position corresponding to Fv (Figure 2) was adsorbed on Dnp-lysine-Sepharose. It is possible that the unadsorbed material represents a fragment composed of C_L and C_H1, but the majority of the half L chain that recombined with H yielded after papain digestion an active Fv. We, therefore, favor the interpretation that V_L is the major component present in the half L chain fragment (Scheme I). The undigested L (Figure 1) also recombined with H and after papain digestion yielded Fab that emerged from Sephadex G-75 in the position indicated in Figure 2. This fraction was not analyzed further, since our major interest was in obtaining Fv which was clearly separated from Fab on Sephadex G-75. The results presented in this paper demonstrate that by this procedure an active Fv can be obtained in a significant yield (~35%), from protein 25. It may be possible to extend this method to other Ig. Obviously the critical step is the limited digestion of L chain, leaving an intact V_L. This may not always be straightforward. Preliminary attempts to prepare in this way an Fv from protein 460, another mouse IgA with anti-Dnp activity, did not yield an Fv fragment. Since L chains of both protein 25 and protein 460 are of the κ type the difference may be in the V_L sequence. At position 18 of V_L, protein 460 has an Arg (Hood et al., 1973) whereas protein 25 has Ser (S. Rudikoff, personal communication). It is possible that trypsin splits the N-terminal 18 residues in protein 460 and thus produces a shorter V_L which is unable to recombine with the H chain. Since this Arg (or Lys) at position 18 is present in a significant number of mouse L chains (Hood et al., 1973), it may be difficult to prepare from them Fv in the way described here. An alternative approach will be to split Fd into halves and to use V_H domains for recombination with L chain. This may be particularly promising in rabbit antibodies where L chain contains an interdomain disulfide bond between V_L and C_L which prevents the isolation of V_L domains in spite of cleavage at the switch region (Poulsen et al., 1972). Procedures to prepare V_H in good yield were recently reported for rabbit immunoglobulin (Mole et al., 1975) and the association of V_H with L chain may provide the suitable recombinant to prepare Fv fragment in these cases. In any event, the preparation of an active Fv 25 reported here indicates that in spite of difficulties in fragmentation, Fv fragment may be obtained from an immunoglobin and that this fragment indeed contains the antibody combining site.

Scheme I: Schematic Diagram of the Preparation of Fv Fragments.



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