Crystallization with Hapten of the Fab' Fragment from a Mouse IgA Myeloma Protein with Antidinitrophenyl Activity

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SUMMARY

Protein 315, a mouse myeloma IgA protein which binds nitrophenyl ligands, and its pepsin-produced Fab' fragment have been purified by affinity chromatography. The Fab' fragments were found to be homogeneous by polyacrylamide electrophoresis and isoelectric focusing, and to possess a uniform binding constant. These fragments were readily crystallizable at low salt concentration near their isoelectric point (pH 4.7). Similarly crystals can be obtained of the Fab'-hapten complex. Such crystals have a 1:1 molar ratio of hapten (ε-N-dinitrophenyl lysine) to protein.

Crystallization of Human Immunoglobulins or Their Active Fragments

Crystals of human immunoglobulins or their active fragments have been obtained in several laboratories (1-4). However, no known binding activity was assigned to these proteins. Protein 315, produced in mice by the plasmacytoma MOPC 315, is a monoclonal mouse IgA which is capable of binding DNP and TNP ligands with high affinity, and exhibits in its reaction with such ligands all the characteristics of binding sites of conventionally prepared anti-DNP and anti-TNP antibodies (6). In addition the binding sites of Protein 315 were localized on its Fab fragment (7). It is therefore desirable to crystallize this protein or its active fragment and in particular to crystallize the active fragment with its ligand. In this communication we report the preparation of the pepsin-produced Fab' of Protein 315 and its crystallization with and without hapten.

MATERIALS AND METHODS

Transplantation of Tumor—Plasmyctoma (MOPC 315)-bearing mice were generously provided by Dr. H. N. Eisen. The tumor was propagated in Balb/C mice by injecting 0.2 ml of tumor homogenate, in Hank's solution, subcutaneously over the anterior dorsal part of the animal. After 14 to 20 days when the tumors reached a sufficiently large size, the mice were exanguinated from the orbits. An average of 90% of injected mice survived to the stage of bleeding. The yield of blood was 1.5 to 2.0 ml per animal, the serum was separated and kept at -20°C.

Purification of Protein 315—The immunoglobulin secreted by the tumor (Protein 315) was isolated immunospecifically as a monomer by a method similar to that described by Goetzl and Metzger (8). The immunoglobulins from 200 ml of serum were precipitated at 45% saturation of (NH₄)₂SO₄. The centrifuged precipitate was dissolved with water (70 to 80 ml) and exhaustively dialyzed against 2 x 6 liters of 0.15 m NaCl, 0.01 m sodium phosphate buffer, pH 7.4, and finally against 0.2 m Tris-HCl, pH 8.2. After dialysis the solution (100 ml) was reduced with 0.01 m dithiothreitol for 1 hour at room temperature, and alkylated with 0.05 m iodoacetamide for 20 min. The alkylated solution was applied to a column, 4 x 16 cm of ε,N-DNP-lysine-Sepharose conjugate, equilibrated, and run with 0.05 m NaCl, 0.003 m sodium phosphate buffer, pH 7.4 (eluting buffer). The column was washed at a flow rate of 300 ml per hour, with the eluting buffer until the absorbance at 280 nm was 0.05. The adsorbed Protein 315 was specifically eluted by 40 ml of 0.05 m DNP-glycine (titrated to pH 1.4) and fractions of 15 ml were collected. Each of the yellow fractions was passed through a column, 2 x 4 cm, of Dowex 1-X8 (200 to 400 mesh) equilibrated with the eluting buffer, to remove DNP-glycine quantitatively, and the protein-containing fractions were pooled. The yield of the eluted protein was 1 g. Fluorescence-quenching titration of this protein (100 μg per ml) with ε-DNP-lysine gave 60% quenching of the protein fluorescence which is close to values obtained by others (7), indicating the purity of the isolated protein.

Preparation of Fab' Fragment—One gram of immunopurified Protein 315 (8.5 mg per ml in the eluting buffer) was adjusted to pH 4.7 by the addition (one-tenth of its volume) of 0.5 m sodium acetate buffer, pH 4.5, and 10 mg of papain (in 1 ml of 0.003 m sodium acetate, pH 4.5) were added. After 6 hours of incubation at 37°C the small amount of precipitate was centrifuged and the supernatant was brought to pH 8 and applied to a column, 3 x 14 cm, of DNP-lysine-Sepharose column. Elution of the Fab' fragment from the column was with 0.05 m DNP-glycine as for the undigested Protein 315. Activity of the purified Fab was assayed by equilibrium dialysis and by fluorescence-quenching titration (9) with ε,N-DNP-L-lysine.

Equilibrium Dialysis—This was performed with dialysis chambers of 1-ml capacity separated by 0.0008-inch Visking

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membranes, at 25° for 24 hours. Hapten (N'-[14C]DNP-lysine) concentration on both sides of the membrane were determined by counting 0.5 ml of solutions for 10 min. The protein concentration was 100 μg per ml and the specific activity of the hapten was 5.3 × 10^5 cpm per μmole.

Materials—Iodoacetamide (Fluka) was recrystallized from hot petroleum ether (b.p. 30-60°). Dithiothreitol was obtained from Calbiochem. DNP-glycine was prepared according to the method of Porter (10). N'-[14C]DNP-lysine was prepared by reacting 2,4-dinitrofluorobenzene (Amersham-Searle, Des Plaines, Illinois) with polylysine (n = 300, a kind gift of Mr. I. Jacobson) in 0.2 M NaHCO₃ for 3 hours at 37°. The solution was extracted with ether to remove excess reagent and the product was precipitated by acidification with HCl, followed by 24 hours of hydrolysis at 108°, with 6 N HCl, in sealed tubes. The hydrolysate was diluted with 5 volumes of water and extracted thoroughly with peroxide-free ether. The [14C]DNP-lysine was extracted into 1-butanol and brought to dryness.

Spectral Analyses—Absorbances were determined with a Zeiss PMQ II spectrophotometer. The extinction coefficient of Protein 315 or its Fab' (0.1%) at 280 nm were taken as 14.0 (7). Spectra and difference spectra were recorded with a Cary model 15 spectrophotometer, and fluorescence measurements were determined with Turner model 210 recording spectrofluorimeter.

Electrophoresis and Sedimentation—Isoelectric focusing was performed with LKB 8100 Ampholine electrofocusing equipment with a 110-ml column with pH gradient between pH 3 to 10. Electrophoresing was for 48 hours with 300 volts, after which the column content was drained at a flow rate of 1 ml per min and fractions of 1.5 ml were collected. The absorbance and pH of each fraction was determined. Electrophoresis in polyacrylamide gel (7% acrylamide) was performed according to the method of Davis (11), at pH 8.6. Sedimentation analyses were performed with Spinco model E ultracentrifuge at a speed of 56,100 rpm.

RESULTS

Preparation of Fab' Fragment—The course of disappearance of the Protein 315 and appearance of the Fab' during the peptic digestion can be followed qualitatively by the difference in their mobilities in acrylamide electrophoresis (Fig. 1). Preliminary studies indicated that 6 hours of digestion were sufficient to split all the monomer Protein 315 to Fab' and Fc peptides. The peptic digest of 900 mg of Protein 315 was applied to a column, 3 × 14 cm, of DNP-lysine-Sepharose conjugate. The elution pattern of this column showed that 34% of the absorbance at 280 nm was not adsorbed by the column and this represents presumably peptides derived from Fc. Elution with DNP-glycine specifically released 55% of the absorbance at 280 nm applied to the column. The protein-containing fractions in the DNP-glycine eluate (108 ml) were pooled, concentrated by vacuum dialysis to a concentration of 30 mg per ml. Portions (0.2 ml) of this material were dialyzed in 8/32 Visking membranes against different buffers (20 ml) of various ionic strength and pH. Crystallization occurred within 1 day upon dialysis at room temperature against dilute buffers near the isoelectric point. The optimal range for crystallization was between pH 4.6 to 4.9 in 0.002 to 0.005 M sodium acetate or sodium propionate buffers. The crystallization depends very much on temperature and it was slower at 4° than at 22°. The crystals formed are needle-like crystals (Fig. 3) and about 50% of the protein was found to be in the crystalline fraction. Further concentration of the...
supernatant of this solution showed that at least 85% of the protein can be crystallized. The crystals were readily dissolved in 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4. When subjected again to dialysis against 0.002 M sodium acetate, pH 4.7, crystals can be obtained at initial protein concentration of 2 to 3 mg per ml.

Crystallization in the presence of the hapten \(\epsilon, N\)-DNP-lysine or \(\epsilon, N\)-DNP-amino caproate was performed under similar conditions except that the hapten was added to the protein solution and to the outside solvent in a concentration of \(10^{-3} \text{ M}\). The Fab'-hapten complex crystallized as yellow crystals. To test the molar ratio of Fab' to hapten in the crystals, crystallization was performed with \(N^\epsilon\text{[H]DNP}-\text{lysine} \) which was diluted with nonradioactive hapten to give 131,000 cpm per pmole. After 2 days the yellow crystals were centrifuged and washed three times with 0.5 ml of 0.002 M sodium acetate buffer, pH 4.7, until no more radioactivity appeared in the supernatant. The crystals were dissolved in 0.15 M NaCl and the amount of protein and hapten present were determined from the absorbance at 280 nm (after subtracting the contribution of DNP-lysine to 280-nm absorbance) and radioactivity, respectively. The absorbances at 280 and 360 nm were 0.91 and 0.22, respectively, and this solution contained 1660 cpm per ml. Hence, the ratio of hapten to protein in the dissolved crystals was 1.05, indicating that the combining sites of all the Fab' molecules in the crystals were occupied by the ligand. The difference spectrum between this solution and a solution of \(\epsilon, N\)-DNP-lysine with identical concentration showed two maxima at 470 and 383 nm similar to the results of Eisen et al. (6). This provides additional evidence that the hapten is present in the combining site of the Fab' in the solution obtained from the yellow crystals and therefore presumably also in the combining site of the Fab' in the crystals. No difference was observed in the general shape of the crystals thus obtained and the crystals obtained without hapten. Moreover, when crystals of Fab' were dialyzed against 0.002 M sodium propionate, 0.001 M \(\epsilon, N\)-DNP-lysine, pH 4.7, the crystals incorporated the hapten and became yellow.

**DISCUSSION**

The procedure described for the isolation by affinity chromatography of Protein 315 from mouse serum on DNP-lysine-Sepharose column is very similar to that described by Goetzl and Metzger (8) for the isolation of this protein from ascites. This is a very simple and rapid procedure and ensures maximal recovery of Protein 315. The yield of Protein 315 (5 mg per ml of serum) is somewhat lower than that reported by Eisen, Simms, and Potter (7) but this may be caused by some degeneration of the tumor line. Under the same conditions we were able to isolate Protein 315 in its polymorphic form (without prior reduction and alkylation) in the same yield. This preparation, although showing ligand-binding activity similar to the monomeric form, was slightly contaminated with red color, probably hemoglobin. Hence, we preferred to isolate the reduced and alkylated monomer of Protein 315. Isoelectric focusing of the isolated monomer showed that it is homogeneous with isoelectric point at pH 4.8. However, attempts to crystallize it near the isoelectric point were unsuccessful, hence we turned to crystallization of the Fab' fragments. Papain- and trypsin-digested Protein 315 also yielded Fab fragments which we were able to crystallize but comparative studies indicated that the pepsin-produced Fab' is more readily crystallizable and we therefore concentrated on this preparation. In some preparations the step of immunospecific isolation of the Fab' on DNP-lysine-Sepharose was omitted and the peptic digest was directly applied to a Sephadex G-100 column. The protein which emerged at a position corresponding to Fab' was pooled concentrated and upon dialysis against 0.002 M sodium acetate buffer, pH 4.7, gave also a good yield of crystals, similar to that obtained with immunospecifically purified Fab'.

Various Fab fragments from human myeloma proteins have been crystallized before (1, 3, 4); none of these have a known antibody activity. Protein 315 is an IgA produced by mouse plasmacytoma. It has high affinity combining sites to DNP ligands and its reaction with such ligand is indistinguishable from that of anti-DNP antibodies induced in experimental animals, as was indicated by the stoichiometry of binding, the quenching of the protein tryptophans fluorescence and the characteristic red shift in absorbance of the ligand upon its binding to Protein 315 (7). The crystallization of the Fab' fragment of this protein is another reflection of the structural homogeneity of this protein. It is clear, therefore, that x-ray analysis of the hapten-protein crystals will provide information pertinent to any antibody-combining site. The crystals obtained so far are
too small for x-ray analysis of a single crystal and attempts are currently being made to grow larger crystals.

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