Isolation and Study of a Fragment of Human Serum Albumin Containing One of the Antigenic Sites of the Whole Molecule

By C. LAPRESLE AND T. WEBB

Service de Chimie microbienne, Institut Pasteur, Paris, France

(Received 14 August 1964)

1. A fragment of human serum albumin called 'inhibitor' has been degraded by trypsin, and one of the degradation products, designated fragment F1, has been isolated. Fragment F1 has a molecular weight of 6600. It contains neither tyrosine nor tryptophan. It is not precipitated with rabbit anti-sera to human serum albumin. 2. Fragment F1 was coupled to *p*-aminobenzylcellulose to form an insoluble conjugate. Rabbit anti-(human serum albumin) antibodies reacting with fragment F1 were specifically adsorbed on this conjugate and were desorbed by glycine-hydrochloric acid buffer. The isolated antibodies are composed of γ -globulin and β_2 -macroglobulin. 3. Human serum albumin and fragment F1 formed with 7s anti-(fragment F1) antibodies soluble complexes that were studied by passive haemagglutination, ultracentrifugation and electrophoresis. Fragment F1 was shown to contain only one of the antigenic sites of albumin molecule. The 7s anti-(fragment F1) antibodies were shown to be bivalent and monospecific.

A fragment of human serum albumin called 'inhibitor' was shown by Webb & Lapresle (1964) to contain at least and probably only two of the antigenic sites of the albumin molecule. The present work describes the isolation and properties of a fragment produced by tryptic degradation of 'inhibitor'. This fragment has been shown to contain only one of the antigenic sites of the albumin molecule. It has been designated fragment F1.

MATERIALS

DEAE-cellulose (Selectacel) was obtained from Carl Schleicher and Schuell Co., Kenne, N.H., U.S.A. It was used in columns provided with a cooling jacket kept at 4°.

Sephadex G-25 and G-200 were obtained from Pharmacia, Uppsala, Sweden. They were used in columns at room temperature.

p-Aminobenzylcellulose (Serva-cellulose) was obtained from Touzart et Matignon, Paris, France.

The molarity of the potassium phosphate buffers used is expressed with respect to phosphate and the pH was measured with a pH-meter.

Human serum albumin (Squibb fraction V), prepared by ethanol fractionation, was kindly given by the American Red Cross.

The 'inhibitor' fragment of human serum albumin was prepared as described by Webb & Lapresle (1964).

Trypsin was once-crystallized Novo-Trypsin obtained from Martinet et Houet, Paris.

Rabbit anti-(human serum albumin) sera were prepared as described by Lapresle & Webb (1960) and were collected after 6 weeks of immunization. The sera of 12 rabbits were pooled and heated for 1 hr. at 56° before use.

Goat anti-(rabbit serum) serum was prepared by repeated intravenous injections of rabbit serum.

METHODS

Electrophoresis and immunoelectrophoresis. Electrophoresis in agar gel and immunoelectrophoresis were carried out according to the technique of Grabar & Williams (1955) under the following conditions: agar gel at 1%; either 50mm-sodium veronal buffer, pH 8.2, in the reservoirs and 25mm-sodium veronal buffer, pH 8.2, in the gel, or 50mm-potassium phosphate buffer, pH 5.5, in the reservoirs and 25mm-potassium phosphate buffer, pH 5.5, in the gel; 7v/cm. for 1 hr. The simple electrophoresis plates were dried and stained with Amido Black according to the technique of Uriel & Scheidegger (1955).

Ultracentrifugation. Solutions for ultracentrifugation were prepared in 5mm-potassium phosphate buffer, pH 8.0, containing NaCl (0.15M), incubated at 37° for 1 hr. and kept at 4° for 48 hr. These solutions were centrifuged at 20° and 59780 rev./min. in a Spinco model E ultracentrifuge. The sedimentation coefficients were calculated by the method described by Schachman (1959).

Molecular weight. The molecular weight of fragment F1 was calculated by the method of Yphantis (1960). The average values obtained at four different concentrations and three different speeds (37020, 31410 and 25980 rev./min.) were extrapolated to zero concentration.

Specific precipitation and inhibition of specific precipitation. Specific precipitation was done according to the technique of Heidelberger & Kendall (1935), and the amount of precipitate was measured with the biuret reagent of Weischelbaum (1946) by using a standard curve prepared with rabbit γ -globulin. Inhibition experiments

were carried out with increasing quantities of fragment F1 and constant amounts of human serum albumin and rabbit anti-(human serum albumin) serum at their equivalence point. Fragment F1 was incubated with the serum for 30 min. at 37° before the addition of albumin.

Passive haemagglutination and inhibition of passive haemagglutination. The tanned-red-cell technique of Boyden (1951) was used under the conditions described by Borduas & Grabar (1953). Inhibition experiments were done by adding a constant amount of fragment F1 to each dilution of purified antibody, incubating for 30 min. at 37° and 30 min. at room temperature before adding the red cells sensitized with human serum albumin. The bisdiazotized-red cell technique was used under the conditions described by Stavitsky & Arquilla (1955).

Isolation of anti-(human serum albumin) antibodies reacting with fragment F1. Diazotized benzylcellulose was prepared as described by Webb & Lapresle (1964). To 6g. of diazotized cellulose, suspended in distilled water, 45mg. of fragment F1 was added with stirring at 4°. After 48hr. of stirring, the pH of the mixture was adjusted to 8.0 by the addition of 1M-K₂HPO₄ and stirring continued for 24hr. The fragment F1-benzylcellulose conjugate was washed with 0.15M-NaCl solution. Less than 5mg. of fragment F1 was recovered in the washing. The unchanged diazo groups were blocked by adding glycine.

The anti-(human serum albumin) antibodies reacting with fragment F1 were isolated by adsorption on and desorption from a column of the fragment F1-benzylcellulose conjugate, as described by Webb & Lapresle (1964) for the isolation of anti-'inhibitor' antibodies by using 'inhibitor'-cellulose conjugates.

Protein determination. The amount of protein in the preparations of human serum albumin, 'inhibitor' and fragment F1 were measured with the biuret reagent of Weichselbaum (1946) by reference to a standard curve made with human serum albumin, and the amount of protein in the antibody preparations with the same reagent by reference to a standard curve made with rabbit γ -globulin.

RESULTS

Degradation of 'inhibitor'. A portion (85mg.) of 'inhibitor' was dissolved in 15ml. of 50mM-potassium phosphate buffer, pH 8.0, containing sodium chloride (0.15M). To this solution 0.17ml. of an aqueous solution of trypsin (1mg./ml.) was added and the mixture incubated at 37° for 2hr. The reaction was stopped by the addition of 2 drops of 0.1M-di-isopropyl phosphorofluoridate. The digest was dialysed for 24hr. against 3l. of 50mM-potassium phosphate buffer, pH 5.5. Fig. 1a shows the electrophoretic pattern of the non-diffusible material. There are two groups of constituents: a minor one on the cathodic side, and a major one on the anodic side of the starting reservoir.

Fractionation of degraded 'inhibitor'. The chromatographic fractionation of degraded 'inhibitor' is shown in Fig. 2. The extinction of the eluates was measured at 220m μ because the absorption at 280m μ was negligible. The dialysed solution of degraded 'inhibitor' was applied to a column

Fig. 1. Photometric tracing of the electrophoretic pattern in agar gel at pH 5.5 given by: (a) non-diffusible degraded 'inhibitor'; (b) chromatographic fraction IV of the degraded 'inhibitor'. R, starting reservoir.

(30cm. long $\times$ 1.7cm. diam.) of DEAE-cellulose equilibrated with 50mM-potassium phosphate buffer, pH 5.5. Three fractions (I-III) were obtained by washing the column with the initial buffer. The passage of the same buffer containing sodium chloride (50mM) eluted fraction IV. The irregularity of the initial part of the elution pattern of fraction IV corresponds to the buffer front. The material eluted in the first tubes of this fraction had the same electrophoretic mobility as the rest of the material in this fraction. Some material remained on the column and could be eluted (fraction V) by the same phosphate buffer containing sodium chloride (0.2M).

Electrophoresis in agar gel (Fig. 1b) shows that fraction IV was the major component of the group of the components migrating to the anodic side of the starting reservoir.

Fraction IV was further purified by filtration through a column (50cm. long $\times$ 2cm. diam.) of Sephadex G-25 suspended in 0.15M-sodium chloride. The component of fraction IV that could be detected by electrophoresis in agar gel was eluted in the exterior volume of the Sephadex column by the passage of the same sodium chloride solution. It was dialysed for 48hr. against two changes of 2l. of distilled water and then freeze-

Fig. 2. Elution diagram of non-diffusible degraded 'inhibitor' on DEAE-cellulose. A 15 ml. sample of a solution of degraded 'inhibitor' was applied at tube 0 and the column was washed with 50 mm-potassium phosphate buffer, pH 5.5. At tube 96 the same phosphate buffer containing NaCl (50 mm) was added. At tube 137 the same phosphate buffer containing NaCl (0.2 M) was added.

dried. We have designated this component fragment F1. Approx. 20 mg. of fragment F1 could be prepared from 85 mg. of 'inhibitor' by the method described.

Properties of fragment F1. (a) By ultracentrifugation, fragment F1 was homogeneous and had a molecular weight of 6600.

(b) The absorption spectrum of fragment F1 showed that there was no tryptophan or tyrosine present.

(c) Fragment F1 was not precipitated in solution or in agar gel with rabbit anti-(human serum albumin) sera and did not inhibit the precipitation of such sera by human serum albumin when tested at fragment F1/albumin molar ratios ranging from 0.1 to 30. Neither tanned red cells nor bisdiazotized red cells treated with fragment F1 were agglutinated by the rabbit anti-(human serum albumin) sera.

Isolation of anti-(human serum albumin) antibodies reacting with fragment F1. Rabbit anti-(human serum albumin) serum was passed through the column of fragment F1-benzylcellulose. All the effluent fractions (1 ml.) gave positive ring-tests with human serum albumin, showing that there were anti-albumin antibodies that were not adsorbed on the column. The antibodies adsorbed on the column were eluted with 0.5 M-glycine-

hydrochloric acid buffer, pH 1.0. The saturation of the column was estimated by passing increasing amounts of the serum and measuring the amount of antibody recovered. The maximum amount of antibody that could be recovered was about 10 mg. for a column of 40 mg. of fragment F1 coupled to 6 g. of benzylcellulose. The amount of anti-serum required to saturate the column varied from 150 to 250 ml., depending on the serum used. The amount of antibody recovered/ml. of serum was about 1% of the amount of anti-albumin antibody measured by specific precipitation. The fragment F1-benzylcellulose preparations could be used repeatedly (at least ten times) without appreciable loss of activity.

Properties of the antibodies isolated from the columns of fragment F1-benzylcellulose. (a) Fig. 3 shows the electrophoretic and immunoelectrophoretic patterns in agar gel given by the isolated antibodies. The major electrophoretic constituent has the mobility of γ -globulin. There are also constituents having the mobilities of β - and α -globulins. Immunoelectrophoretic analysis revealed two lines of precipitation: one given by γ -globulins extends into the zone of β - and α -globulins, the other, which is close to the antigen reservoir, given by β_2 -macroglobulin.

Fig. 3. (a) Photometric tracing of the electrophoretic pattern in agar gel at pH 8.2 given by the antibodies isolated from the column of fragment F1-benzylcellulose. (b) Immunelectrophoretic pattern of the same antibodies. R, Starting reservoir.

(b) Fig. 4 shows the gel-filtration pattern of the isolated antibodies on Sephadex G-200. The material was eluted in two fractions and the eluates were pooled to form two fractions, as shown in Fig. 4. Fraction I was shown by immunelectrophoresis and ultracentrifugation to be β_2 -macroglobulin and was not studied further. Fraction II was shown by immunelectrophoresis and ultracentrifugation to be γ -globulins with uncorrected sedimentation coefficient 6.7s. This fraction, which represented more than 90% of the isolated antibodies, was used in the experiments reported below and was designated as anti-(fragment F1) antibodies.

Immunological behaviour of human serum albumin and fragment F1 with isolated anti-(fragment F1) antibodies. Human serum albumin or fragment F1 was not precipitated in solution or in agar gel with anti-(fragment F1) antibodies. Tanned red cells sensitized with human serum albumin were agglutinated by the anti-(fragment F1) antibodies, which shows that albumin reacts with these antibodies. Tanned or bisdiazotized red cells sensitized with fragment F1 were not agglutinated by the anti-(fragment F1) antibodies. However, as shown in Table 1, fragment F1 inhibited the agglutination by anti-(fragment F1) antibodies of tanned red cells sensitized with albumin. This shows that fragment F1 reacted with the anti-(fragment F1) antibodies.

The complexes formed between albumin or fragment F1 and the anti-(fragment F1) antibodies were analysed by ultracentrifugation. The ultracentrifugal patterns are given in Fig. 5 and the concentrations of the reagents and the sedimenta-

Fig. 4. Elution diagram of the gel filtration on Sephadex G-200 of the antibodies isolated from the columns of fragment F1-benzylcellulose. A 9 ml. sample of a solution of isolated antibody (4.7 mg./ml.) was applied to the column (85 cm. long $\times$ 2 cm. diam.). The column was washed with 50 mM-phosphate buffer, pH 8.0, containing NaCl (0.15 M). The volume of the eluate fractions was 5 ml.

Table 1. *Inhibition with fragment F1 of the agglutination by anti-(fragment F1) antibodies of tanned red cells sensitized with human serum albumin*

The tanned red cells were sensitized with 100 μ g. of human serum albumin/ml. of 2.5% (v/v) suspension of red cells. They were tested with serial 1:2 dilutions of a solution of anti-fragment F1 antibodies.

Amount of F1 (μ g./tube)	Minimum amount of antibody giving a positive reaction (μ g./tube)
0	0.2
10	1.6
20	3.2
40	3.2
80	6.4
160	12.8

tion constants of the observed components are in Table 2.

Fig. 5(a) was given by a mixture of anti-(fragment F1) antibody and an excess of human serum albumin. There are two components: one having sedimentation coefficient 4.4s represents free

albumin; the other having sedimentation coefficient 8.9s represents an albumin-antibody complex. There is no free antibody.

Fig. 5(b) was given by a mixture of human serum albumin and an excess of anti-(fragment F1) antibodies. There is a major peak that with further centrifugation separated into two constituents: one having sedimentation coefficient 6.7s represents free antibody; the other having sedimentation coefficient 7.7s represents an albumin-antibody complex. There are also traces of a minor component having sedimentation coefficient 11.4s. There is no free albumin.

Fig. 5(c) was given by a mixture of fragment F1 and an excess of anti-(fragment F1) antibody. There is a major peak that corresponds to com-

ponents having sedimentation coefficient 6.7s. There are also traces of a minor component having sedimentation coefficient 10.0s.

Electrophoresis of the solution of fragment F1 and anti-(fragment F1) antibody. The solution of fragment F1 and anti-(fragment F1) antibody studied by ultracentrifugation was also submitted to electrophoresis in agar gel. Fig. 6 shows the electrophoretic pattern obtained. The major peak is given by material that has the electrophoretic mobility of the free antibody. On the anodic side of this peak there is a significant amount of material that has a mobility intermediate between that of free antibody and that of fragment F1. These results indicate that there was in this solution free antibody and an appreciable amount of antibody combined as a complex with fragment F1.

Fig. 5. Ultracentrifugal patterns of anti-(fragment F1) antibodies mixed with human serum albumin or fragment F1. The concentrations of the reagents and the sedimentation constants of the observed components are given in Table 2. The arrow indicates the direction of the sedimentation. The times after full speed was obtained were: (a) 40 min.; (b) and (c), 48 min.

Fig. 6. Photometric tracing of the electrophoretic pattern in agar gel at pH 8.2 given by the mixture of fragment F1 and anti-(fragment F1) antibodies used in ultracentrifugal analysis. The broken line represents the pattern given under the same conditions by the anti-(fragment F1) antibodies alone. R, Starting reservoir.

Table 2. Sedimentation coefficients of components observed by ultracentrifugation

The sedimentation coefficients were calculated from the peaks and are given without correction for protein and salt concentrations. The molecular weights used for the calculation of the molar ratios were: 160000 for the antibodies; 70000 for human serum albumin; 6600 for fragment F1. (a), (b) and (c) refer to the patterns obtained in Figs. 5(a), 5 (b) and 5(c) respectively.

	Concn. of antigen (mg./ml.)		Concn. of antibody (mg./ml.)	Antigen/antibody molar ratio	Sedimentation coefficients of observed components (s)			
	Human serum albumin	Fragment F1			4.4	6.7	7.7	8.9 10.0 11.4
(a)	4.4		1.0	10				
(b)	0.9		5.0	0.4				
(c)		0.08	5.0	0.4				

DISCUSSION

The molecular weight of 'inhibitor' being 11 000 (Lapresle & Webb, 1964) and that of fragment F1 being 6600, the yield of fragment F1 from 'inhibitor' is 50% of the theoretical maximum.

The immunological activity of fragment F1 was demonstrated by the fact that antibodies reacting with fragment F1 could be isolated from rabbit anti-(human serum albumin) sera by using an insoluble fragment F1-benzylcellulose conjugate. The fact that these antibodies corresponded to only 1% of the total anti-albumin antibodies indicates that a very small proportion of the anti-albumin antibodies react with fragment F1. This must explain why fragment F1 did not inhibit the specific precipitation of human serum albumin with rabbit anti-(human serum albumin) sera.

The number of antigenic groups of human serum albumin present on fragment F1 was studied by ultracentrifugal analysis of the complexes formed between albumin or fragment F1 and the 7s anti-(fragment F1) antibodies.

In the experiment shown in Fig. 5(a) anti-(fragment F1) antibody was mixed with a molar excess of albumin to estimate the valence of the antibody. The presence of free antigen and the absence of free antibody indicate that there was in fact an excess of antigen and that all the antibody molecules could react with human serum albumin. The composition of the only antigen-antibody complex formed that is most compatible with its sedimentation coefficient 8.9s is 1mol. of antibody and 2mol. of albumin. This composition agrees with that found by Webb & Lapresle (1964) for a similar complex formed between anti-'inhibitor' antibodies and a great excess of albumin, and indicates that the anti-(fragment-F1) antibodies are bivalent.

In the experiment shown in Fig. 5(b) human serum albumin was mixed with a molar excess of anti-(fragment F1) antibodies. There is an excess of antibody, as shown by the presence of free antibody and the absence of free albumin. The major complex, which has sedimentation coefficient 7.7s, sediments faster than free antibody but more slowly than the complex of 1mol. of antibody and 2mol. of albumin formed in excess of albumin. Its sedimentation coefficient is therefore compatible only with that of a complex containing 1mol. of antibody and 1mol. of albumin. The sedimentation coefficient 11.4s given by the traces of heavier complex is compatible only with a complex containing at least 2mol. of antibody. Since it is formed in excess of antibody its most probable composition is 2mol. of antibody and 1mol. of albumin. These results indicate that almost all the isolated anti-(fragment F1) antibodies react with a single site on

the albumin molecule and that there are traces of another antibody reacting with another site.

In the experiment shown in Fig. 5(c) fragment F1 was mixed with anti-(fragment F1) antibody to give the same molar excess of antibody as in the preceding experiment. Almost all the material sedimented with sedimentation coefficient 6.7s, which is that of free antibody. However, owing to the low molecular weight of fragment F1, this sedimentation coefficient can also be given by complexes of 1mol. of antibody and 1 or 2mol. of fragment F1. The electrophoretic analysis of this solution showed that it contained free antibody and much antibody in the form of a complex with fragment F1. This indicates that the material that sedimented with sedimentation coefficient 6.7s was in fact a mixture of free antibody and antibody combined as a complex with fragment F1. This complex, being formed in an excess of antibody, is probably composed of 1mol. of antibody and only 1mol. of fragment F1. Traces of other material had sedimentation coefficient 10.0s, which is compatible only with a complex containing 2mol. of antibody. Since there is an excess of antibody its probable composition is 2mol. of antibody and 1mol. of fragment F1.

The fact that the rabbit anti-(human serum albumin) antibodies isolated from the fragment F1-benzylcellulose conjugate were essentially composed of antibodies reacting with a single site of either the albumin or fragment F1 molecules shows that fragment F1 contains only one of the antigenic sites of the albumin molecule. The presence of traces of another anti-(human serum albumin) antibody can be due either to the contamination of fragment F1 with traces of material carrying another antigenic site or to the presence on fragment F1 of another almost completely inactive site.

The absence of specific precipitation of fragment F1 with either rabbit anti-(human serum albumin) sera or isolated anti-(fragment F1) antibody and the absence of specific precipitation of human serum albumin with the anti-(fragment F1) antibodies is explained by the univalence of the antigen in each of these systems.

Recent findings reviewed by Lapresle & Webb (1964) indicate that an antigenic site of a protein molecule should not correspond to a structure having a molecular weight of more than 3500. It is probable therefore that the active site of fragment F1 corresponds to only a part of its structure. The absence of tryptophan and tyrosine shows that these amino acids are not indispensable to the structure of an antigenic site of a protein molecule.

The purification of a univalent fragment of human serum albumin permitted the isolation of monospecific antibody reacting with one site of the albumin molecule. These antibodies were bivalent,

which confirms the fact, already shown by anti-hapten antibodies, that the two active sites of an antibody molecule have the same specificity.

Pepe & Singer (1956, 1959) have prepared an artificial univalent antigen by allowing the single thiol group of bovine serum mercaptalbumin to react with *N*-(*p*-benzenearsonic acid)-iodoacetamide [*p*-(2-iodoacetylamino)benzenearsonic acid]. The combination of this antigen and purified anti-(benzenearsonic acid) antibodies was analysed by ultracentrifugation. For a wide range of antigen/antibody ratios, in addition to the complexes formed, both free antigen and free antibody were always present. In our results obtained with human serum albumin and the anti-(fragment F1) antibodies we did not observe free antigen and free antibody together. These results indicate that the anti-(benzenearsonic acid) antibodies had a lower affinity for the benzenearsonic acid hapten than the anti-(fragment F1) antibodies had for albumin. This may be due to the fact that the anti-hapten antibodies were induced by the injection of bovine γ -globulin-benzenearsonic acid and were in fact heterogeneous with respect to their specificity (Singer, 1964).

We thank Mr R. Mangalo and Mr S. Mangalo for performing the ultracentrifugation and Mrs J. Cavillon and Mrs M. Ciolek for their technical assistance. This work was supported in part by Grant E-3225 from the National

Institutes of Health, Bethesda, Md., U.S.A., and by a Grant from World Health Organization.

REFERENCES

- Borduas, A. F. & Grabar, P. (1953). *Ann. Inst. Pasteur*, **84**, 903.
 Boyden, S. V. (1951). *J. exp. Med.* **93**, 107.
 Grabar, P. & Williams, C. A., jun. (1955). *Biochim. biophys. Acta*, **17**, 67.
 Heidelberger, M. & Kendall, F. E. (1935). *J. exp. Med.* **62**, 467.
 Lapresle, C. & Webb, T. (1960). *Ann. Inst. Pasteur*, **99**, 523.
 Lapresle, C. & Webb, T. (1964). *Volume du Cinquantenaire de la Société de Chimie biologique*, p. 157. Paris: Masson et Cie.
 Pepe, F. A., & Singer, S. J. (1956). *J. Amer. chem. Soc.* **78**, 4583.
 Pepe, F. A. & Singer, S. J. (1959). *J. Amer. chem. Soc.* **81**, 3878.
 Schachman, H. K. (1959). *Ultracentrifugation in Biochemistry*, p. 75. New York: Academic Press Inc.
 Singer, S. G. (1964). *Immunochemistry*, **1**, 15.
 Stavitsky, A. B. & Arquilla, E. R. (1955). *J. Immunol.* **74**, 306.
 Uriel, J. & Scheidegger, J. J. (1955). *Bull. Soc. Chim. biol., Paris*, **37**, 165.
 Webb, T. & Lapresle, C. (1964). *Biochem. J.* **91**, 24.
 Weichselbaum, T. E. (1946). *Amer. J. clin. Path. tech. Suppl.* **10**, 40.
 Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.