Preparation and Characterization of Poly-dL-alanyl Rabbit γ-Globulin

SARA FUCHS AND MICHAEL SELA

From the Section of Chemical Immunology, The Weizmann Institute of Science, Rehovoth, Israel

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The solubility properties of poly-dL-alanyl rabbit γ-globulin and the A chain derived from it were investigated. The non-alanylated A chain obtained upon partial reduction of disulfide bridges in γ-globulin (2) is hardly soluble between pH 3 and 10 (2, 18). The antigenic and antibody properties of the modified molecules were studied, as the attachment of peptide chains to the γ-globulin molecule might affect both its antigenic determinants and the antibody combining sites.

EXPERIMENTAL PROCEDURE

Rabbit γ-globulin either from normal or from immune serum was prepared by precipitation of a globulin fraction at 37% saturation of (NH₄)₂SO₄ at 5°C, subsequent DEAE-cellulose chromatography (19), and concentration by ultrafiltration (20). The γ-globulin solutions were kept frozen until used. Ovalbumin, twice crystallized, and papain, twice crystallized, were obtained from Worthington. Diethylaminoethyl cellulose was obtained from Eastman Kodak. Carboxymethyl cellulose was obtained from Bio-Rad; Sephadex (G-50 and G-100, medium) was obtained from Pharmacia, Sweden. N-Carboxy-dL-alanine anhydride was prepared according to Sela and Berger (21). All other chemicals used were of analytical reagent grade.

Preparation of Poly-dL-alanyl γ-Globulin—Three samples of this derivative were prepared. (a) Poly-dL-ala-RGG 1: 0.25 g of rabbit γ-globulin (RGG 1) was dissolved in 25 ml of cold 0.05 M phosphate buffer, pH 7 (1 g of protein per 100 ml of buffer). The solution was kept cold in an ice bath; then 1 g of N-carboxy-dL-alanine anhydride (4 g of anhydride per g of protein or 78 moles of alanine anhydride per mole of lysine, assuming a molecular weight of 140,000 for γ-globulin and 63 lysine residues per molecule of γ-globulin (4)) dissolved in 10 ml of anhydrous dioxane was added, and the mixture was stirred in the cold overnight. The solution was then dialyzed against several changes of cold 0.9% NaCl for 4 days and concentrated by ultrafiltration.
(b) Poly-dL-ala-RGG 2 was prepared in an identical manner with 1.6 g of rabbit normal γ-globulin and 8 g of the anhydride (5 g of the anhydride per g of protein or 96 moles of alanine anhydride per mole of lysine). (c) Poly-dL-ala-oV-RGG was prepared in an identical manner from 1.6 g of rabbit anti-ovalbumin γ-globulin and 6.4 g of the anhydride.

Purification of Poly-dL-alanyl γ-Globulin—Gel filtration on Sephadex G-50 (medium) was performed to remove any linear poly-dL-alanine chains which did not diffuse during the prolonged

* The abbreviations used are: OV, ovalbumin; RGG, rabbit γ-globulin; poly-dL-ala-RGG, poly-dL-alanyl rabbit γ-globulin; oV RGG, rabbit γ-globulin isolated from an anti-ovalbumin serum; poly-dL-ala-oV-RGG, poly-dL-alanyl-oV-RGG.
dialysis. Approximately 150 mg of the preparation were applied to a column (2.7 x 65 cm) in 10 ml of 0.9% NaCl, and the column was developed with 0.9% NaCl. The optical density of the effluent solution was measured at 280 m and the Lowry (22) modification of the Folin biuret reaction was used to detect the DL-alanine polypeptides.

Hydrolysis of Poly-DL-alanyl γ-Globulin with Pepsin and Separation of Fragments by Column Chromatography Pepsin digestion of poly-DL-alanyl γ-globulin was performed in a manner similar to that described by Porter (23) for the fragmentation of γ-globulin. Poly-DL-alanyl-αOV-RGG (240 mg) in 10 ml of 0.9% NaCl solution was brought to a final volume of 20 ml in buffer solution (0.1 M sodium phosphate, pH 7.0, 0.02 M cysteine, and 0.005 M ethylenediaminetetraacetate). Pepsin (2.5 mg) was added and the solution was maintained at 37° for 4 hours. After 3 hours, an aliquot was taken for ultracentrifugation, and no residual intact protein could be seen. Following the incubation, the pepsin digest was dialyzed for 3 hours against three changes of distilled water and then overnight against 0.01 M sodium acetate buffer, pH 5.5.

Chromatography of the digest was carried out on a CM-cellulose column (2.7 x 35 cm) with pH 5.5 sodium acetate buffer and a linear gradient from 0.01 M to 0.9 M. The initial volume in the mixing chamber was 1000 ml, and the gradient was commenced after the first fragment was eluted.

Normal γ-globulin was hydrolyzed and fractionated under the same conditions for comparison.

Reduction and Fractionation of Poly-DL-alanyl γ-Globulin—Reduction and fractionation of poly-DL-alanyl-RGG 2 was carried out according to the method of Fleischman et al. (2, 4) for the reduction of γ-globulin. Mercaptoethanol (in a final concentration of 0.2 M) was added to a 1% (w/v) solution of poly-DL-alanyl-RGG 2 in 0.5 M Tris buffer, pH 8.2. After 1 hour at room temperature, the solution was cooled in ice water and an equal volume of 0.2 M iodosacetamide, cooled to 0°, was added to the reduced protein. After 1 hour the solution was dialyzed overnight against 100 volumes of cold 0.9% NaCl solution. Before fractionation, the reduced protein solution was dialyzed against cold 1 N propionic acid overnight (any slight precipitate was removed) and then put on a Sephadex G-100 column (3 x 65 cm). The slow peak (B) was concentrated by ultrafiltration and rerun on the same column. The fractions obtained were concentrated by ultrafiltration and kept in the cold room. Normal γ-globulins were reduced and fractionated under identical conditions for comparison.

Chemical Characterization—Amino acid analyses were performed on the Beckman/Spinco automatic amino acid analyzer, model 120 B, after hydrolysis under reduced pressure in constant boiling hydrochloric acid (6 N) for 22 hours at 110° (24).

In order to determine the number of amino groups which reacted as initiators in the polymerization reaction to yield the poly-DL-alanyl-RGG derivatives, these derivatives were treated with sodium nitrite in glacial acetic acid (10). The number of alanylated lysine residues (which are not deaminated) was determined by quantitative amino acid analysis (24).

Physical Methods—Spectrophotometric measurements were made on a Zeiss model PMQ II spectrophotometer, at approximately 25°, with quartz cells of 1-cm light path. The specific extinction coefficients, $E^p_{280}$, at 280 m, used in this work, are 13.5 for rabbit γ-globulin, 13.7 for rabbit γ-globulin chain A, and 11.8 for rabbit γ-globulin chain B (25). The same coefficients were also used for determining the original globulin or chains in the polyalanyl derivatives.

Sedimentation measurements were carried out in a Spinco model E ultracentrifuge at 20-22° with the schlieren optical system. The samples were sedimented at 56,100 rpm. The results were corrected to 20°. Diffusion measurements were performed in the same Spinco model E ultracentrifuge (26). The boundary between the solvent and the solution was obtained with a synthetic boundary cell and operating at low gear (9,341 rpm). At this speed the sedimentation of the samples investigated was practically negligible.

Starch gel electrophoresis in 8 M urea and formate buffer was carried out as described by Edelmann and Poulik (6). Electrophoresis on cellulose acetate strips was carried out as described by Kohn (27).

Immunological Methods—Immunization of rabbits against ovalbumin was accomplished by two intramuscular injections of 15 mg each in complete Freund’s adjuvant with an interval of 10 days. Pooled antisera were used. Immunization of goats against rabbit γ-globulin, or against Fragment I obtained by papain digestion of rabbit γ-globulin, was accomplished by three intramuscular injections of 30 mg of antigen, each in complete Freund’s adjuvant, at intervals of 10 days. Goat antiserum to rabbit papain digest Fragment III was a gift from Dr. J. B. Fleischman.

The antibody activity in rabbit immunoglobulins was followed by the quantitative precipitin reaction (28, 29), by the inhibition of precipitation caused on the addition of antibody fragments or derivatives to immune globulin and the homologous antigen in optimum amount (23), or by antigen-binding capacity. In the last technique the rabbit immunoglobulin (anti-ovalbumin) or poly-DL-alanyl immunoglobulin tested (0.0025 μg in 0.5 ml of 0.9% NaCl) was first incubated with 3H-labeled (30) ovalbumin (2 or 6 μg in 0.1 ml of 0.9% NaCl; the labeled ovalbumin had a radioactivity of 2,000,000 cpm per mg) and then precipitated by goat antiserum to rabbit γ-globulin (0.3 ml of an antiserum containing 11 mg of antibodies to RGG per ml).

Any radioactivity (measured in a well-type Tracerlab scintillation counter) found in the final precipitates was due to specific binding of the antigen to the immunoglobulin or the polyalanyl immunoglobulin.

Antigenic activity was followed by the quantitative precipitin tests with goat antiserum to rabbit γ-globulin or goat antiserum to rabbit papain digest Fragment I or III as well as by double diffusion in agar plates (31).

RESULTS

Purification of Poly-DL-alanyl γ-Globulins—All the polyalanyl globulin samples prepared were passed through Sephadex G-50 columns in order to remove free poly-DL-alanine chains. A typical elution curve obtained for poly-DL-alanyl-αOV-RGG is illustrated in Fig. 1. The polyalanyl γ-globulin came directly after the void volume of the channel, while the second peak, which had no absorbance at 280 m and was detected by the Folin biuret method, contained free poly-DL-alanine. No further purification of the protein fraction was obtained when it was rerun on a Sephadex G-100 column.

Chemical and Physicochemical Characterization of Poly-DL-alanyl γ-Globulins—

We are grateful to Dr. H. O. McDevitt for suggesting this technique.
The rabbit y-globulin preparations used in this study gave an amino acid analysis very close to that reported by Fleischman et al. (4). Deamination experiments summarized in Table I show that over 75% of the ε-amino groups of RGG reacted with N-carboxy-DL-alanine anhydride. Thus, approximately 48 alanine peptide chains were attached, on the average, to each y-globulin molecule. The enrichment of y-globulin with alanine, as a result of the alanylation reaction, is given in Table II. The reaction of y-globulin with more alamine monomer (poly-DL-al-aOV-RGG 2 in Table II against poly-DL-al-aOV-RGG) resulted in greater enrichment (755 alanine residues per poly-DL-al-aOV-RGG 2 molecule versus 640 per poly-DL-al-aOV-RGG molecule) but not in an increase in the number of alamine peptide chains attached.

The ultraviolet spectra of y-globulin before and after poly-DL-alanylation were compared. Assuming that the native and the modified protein have the same molar absorbance at 280 nm in 0.9% sodium chloride, it was found that the spectra were identical for both proteins between 240 and 320 nm, both in the neutral pH range and at pH 13. It was therefore concluded that alanylation did not change the spectral properties of y-globulin and that spectral measurements can be used to determine the content of original globulin in solutions of polyalanyl globulins. Thus, knowing the enrichment with alamine residues, the concentration of any polyalanyl globulin derivative may be calculated from spectral data.

In contrast to unmodified rabbit y-globulin, which is precipitated by aqueous solutions of ammonium sulfate at 37% saturation, the poly-DL-alanyl globulins investigated are soluble at this salt concentration, and start to precipitate only at 50% saturation.

A comparison of the electrophoretic behavior on a cellulose acetate strip of the same sample of rabbit y-globulin before and after alanylation (Fig. 2) shows that, at pH 8.6, the alanylated derivative gave a less diffuse band and moved more quickly toward the anode than the native globulin. This would be expected, as the attachment of alamine peptides to globulin results in the replacement of most of the ε-amino groups of lysine, with a pK around 10.4 (32), by the ε-amino groups of alanine, which display a pK value of 7.9 (33), and, therefore, in a decrease in the number of positive charges per molecule. It is also seen from Fig. 2 that the exposure of rabbit y-globulin to the conditions of the alanylation reaction, but in the absence of N-carboxy-DL-alanine anhydride, did not cause any changes in the electrophoretic mobility of the molecule. Electrophoretic patterns of RGG and poly-N-DL-al-RGG in 8 M urea-formic acid-starch gel (Fig. 3) show that under these conditions the alanylated globulin moves toward the cathode more slowly than the unmodified globulin. This would be expected, as the attachment of alanine peptides to globulin results in the replacement of most of the ε-amino groups of lysine, with a pK around 10.4 (32), by the ε-amino groups of alanine, which display a pK value of 7.9 (33), and, therefore, in a decrease in the number of positive charges per molecule. It is also seen from Fig. 3 that the exposure of rabbit y-globulin to the conditions of the alanylation reaction, but in the absence of N-carboxy-DL-alanine anhydride, did not cause any changes in the electrophoretic mobility of the molecule.

**Table I**

Deamination of polyalanyl y-globulins

Samples of polyalanyl y-globulins were deaminated by treatment with nitrous acid, which altered the unprotected ε-amino groups of lysine. The deaminated samples were hydrolyzed, and the amino acid content was determined. The number of alanylated lysine residues was assumed to be equal to the number of unaltered lysine residues found after deamination.

<table>
<thead>
<tr>
<th>Deaminated sample</th>
<th>Lysine</th>
<th>Histidine</th>
<th>Alanylated lysine</th>
<th>Free lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmole/sample</td>
<td>µmole/sample</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>0.214</td>
<td>0.075</td>
<td>48</td>
<td>15c</td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>0.208</td>
<td>0.085</td>
<td>48</td>
<td>15c</td>
</tr>
</tbody>
</table>

a Assuming 18 histidine residues per molecule of RGG (4).
b Assuming 63 lysine residues per molecule of RGG (4).
c Average of the two determinations.

**Table II**

Alanine enrichment of polyalanyl y-globulins

Samples of polyalanyl y-globulins were hydrolyzed, and the amino acid content was determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alanine</th>
<th>Aspatic acid</th>
<th>Total alamine</th>
<th>Added alanine</th>
<th>Average alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmole/sample</td>
<td>µmole/sample</td>
<td>resid./molecule</td>
<td>resid./molecule</td>
<td>resid./chain</td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>2.218</td>
<td>0.345</td>
<td>680</td>
<td>607</td>
<td>13.4c</td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>3.6</td>
<td>0.51</td>
<td>745</td>
<td>762</td>
<td></td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>1.780</td>
<td>0.233</td>
<td>805</td>
<td>732</td>
<td>15.6c</td>
</tr>
<tr>
<td>Poly-DL-al-aOV-RGG</td>
<td>5.89</td>
<td>0.617</td>
<td>850</td>
<td>777</td>
<td></td>
</tr>
</tbody>
</table>

a Assuming 106 aspartic acid residues per molecule of RGG (4).
bAssuming 73 alanine residues per molecule of RGG (4).
c The number of chains per molecule is assumed to be identical with the number of the alanylated lysine residues in Table I, and ε-amino groups are not taken into account, as there is less than one ε-amino group per molecule of RGG (1).

d Average of the two determinations.
FIG. 2. Electrophoretic behavior on a cellulose acetate strip of aOV-RGG (rabbit γ-globulin isolated from an anti-ovalbumin serum), of poly-DL-ala-aOV-RGG, and of a sample of aOV-RGG exposed to aqueous dioxane under the conditions of the alanylation reaction but in the absence of alanine monomer (denoted aOV-RGG d). The electrophoresis was carried out in 0.06 M sodium Verona buffer, pH 8.6, for 2 hours at 9 volts per cm.

FIG. 3. Electrophoresis in 8 M urea-formic acid-starch gel (2) of: A, RGG; B, poly-DL-ala-RGG 2; C, A chain (2) of RGG; D, A chain of poly-DL-ala-RGG 2; E, B chain of RGG; F, B chain of poly-DL-ala-RGG 2.

The poly-DL-alanyl rabbit γ-globulin preparations investigated gave upon sedimentation in the ultracentrifuge in 0.05 M phosphate buffer, pH 6.8, single symmetrical peaks. Poly-DL-ala-RGG 2 had an intrinsic sedimentation coefficient $s_{20,w} = 7.08$, and an intrinsic diffusion coefficient of $3.9 \times 10^{-7}$ cm$^2$ sec$^{-1}$.

Reductive Cleavage of Poly-DL-alanyl γ-Globulin and Characterization of Products—Poly-DL-ala-RGG 2 was reduced with mercaptoethanol in the absence of urea. The polyalanyl A chain was separated from the polyalanyl B chain under conditions used (2-4) for the separation of the unmodified chains. As seen in Fig. 4, the alanylated chains behaved on Sephadex G-100 in 1 M propionic acid similarly to the unmodified chains. In both cases the A chain was distributed as a double peak. The two fractions under this double peak gave indistinguishable electrophoretic patterns in 8 M urea-formic acid-starch gel, and had the same amino acid composition.

The enrichment with alanine of the poly-DL-alanyl A and B chains is given in Table III. The alanine attached distributed equally between A and B chains, when the enrichment was calculated per lysine residue. The relative enrichment was based on the total lysine content, as no analysis of the number of alanine peptides attached per globulin chain was carried out. The behavior of the separated A and B chains, before and after alanylation, upon electrophoresis on starch gel in 8 M urea-formic acid, is shown in Fig. 3. Just as poly-DL-alanyl globulin had an increased mobility as compared with unmodified globulin, so did the alanylated chains move faster under these conditions toward the cathode than did the unmodified chains.

Both the polyalanyl A chain and the polyalanyl B chain are water-soluble. A comparison of the solubility in aqueous solutions at various pH values of A chain derived from RGG and of one derived from poly-DL-ala-RGG 2 is given in Fig. 5. Under
the experimental conditions used, the alanylated A chain is completely soluble, while the A chain derived from RGG is only partially soluble between pH 4 and 10. The increased solubility of the A chain after alanylation is also apparent from the observation that it moves as a diffuse band toward the cathode on starch gel electrophoresis in formate buffer, pH 3.5.

FIG. 4. Fractionation of reduced RGG (bottom) and reduced poly-DL-ala-RGG 2 (top) on a Sephadex G-100 column (3 x 65 cm) in 1 M propionic acid.

FIG. 5. Solubility of RGG chain A (O—O) and poly-DL-ala-RGG 2 chain A (△—△) as a function of pH. One volume of a solution of either of the two different chains in 0.9% sodium chloride and 0.01 M propionic acid (pH 3.25) was maintained for 48 hours at 3° with 3 volumes of a 0.05 M Tris-malonic acid solution adjusted with sodium hydroxide to the desired pH and with sodium chloride to an ionic strength of 0.15. The pH values plotted are those measured in the final mixtures before incubation. The final concentration of chain A in each experiment was 1.8 mg per ml. The solution of the alanylated chain had the same content of the chain A moiety and, therefore, a somewhat higher total concentration. The extent of precipitation was followed by measurements of the absorbance of supernatant solutions diluted with 0.1 M propionic acid, taking the absorbance of a solution of the A chain at pH 3.25 as 0% precipitation.

TABLE III
Alanine enrichment of polyalanyl chains

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alanine</th>
<th>Aspartic acid</th>
<th>Total alanine 4</th>
<th>Added alanine 4</th>
<th>Added alanine residues/lysine residue 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-DL-ala-RGG, chain A</td>
<td>11.27</td>
<td>1.48</td>
<td>251</td>
<td>227</td>
<td>9.9</td>
</tr>
<tr>
<td>Poly-DL-ala-RGG, chain B</td>
<td>7.17</td>
<td>1.25</td>
<td>192</td>
<td>79</td>
<td>10.1</td>
</tr>
</tbody>
</table>

4 Assuming 33 and 16 aspartic acid residues per molecule of RGG chain A and RGG chain B, respectively (4).

Hydrolysis of Poly-DL-alanyl γ-Globulin with Papain and Characterization of Products—Poly-DL-ala-aOV-RGG was hydrolyzed with papain as described in “Experimental Procedure.” Like unmodified rabbit γ-globulin, the polyalanyl globulin preparation was cleaved by papain, yielding a mixture of fragments with a sedimentation coefficient of 3.2S. Since poly-DL-alanine is digested slowly (to the extent of approximately 12% of its peptide bonds) by papain,4 the reaction of poly-DL-ala-aOV-RGG with papain could result not only in fragmentation of the γ-globulin backbone, but also in partial hydrolysis of the polyalanine side chains. This could cause release of free alanine peptides. Indeed, as seen in Table IV, the alanine content of the papain digest of poly-DL-ala-aOV-RGG decreased significantly after dialysis. The nondiffusible papain digest was still enriched with 50% of the alanine residues attached to the globulin before the enzymatic hydrolysis.

The chromatographic pattern of the nondiffusible papain digest of poly-DL-ala-aOV-RGG on CM-cellulose is shown in Fig. 6. In contrast to the papain digest of RGG, which gave under identical conditions a good separation of Fractions I, II, and III (23), polyalanyl Fragment I was separated from polyalanyl Fractions II and III, but no clear separation between the last

4 T. Schechter, M. Sela, and A. Berger, unpublished observations.
two was obtained even after rechromatography of the second peak either under identical conditions or with a logarithmic buffer gradient. The tubes representing the second peak (Fig. 6) were therefore tested by gel diffusion for their content of antigenic determinants characteristic of Fragment II and Fragment III. Those giving a positive reaction, respectively, with goat anti-

rabbit papain digest Fragment I and goat antiserum to rabbit papain digest Fragment III were pooled and were considered to represent, respectively, polyalanyl Fragment II and polyalanyl Fragment III. The intermediate tubes, which gave a positive reaction with both goat antisera, were not used in further studies. The separated polyalanylated Fragments I, II and III were analyzed for their alanine content (Table IV).

**TABLE IV**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Alanine</th>
<th>Aspartic acid</th>
<th>Total alanine</th>
<th>Added alanine</th>
<th>Added alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles/sample</td>
<td>residues/molecule</td>
<td>added alanine</td>
<td>residue/lysine</td>
<td></td>
</tr>
<tr>
<td>Intact poly-DL-ala-aOV-RGG...</td>
<td>2.218</td>
<td>0.345</td>
<td>680</td>
<td>607</td>
<td>9.6</td>
</tr>
<tr>
<td>Digest of poly-DL-ala-aOV-RGG (before dialysis)</td>
<td>1.797</td>
<td>0.282</td>
<td>675</td>
<td>602</td>
<td>9.6</td>
</tr>
<tr>
<td>Digest of poly-DL-ala-aOV-RGG (after dialysis)</td>
<td>0.882</td>
<td>0.230</td>
<td>436</td>
<td>363</td>
<td>5.6</td>
</tr>
<tr>
<td>Fragment I of poly-DL-ala-aOV-RGG</td>
<td>1.255</td>
<td>0.361</td>
<td>102</td>
<td>75</td>
<td>5.4</td>
</tr>
<tr>
<td>Fragment II of poly-DL-ala-aOV-RGG</td>
<td>1.489</td>
<td>0.345</td>
<td>125</td>
<td>96</td>
<td>6.0</td>
</tr>
<tr>
<td>Fragment III of poly-DL-ala-aOV-RGG</td>
<td>0.419</td>
<td>0.139</td>
<td>90</td>
<td>81</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Assuming 63, 14, 16, and 32 lysine residues per molecule of RGG, RGG Fragments I, II, and III, respectively (4, 23, 34).

* Assuming 73, 27, 29, and 18 alanine residues per molecule of RGG, RGG Fragments I, II, and III, respectively (4, 23, 34).

* Assuming 106, 27, 29, and 39 aspartic acid residues per molecule of RGG, RGG Fragments I, II, and III, respectively (4, 23, 34).

It seems that polyalanylation has covered some antigenic specificity determinants on the A chain, as illustrated in Fig. 8D. On the other hand, polyalanylation does not seem to have similarly affected the B chain (Fig. 8E).

**Antibody Activity of Poly-DL-ala-Derivative of γ-Globulin**

Isolated from an Antiserum—In contrast to the positive precipitin reaction obtained between ovalbumin and the rabbit γ-globulin which was isolated from an anti-ovalbumin serum (aOV-RGG) and exposed to the conditions of the alanylation reaction in the absence of the alanine monomer, no precipitation was observed upon reaction of ovalbumin with poly-DL-ala-aOV-RGG in the usual manner. There were two possible explanations for this observation: Either polyalanylation had abolished the capacity of the antibody molecule to combine with the antigen, or although antibody-antigen interaction still occurred, polyalanylation prevented precipitation of the complex. In order to distinguish between these two possibilities, experiments were performed to...
Fig. 7. Comparison of the antigenic properties of RGG before and after alanylation. Precipitin curves of: A, goat anti-RGG with RGG (●) and poly-DL-ala-RGG 2 (○); B, goat antiserum to rabbit papain digest Fragment I with RGG Fragment I (●), poly-DL-ala-RGG 2 Fragment I (○), and poly-DL-ala-RGG 2 Fragment II (□); C, goat antiserum to rabbit papain digest Fragment III with RGG Fragment III (●) and poly-DL-ala-RGG 2 Fragment III (○).

Fig. 8.
find out whether polyalanylated antibodies may still bind the antigen. As seen in Fig. 9, poly-nn-ala-aOV-RGG almost completely inhibited the homologous ovalbumin anti-ovalbumin reaction, whereas poly-nn-alanyl normal rabbit γ-globulin (poly-nn-ala-RGG) did not affect the homologous reaction at all.

In another series of experiments, the antigen-binding capacity of aOV-RGG, of poly-DL-ala-aOV-RGG, and of normal rabbit γ-globulin before and after alanylation was checked by mixing them with 3H-labeled ovalbumin and adding goat antiserum to rabbit γ-globulin to precipitate the rabbit globulins in the mixtures. When the antigen-binding capacity of anti-ovalbumin (aOV-RGG) was taken as 100%, poly-DL-ala-aOV-RGG bound the antigen to the extent of 79%, while RGG and poly-DL-ala-RGG 2 bound 1.3% and 6.0%, respectively (average of four experiments). It may be thus concluded that polyalanylation interferes with the ability of an antibody to precipitate its homologous antigen, but not with its capacity to bind the antigen.

The so called Fragment I and II obtained upon papain hydrolysis of immune globulins still possess antibody activity, in contrast to Fragment III, as demonstrated by their capacity to inhibit the homologous immunological reaction (22). This property is kept by the fragments derived from poly-DL-alanyl immune γ-globulin, as shown in Fig. 10. Fragments I and II obtained from poly-DL-ala-aOV-RGG are inhibitory, while Fragment III is not.

DISCUSSION

Alanylation of rabbit γ-globulin yielded derivatives in which 600 to 780 alanine residues were attached, on the average, to each globulin molecule (Table II). Any nondiffusible poly-nn-alanine formed during the reaction was removed by gel filtration (Fig. 1). No unmodified protein was expected to be present in the reaction mixture as polyalanyl ribonuclease preparations had previously been shown to contain no unmodified ribonuclease (10). Growing experimental evidence indicates that polypeptidyl proteins obtained by the polymerization procedures used in this study possess, as predicted by statistical analysis (35), a relatively sharp molecular weight distribution (9). Thus, for example, polytyrosyl trypsin (36) and polyalanyl ribonuclease (10) gave single symmetrical peaks on sedimentation and yielded symmetrical effluent peaks on column chromatography. The same is also true for the polyalanyl RGG preparations described here.

Fig. 9. Inhibition of the homologous precipitin reaction of ovalbumin with anti-ovalbumin by polyalanyl antibodies. Increasing amounts of poly-DL-ala-aOV-RGG or of poly-DL-ala-RGG 1 in 1.2 ml of 0.9% NaCl were added to 0.3 ml of a 0.015% solution of ovalbumin in 0.9% NaCl; the tubes were kept for 30 min at 37°, following which 0.2 ml of a 1% solution of aOV-RGG in 0.9% NaCl (corresponding to equivalence) were added. ---, poly-DL-ala-aOV-RGG (three different experiments, denoted A, C, and Δ); ---, poly-DL-ala-RGG 1 (□).

Fig. 10. Inhibition of the homologous precipitin reaction of ovalbumin by fragments obtained upon papain hydrolysis of poly-DL-ala-aOV-RGG. Increasing amounts of the alanylated anti-ovalbumin Fragment I (○), Fragment II (□), or Fragment III (△) in 0.8 ml of 0.9% NaCl were added to 0.2 ml of a 0.15% solution of ovalbumin in 0.9% NaCl; the tubes were kept for 30 min at 37°, following which 0.2 ml of a 1% solution of aOV-RGG in 0.9% NaCl (corresponding to equivalence) were added.
Almost 25% of the ε-amino groups of RGG were unavailable for the reaction with N-carboxy-DL-alanine anhydride under the conditions described (Table I).

Poly-DL-ala-RGG was degraded both by papain hydrolysis and by reductive cleavage. While the alanylated A and B chain were well separated under the conditions described for A and B chains derived from RGG (Fig. 4), the alanylated papain-produced fragments behaved on CM-cellulose somewhat differently from the fragments derived from unalanylated RGG (Fig. 6). Polyalanyl Fragment I separated well from polyalanylated Fragments II and III, but the last two could be obtained in a pure form only after recourse was made to immunological techniques to follow their order of elution from the column.

The extent of attachment of alanine residues per ε-amino group of chain A or B was essentially the same (Table III). During the papain fragmentation of poly-DL-ala-RGG, some alanyl peptides were released by enzymic hydrolysis of the side chains. The content of the remaining alanyl residues attached to the fragments was similar for I and II, but much lower for III (Table IV). It is not clear whether this part of the RGG bound fewer alanyl residues during the alanylation reaction, or whether the action of papain removed more alanyl residues from Fragment III than from the other two fragments.

Poly-DL-alanylation considerably increased the solubility properties of RGG as is apparent from the fact that RGG is soluble in aqueous ammonium sulfate at 37% saturation, and from the observation that the polyalanyl A chain is soluble in aqueous solutions in the pH range of 3 to 10 (Fig. 5), in contrast with its nonalanylated analogue.

Antibody molecules kept their capacity to bind the antigen after alanylation, but lost their ability to precipitate the antigen. Polyalanyl Fragments I and II, derived from poly-DL-ala-nOV RGG after papain hydrolysis, inhibited the homologous ovalbumin-anti-ovalbumin reaction in a manner similar to nonalanylated RGG, but lost their ability to precipitate the antigen. Polyalanyl globulin, as well as chains and fragments derived from it, gave positive reactions with goat antiserum prepared against rabbit γ-globulin or against papain-produced fragments of rabbit γ-globulin. Thus polyalanylation of rabbit immune γ-globulin increased its solubility significantly without drastically affecting its antigenic or antibody properties.

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Sara Fuchs and Michael Sela


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