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References

(1) B. P. Das, J. A. Campbell, F. B. Samples, R. A. Wallace, L. K. Whisenant, R. W. Woodard, and D. W. Boykin, Jr., *J. Med. Chem.*, 15, 370 (1972).
(2) C. E. Loader and C. J. Timmons, *J. Chem. Soc. C*, 1677 (1967).
(3) G. De Luca, G. Martelli, P. Spalgnolo, and M. Tiecco, *ibid.*, 2504 (1970).
(4) G. M. Badger, J. H. Elix, and G. E. Lewis, *Aust. J. Chem.*, 18, 70 (1965).
(5) D. S. Rao and B. D. Tilak, *J. Sci. Ind. Res., Sect. B*, 16, 65 (1957); *Chem. Abstr.*, 51, 13, 841 (1957).
(6) D. D. Morgan, S. W. Horgan, and M. Orchin, *Tetrahedron Lett.*, 1789 (1972).
(7) W. H. Laarhoven, Th. J. H. Cuppen, and R. J. F. Nivard, *Recl. Trav. Chim. Pays-Bas*, 87, 687 (1968).
(8) R. E. Lutz, *et al.*, *J. Amer. Chem. Soc.*, 68, 1813 (1946).
(9) T. S. Osdene, P. B. Russell, and L. Rane, *J. Med. Chem.*, 10, 431 (1967).
(10) A. I. Vogel, "Practical Organic Chemistry," 3rd ed, Longmans, Green and Co., London, 1964, p 969.

Studies on the Specificity of Antibodies Produced by Linear Antigenic Polypeptides of a Known Primary Structure. Synthesis and Use of Poly(L-tyrosyl-L-aspartyl-L-alanylglycyl)glycine Methyl Ester†,‡

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It has been previously reported that the antibodies produced by rabbits against the polypeptide poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester[1-3] (**1**) are most probably dependent upon the conformation of the antigen.[4-7] Also, these antibodies have been shown to possess a specificity for the phenolic hydroxyl group and the aromatic moiety of the tyrosyl residue.[8] However, no such specificity has been shown for the alanyl residue.[9] The next phase of this work has been to study the specificity of these antibodies pertaining to the role of the glutamyl residue. In this paper we wish to report the characterization of the specificity of the antisera produced by rabbits against the antigen **1** as studied by cross reactions and absorption studies. For this purpose the following polymer was prepared and used, poly(Tyr-Asp-Ala-Gly)Gly (**2**).

Chemistry. The synthesis of the polymerizing unit *O-tert*-Bu-Tyr-*β-tert*-Bu-Asp-Ala-Gly pentachlorophenyl ester · HCl (**6**) and the necessary intermediates for its preparation are outlined in the Experimental Section. The polymerization of **6** was performed by the procedure which has been shown to produce linear high-molecular-weight polypeptides[1,2,4-8] to give poly(*O-tert*-Bu-Tyr-*β-tert*-Bu-Asp-Ala-Gly)Gly Me ester. The protecting *tert*-Bu groups were removed by the use of 90% $F_3C \cdot CO_2H$ to yield the polymer **2**. After extensive dialysis, the polymer was fractionated by successive diafiltrations through Diaflo membranes into four different molecular weight fractions: $>5 \times 10^4$; $2\text{-}5 \times 10^4$; $1\text{-}2 \times 10^4$; and $<1 \times 10^4$.

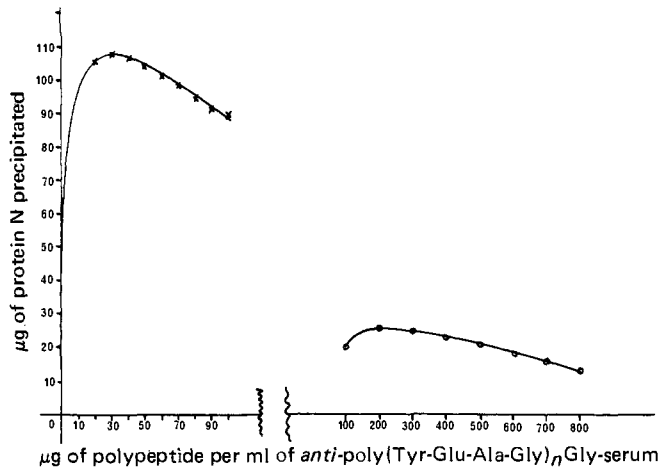

Figure 1. Relative precipitin curves for poly(Tyr-Glu-Ala-Gly)$_n$Gly (1, X) and poly(Tyr-Asp-Ala-Gly)$_n$Gly (**2**, O).

Immunochemistry. Eight rabbits were immunized against poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester (**1**) using the previously reported protocol.[3] It was found that each serum gave a positive precipitin reaction with the homologous polymer **1**. The serum from each animal was pooled, it being assumed that in this time interval that each rabbit had responded to the same antigenic determinants.[10] Incremental amounts of the polypeptide **2** were added to 1-ml aliquots of this pooled antisera and it was observed that a cross reaction occurred. The relative precipitin curves for the polypeptides **1** and **2** are shown in Figure 1. It was noted that the heterologous polypeptide **2** does not precipitate as much antibody as the homologous polypeptide **1**. In order to quantitate the amount of antibody not precipitated by polymer **2**, a separate experiment was performed. A quantity equal to the equivalent point amount of the heterologous polypeptide **2** was allowed to react with the pooled sera. After removal of the precipitate, 30 μg of the homologous polypeptide **1** was added to the resulting supernatant liquid. Further precipitation was obtained and quantitated by analysis for N (Kjeldahl) as shown in Table I.

Conclusions

It has been found that the heterologous polypeptide **2** cross reacts with *anti*-1-sera, precipitating less antibody than the homologous antigen **1**. Also, the amount of the heterologous polymer **2** required to attain the equivalent point is larger than that necessary for the homologous material. It would appear from these cross reactions that the conformation of polymer **2** is similar to that of the homologous antigen **1**; however, these results indicate a lower affinity of the antibody-combining sites for the heterologous polypeptide **2**. Using the rationale that the determinants of the heterologous polypeptide are in the same orientation as those of the antigen **1**, it is suggested that the observed differences in the binding ability of the heterologous polymer **2** are due only to the modification of the glutamyl residue. Thus, it would appear that the antibody-combining sites have a high affinity for the γ-carboxyl group of the glutamyl residue.

Experimental Section

Z-*β-tert*-Bu-Asp-Ala-Gly Me Ester (3).§ To a solution of 11.1 g (0.0565 mol) of Ala-Gly Me ester · HCl and 5.6 g (0.056 mol) of

†All amino acids are of the L variety.

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§ Z = benzyloxycarbonyl.

Table I

Polypeptide	μg of protein N pptd at equiv pta	μg of protein N pptd by 1 after absorption[a]	% of protein N pptd by polypeptide[a]
(Tyr-Glu-Ala-Gly)$_n$Gly (1)	106	0	100
(Tyr-Asp-Ala-Gly)$_n$Gly (2)	26	78	25

[a] Per milliliter of *anti*-poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester-sera.

Et_3N in 200 ml of CH_2Cl_2 was added 32.3 g (0.0565 mol) of Z-β-*tert*-Bu-Asp pentachlorophenyl ester. The mixture was stirred overnight at room temperature and concentrated, and the product was dissolved in EtOAc, washed with 1 *N* HCl and H_2O, then dried (Na_2SO_4), and concentrated under reduced pressure to give an oil. This material was chromatographed on a column of Silicar CC-7 using $CHCl_3$–EtOAc (1:1) as eluent to give the fully blocked tripeptide; crystallization from EtOAc–hexane yielded 16.3 g (62%): mp 122.5°; $[\alpha]^{24}D$ −7.7° (*c* 4.2, DMF). *Anal.* ($C_{22}H_{31}N_3O_8$) C, H, N.

Z-*O*-*tert*-Bu-Tyr-β-*tert*-Bu-Asp-Ala-Gly Me Ester (4). A suspension of 15.0 g (0.0322 mol) of 3 and 0.7 g of 10% Pd/C in 200 ml of MeOH was treated with 1.168 g (0.0322 mol) of dry HCl in MeOH, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate concentrated under reduced pressure. The residue was dissolved in 200 ml of CH_2Cl_2 and 3.58 g (0.0354 mol) of Et_3N and 20.0 g (0.0324 mol) of Z-*O*-*tert*-Bu-Tyr pentachlorophenyl ester[‡] was then added. The reaction mixture was stirred overnight at room temperature and concentrated under reduced pressure, and the product was dissolved in EtOAc, washed with 1 *N* HCl and H_2O, then dried (Na_2SO_4), and concentrated under reduced pressure to give a solid. This material was chromatographed on a column of Silicar CC-7 using $CHCl_3$–EtOAc (1:1) as eluent to give the fully blocked tetrapeptide; crystallization from EtOAc–hexane yielded 13.0 g (58.7%): mp 131°; $[\alpha]^{24}D$ −20.2° (*c* 5.7, DMF). *Anal.* ($C_{35}H_{48}N_4O_{10}$) C, H, N.

Z-*O*-*tert*-Bu-Tyr-β-*tert*-Bu-Asp-Ala-Gly Pentachlorophenyl Ester (5). To a solution of 12.0 g (0.0175 mol) of 4 in 250 ml of MeOH was added 17.5 ml of 1 *N* KOH and the solution was stirred for 90 min at room temperature and then concentrated under reduced pressure. The residue was flooded with H_2O, acidified with 10% citric acid solution, and extracted into EtOAc. The EtOAc solution was dried (Na_2SO_4) and concentrated under reduced pressure to give 11.7 g (100%) of the tetrapeptide free acid. This material was dissolved in 250 ml of CH_2Cl_2, and 4.5 g (0.0167 mol) of pentachlorophenol and 7.1 g (0.0167 mol) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methotoluene-*p*-sulfonate were added. The mixture was shaken for 2 days at room temperature and then concentrated under reduced pressure to give a solid. This material was washed with H_2O and crystallized from MeOH to yield 1.1 g (8%): mp 188°; $[\alpha]^{24}D$ −19.8° (*c* 1.64, DMF). *Anal.* ($C_{40}H_{45}Cl_5N_4O_{10}$) C, H, N.

***O*-*tert*-Bu-Tyr-β-*tert*-Bu-Asp-Ala-Gly Pentachlorophenyl Ester·HCl (6).** A suspension of 3.3 g (0.0036 mol) of the tetrapeptide active ester 6 and 0.7 g of 10% Pd/C in 200 ml of MeOH was treated with 0.1314 g (0.0036 mol) of dry HCl in MeOH, and the suspension was hydrogenated for 2 hr. The reaction mixture was filtered and the filtrate concentrated. The residue was crystallized from MeOH–Et_2O to give 2.3 g (78%): mp 152°; $[\alpha]^{24}D$ −7.5 (*c* 2.7, DMF). *Anal.* ($C_{12}H_{40}Cl_6 \cdot N_4O_8 \cdot H_2O$) C, H, N.

Poly(Tyr-Asp-Ala-Gly)Gly Me Ester (2). To a solution of 0.9 g (0.00896 mol) of Et_3N and 0.64 mg of Gly Me ester·HCl in 5 ml of DMSO was added a solution of 2.1 g (0.00256 mol) of the polymerization unit 6 in 20 ml of DMSO. The mixture was shaken for 1 week and then centrifuged to yield the fully protected polymer which was washed with three 35-ml portions of H_2O, three 35-ml portions of MeOH, and three 35-ml portions of Et_2O and dried to give the blocked polymer. This material was treated with 30 ml of 90% $F_3C \cdot CO_2H$ and stirred for 50 min and then concentrated under reduced pressure to yield the crude polypeptide 2. This material was suspended in 20 ml of H_2O and dissolved by the addition of 1 *N* NaOH to pH 7.5. The solution was dialyzed against distilled H_2O overnight and acidified with 6 *N* HCl to pH 2.5 and dialyzed against distilled H_2O for a day. The precipitated polypeptide 2 was collected by centrifugation and then lyophilized to yield 0.1 g (10%); amino acid ratios of an acid hydrolysate, $Tyr_{1.0}$ $Asp_{1.0}$ $Ala_{1.0}$ $Gly_{1.0}$.

Molecular Weight Determination. The polypeptide 1 (0.1 g) was dissolved in 50 ml of H_2O by the addition of 1 *N* NaOH to pH 7.4. This solution was separated into four different molecular weight ranges by successive diafiltrations through Diaflo membranes XM 50, UM 20E, and UM 10.[#] Each fraction was acidified with 6 *N* HCl, dialyzed, and lyophilized to yield 0.05 g, mol wt >50,000; 0.01 g, mol wt 20–50,000; 0.01 g, mol wt 10–20,000; 0.014 g, mol wt <10,000.

Immunochemical Procedures. Eight rabbits were treated with poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester (1) at weekly intervals, using the immunization schedule previously described;[3] 25 days after the last injection all rabbits were bled using the standard heart puncture technique. Serum from each rabbit was tested for the precipitin reaction with the homologous antigen 1; serum from each animal gave a positive precipitin reaction. The serum from each animal was pooled and this combined serum was used for the following experiments. It was assumed that the antibodies produced by each rabbit after the same time interval were directed against the same antigenic determinants of poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester (1).

Quantitative Precipitin Reactions. To 1-ml aliquots of the pooled rabbit serum was added incremental amounts of the polypeptide **2.** Each tube was made up to a total of 2 ml with buffer (0.1 *M* NaCl–0.05 *M* $NaHCO_3$) and incubated for 1 hr at 37° and then kept at 4° for 48 hr. The tubes were centrifuged in the cold and the precipitates were washed twice with 1 ml of buffer (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The total amount of protein precipitate was estimated by analysis for N (Kjeldahl). An identical procedure was used for the homologous polymer 1 which was run simultaneously with that used for the polypeptide **2.** The comparative precipitin curves are shown in Figure 1.

Absorption Studies. The pooled rabbit serum was treated with the equivalent point amount of the heterologous polypeptide **2** as described above. The precipitate was centrifuged out and the supernatant liquid was poured off into a separate tube. To this was added 30 μg of the homologous antigen, poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C Et ester **(1).** The mixture was incubated at 37° for 1 hr and then stored at 4° for 48 hr. The precipitate was collected by centrifugation and washed twice with 1 ml of buffer solution (0.05 *M* K_2HPO_4–NaOH), pH 7.0. The amount of protein precipitate was estimated by analysis for N (Kjeldahl). The amount of precipitate obtained using this procedure is shown in Table I. A control in which the serum was first absorbed with the homologous antigen 1 ascertained that the homologous antigen precipitated all of the antibody, since the supernatent liquid gave no further precipitation reaction when 30 μg of 1 was added.

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References

(1) B. J. Johnson and E. G. Trask, *J. Chem. Soc. C*, 2644 (1969).
(2) B. J. Johnson, *J. Pharm. Sci.*, **59**, 1849 (1970).
(3) B. J. Johnson and E. G. Trask, *ibid.*, 59, 724 (1970).
(4) B. J. Johnson, *J. Med. Chem.*, **15**, 423 (1972).
(5) B. J. Johnson, C. Cheng, and N. Tsang, *ibid.*, 15, 95 (1972).
(6) B. J. Johnson, *ibid.*, 14, 488 (1971).
(7) B. J. Johnson and E. G. Trask, *ibid.*, 14, 251 (1971).
(8) B. J. Johnson and N. Tsang, *ibid.*, **15**, 488 (1972).
(9) B. J. Johnson, *J. Pharm. Sci.*, **60**, 332 (1971).
(10) E. Maron, R. Arnon, and B. Bonavida, *Eur. J. Immunol.*, 1, 181 (1971).

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Synthesis of 3,4-Dihydroxy-5-fluoro-DL-phenylalanine and 3,4-Dihydroxy-5-[^{18}F]fluoro-DL-phenylalanine

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Radioactive agents ranging from simple ions to protein molecules have been used in an attempt to visualize malignant or vascular disease in the brain by means of external detectors. These agents are nonspecific and detect only