Investigations on the Antigenic Determinants of the Polypeptide, Poly(L-tyrosyl-L-glutamyl-L-alanylglycyl)glycine-*1*-^{14}C Ethyl Ester

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The synthesis of poly(Tyr-Glu-Ala-β-Ala)Gly methyl ester (2) and the oligopeptides Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (3), Glu-Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (4), and (Try-Glu-Ala-Gly)$_4$Gly (5) is described. It has been found that none of these polypeptides cross react with antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester (1). However, it has been found that the oligopeptides 4 and 5 do inhibit the precipitin reaction of anti-1-sera and its homologous antigen. From this investigation it has been concluded that the higher efficiency of inhibition produced by the peptide 5 relative to peptide 4 and smaller peptides probably is a reflection of the chain length requirements for acquisition of a conformation capable of interacting with antibody to 1.

The molecular aspects of the antigenicity of proteins and the concomitant specificity of their antibodies are not well understood. In this respect we have been using the sequential polypeptide, poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester[1-3] (1), as a protein model. It has been found that the antibodies raised against this material are dependent upon its conformation[4-6] and are also directed toward the tyrosyl[7-9] and glutamyl residues.[10,11] It has been shown that antibodies to *Bacillus anthracis* are directed toward the carboxyl groups of the γ-D-glutamyl residues which constitute the capsular material of this organism. Kovacs[12-16] has shown by the judicious use of sequential polypeptides that the distance between the repeating carboxyl groups is of paramount importance in order to affect precipitation or inhibition of precipitation in this system. It was considered of interest to investigate the possibility that an analogous situation may occur with the model polypeptide, poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester (1). It is known that the tyrosyl[7-9] and glutamyl[10,11] residues are part of the antigenic site of this polypeptide, whereas the alanyl residue is only necessary for maintaining the conformation of this antigen.[17] The tyrosyl and glutamyl residues are adjacent in the primary structure of the molecule; however, their relationship vis-à-vis other repeating units along the peptide backbone is not known. It was considered that this could be investigated by increasing the distance between each repeating tyrosyl-glutamyl dipeptidyl unit. This has been achieved by replacing the glycyl residue with a β-alanyl moiety. This substitution causes the distance between each tyrosyl-glutamyl moiety to be increased by one methylene group per repeating unit. It would be expected that if the antigenic site of the model polypeptide required only the sequence tyrosyl-glutamyl then this substitution would have little effect on the precipitin reaction with antisera to the homologous antigen. However, if the relationship between several tyrosyl-glutamyl residues is very stringent, then such a substitution would be expected to effect the precipitin reaction greatly. For this purpose we wish to report the synthesis and immunochemical properties of poly(Tyr-Glu-Ala-β-Ala)Gly methyl ester (2).

Another approach that was considered in order to elucidate the spacial requirements of the antigenic determinants of the polypeptide, poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester (1), was to find the smallest oligopeptide which would affect the precipitin reaction between the model polypeptide and its antisera. For this purpose we wish to report the synthesis and immunochemical properties of the following oligopeptides: Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (3), Glu-Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (4), and (Tyr-Glu-Ala-Gly)$_4$Gly (5).

Chemistry. The polymerizing unit *O-tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanyl-β-alanyl pentachlorophenyl ester hydrochloride (12) and the necessary intermediates for its preparation were synthesized as detailed in the Experimental Section. The polymerization was performed in the presence of a preformed monomer since this has been shown to produce linear high molecular weight polypeptides.[1-11,17] Following this established procedure, the insoluble polymer, poly(*O-tert*-butyl-L-tyrosyl-γ-*tert*-butyl-L-glutamyl-L-alanyl-β-alanyl)glycine methyl ester, was prepared, from which the protecting *tert*-butyl groups were removed by use of 90% trifluoroacetic acid to yield poly(L-tyrosyl-L-glutamyl-L-alanyl-β-alanyl)glycine methyl ester (2). After extensive dialysis, the polymer 2 was fractionated by successive diafiltration through Diaflo membranes into four different molecular weight fractions: 5×10^4; $2\text{-}5 \times 10^4$; $1\text{-}2 \times 10^4$; and 1×10^4.

The oligopeptides 3-5 were prepared by the solid-phase method.[18,19] The fully protected resin bound oligopeptides (3, 4, or 5) were synthesized by the stepwise addition of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid to an insoluble polystyrene resin. The cycle for the addition of each amino acid residue consisted of the following steps: removal of the *N-tert*-butyloxycarbonyl protecting groups by treatment with excess anhydrous trifluoroacetic acid in methylene chloride, neutralization of the resulting salt with triethylamine in methylene chloride, and then coupling the resulting free amino group with 4 equiv of the appropriate *N-tert*-butyloxycarbonyl-L-amino acid for a 12-hr period. Any unreacted N-terminal amino groups were acetylated before continuing with the next cycle of deprotection and coupling. At the end of the chain lengthening sequence each protected peptide was cleaved from its polymer support by the action of anhydrous hydrogen bromide in anhydrous trifluoroacetic acid. The peptides were purified by ion-exchange chromatography and gel filtration.

Immunochemistry. To aliquots of antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester (1) was added in separate experiments incremental amounts of the polypeptide 2 and the oligopeptides 3-5. No precipitin reaction was observed in each case. The possibility that some of these polypeptides could cover part of the active site of the antibodies to 1 was also investigated. In separate experiments incremental amounts of the polypeptide 2 and the oligopeptides 3-5 were added to aliquots of antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C ethyl ester containing the equivalence point amount of the homologous antigen. It was found that inhibition of the precipitin reaction was observable with only the oligopeptides 4 and 5 when 10,000 μg of these materials was used as shown in Table I.

Discussion

The inability of the polypeptide, poly(Tyr-Glu-Ala-β-Ala)Gly (2), to cross react with antisera to the model anti-

Table I

Polypeptide	Amt of protein N ppt in μg at equiv pt	Amt of protein N ppt by **1** in the presence of each polypeptide[a,b]	% inhibition of ppt rxn of **1**[a] at equiv pt
$(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_nGly$ (**1**)	106	NA	0
$(Tyr\text{-}Glu\text{-}Ala\text{-}\beta\text{-}Ala)_nGly$ (**2**)	0	106	0
$Ala\text{-}Gly(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_3Gly$ (**3**)	0	106	0
$Glu\text{-}Ala\text{-}Gly(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_3Gly$ (**4**)	0	88	17
$(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_4Gly$ (**5**)	0	53	50

[a]Per milliliter of antisera poly(Tyr-Glu-Ala-Gly)Gly-1-^{14}C ethyl ester. [b]When up to 10,000 μg of each polypeptide is used. NA, not applicable.

gen 1 nor even to cause inhibition of the precipitin reaction between anti-1-sera and its homologous antigen is indicative of the very strict spacial relationship between the known residues that are implicated in the antigenic site. It has already been reported that the peptides $(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_nGly$,[9,20] where n = 1, 2, and 3, do not cross react with nor do they inhibit the precipitin reaction between anti-1-sera and its homologous antigen. From the immunochemical results obtained with the oligopeptides 3–5, it is seen that there is a very dramatic increase in the inhibitory properties of the heptadecapeptide 5 when compared to that shown by the hexadecapeptide 4 and smaller peptides. This is indicative that the extra tyrosyl residue of 5 plays an important role in covering part of the antibody active site and thereby inhibits the precipitin reaction between the antigen 1 and its antisera. An explanation for this behavior of the higher efficiency of inhibition produced by peptide 5 relative to peptide 4 and smaller peptides may simply be a reflection of the chain length requirements for acquisition of the conformation capable of interacting with the antibody.

An interesting corollary from these experiments is the apparent similarity in conformation of the small peptide $(Tyr\text{-}Glu\text{-}Ala\text{-}Gly)_4Gly$ (5) and the model antigen 1. As an explanation, it is possible that the conformation of the antibodies helps the heptadecapeptide 5 to assume a conformation that is most conducive for coverage of the antibody active site. Evidence for this suggestion has been reported[21] for a peptide derived from myoglobin which, although it is a random coil form, was capable of reacting with antibodies against a helical segment within this protein. Similarly, it has been reported[22] that the peptide $(Try\text{-}Ala\text{-}Gly)_{13}$, which is very low in helical content, assumes a far larger helical content when in association with the F_{ab} fragment of the antibodies derived from poly(Tyr-Ala-Glu).

Experimental Section

Melting points were taken with a Mel-Temp apparatus and are uncorrected.

Boc-Ala-β-Ala Methyl Ester (6).† To a solution of 38 g (0.2 mol) of Boc-Ala in 350 ml of CH_2Cl_2 was added 30.7 g (0.22 mol) of β-Ala methyl ester hydrochloride, 22.2 g (0.22 mol) of Et_3N, and 43.5 g (0.22 mol) of DCC. The mixture was stirred overnight at room temperature and the precipitated urea was filtered and the filtrate was concentrated. The residue was dissolved in EtOAc, washed with 10% citric acid solution and H_2O, and then dried (Na_2SO_4) and concentrated *in vacuo* to give a solid which was crystallized from EtOAc–hexane to yield 41.4 g (78%): mp 75–75.6°; $[\alpha]^{25}D$ −4.1° (*c* 2.69, DMF). *Anal.* ($C_{12}H_{22}N_2O_5$) C, H, N.

Ala-β-Ala Methyl Ester Hydrochloride (7). To 38.4 g (0.14 mol) of Boc-Ala-β-Ala methyl ester was added 460 ml of 0.92 *N* HCl in AcOH. The mixture was stirred for 45 min and then concentrated *in vacuo* to give a solid. This material was crystallized from MeOH–Et_2O to yield 25.4 g (83%): mp 135–136.5°; $[\alpha]^{25}D$ 18.2° (*c* 4.30, DMF). *Anal.* ($C_7H_{15}ClN_2O_3$) C, H, N.

Z-γ-*t*-Bu-Glu-Ala-β-Ala Methyl Ester (8).‡ To a solution of 33.4 g (0.057 mol) of Z-γ-*t*-Bu-Glu pentachlorophenyl ester in 300 ml of CH_2Cl_2 was added 12.0 g (0.057 mol) of Ala-β-Ala methyl ester hydrochloride and 6.1 g (0.06 mol) of Et_3N. The mixture was stirred overnight at room temperature and concentrated, and the product was dissolved in EtOAc, washed with 10% citric acid solution and H_2O, and then dried (Na_2SO_4) and concentrated *in vacuo* 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 tripeptide. Crystallization from EtOAc–hexane yielded 12.2 g (43.4%): mp 126–127.5°; $[\alpha]^{25}D$ −3.1° (*c* 2.9, DMF). *Anal.* ($C_{24}H_{35}N_3O_8$) C, H, N.

γ-*t*-Bu-Glu-Ala-β-Ala Methyl Ester Hydrochloride (9). To a solution of 11.2 g (0.023 mol) of Z-γ-*t*-Bu-Glu-Ala-β-Ala methyl ester in 300 ml of MeOH was added 0.5 g of 10% Pd/C and 0.84 g of dry HCl in MeOH. The mixture was hydrogenated for 2 hr and filtered, and the filtrate was concentrated *in vacuo*. The residue was crystallized from MeOH–Et_2O to give 8.0 g (97%): mp 137–139°; $[\alpha]^{25}D$ 14.4° (*c* 2.43, DMF). *Anal.* ($C_{16}H_{30}ClN_3O_6$) C, H, N.

Z-*O*-*t*-Bu-Tyr-γ-*t*-Bu-Glu-Ala-β-Ala Methyl Ester (10). To a solution of 7.99 g (0.021 mol) of γ-*t*-Bu-Glu-Ala-β-Ala methyl ester hydrochloride in 300 ml of CH_2Cl_2 was added 13.0 g (0.021 mol) of Z-*O*-*t*-Bu-Tyr pentachlorophenyl ester[1] and 2.32 g (0.023 mol) of Et_3N. The mixture was stirred overnight at room temperature and then concentrated under reduced pressure. The product was dissolved in EtOAc and washed with 10% citric acid solution and H_2O, then dried (Na_2SO_4), and then concentrated *in vacuo* 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 9.0 g (61%): mp 162°; $[\alpha]^{25}D$ −10.7° (*c* 3.05, DMF). *Anal.* ($C_{37}H_{52}N_4O_{10}$) C, H, N.

Z-*O*-*t*-Bu-Tyr-γ-*t*-Bu-Glu-Ala-β-Ala Pentachlorophenyl Ester (11). To a solution of 9.0 g (0.0126 mol) of Z-*O*-*t*-Bu-Tyr-γ-*t*-Bu-Glu-Ala-β-Ala methyl ester in 300 ml of MeOH was added 12.6 ml of 1 *N* KOH. 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 the tetrapeptide free acid; crystallization from EtOAc–hexane yielded 8.8 g (100%). To this product, dissolved in 300 ml of CH_2Cl_2, was added 3.71 g (0.013 mol) of pentachlorophenol and 5.9 g (0.0139 mol) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. The mixture was stirred overnight 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 2.8 g (23.5%): mp 174–176°; $[\alpha]^{25}D$ −3.3° (*c* 4.56, DMF). *Anal.* ($C_{42}H_{49}Cl_5N_4O_{10}$) C, H, N.

***O*-*t*-Bu-Tyr-γ-*t*-Bu-Glu-Ala-β-Ala Pentachlorophenyl Ester Hydrochloride (12).** To a suspension of 5.4 g (0.0057 mol) of Z-*O*-*t*-Bu-Tyr-γ-t-Bu-Glu-Ala-β-Ala pentachlorophenyl ester in 500 ml of MeOH was added 0.21 g of dry HCl in MeOH and 0.5 g of 10% Pd/C. The mixture was hydrogenated for 2 hr and filtered, and the filtrate was concentrated. The residue was crystallized from MeOH–Et_2O to give 3.8 g (80%): mp 146°; $[\alpha]^{25}D$ 4.33° (*c* 2.31, DMF). *Anal.* ($C_{34}H_{44}Cl_6N_4O_8$) C, H, N.

Poly(Tyr-Glu-Ala-β-Ala)Gly Methyl Ester (2). To a solution of 1.2 mg of glycine methyl ester hydrochloride containing 1.54 g (0.0152 mol) of Et_3N in 5 ml of DMSO was added 3.7 g (0.0044 mol) of *O*-*t*-Bu-Tyr-γ-*t*-Bu-Glu-Ala-β-Ala pentachlorophenyl ester hydrochloride in 38.6 ml of DMSO. The mixture was shaken for 1 week and then centrifuged to yield the product 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 1.7 g

† Boc = *tert*-butyloxycarbonyl.

‡ Z = benzyloxycarbonyl.

(70%) of the fully protected polymer. This material was treated with 50 ml of 90% CF_3COOH and stirred for 50 min and then concentrated under reduced pressure to yield the crude polypeptide **2**. This material was suspended in 40 ml of H_2O and dissolved by the addition of 4 *N* NaOH to pH 7.5. The solution was dialyzed against distilled H_2O for 2 days and acidified with 6 *N* HCl to pH 2.5 and dialyzed against distilled H_2O overnight. The precipitated polypeptide **1** was collected by centrifugation and then lyophilized to yield 0.6 g (30%); amino acid ratios in an acid hydrolysate were $Tyr_{1.0}Glu_{1.0}Ala_{1.0}\beta\text{-}Ala_{1.0}$. *Anal.* ($C_{20}H_{27}N_4O_8$) C, H, N.

Molecular Weight Determination. The polypeptide **2** (0.5 g) was dissolved in 50 ml of H_2O by the addition of 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* MCl, dialyzed, and lyophilized to yield 0.33 g, mol wt >50,000; 0.08 g, mol wt 20–50,000; 0.04 g, mol wt 10–20,000; 0.2 g, mol wt <10,000.

Synthesis of Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (3), Glu-Ala-Gly(Tyr-Glu-Ala-Gly)$_3$Gly (4), and (Tyr-Glu-Ala-Gly)$_4$Gly (5). The peptides were synthesized by the solid-phase method.[18,19] Boc-Gly esterified to 2% cross-linked chloromethylated polystyrene such that 0.483 mmol of Gly was incorporated per gram of resin. Chain elongation was acheived by using the following cycle of operations. The resin carrying the *tert*-butyloxycarbonylamino acid or peptide moiety was washed with four 20-ml portions of CH_2Cl_2; cleavage of the protective Boc group was achieved by the addition of 20 ml of 40% CF_3COOH in CH_2Cl_2 and shaking at room temperature for 20 min, followed by three washings with 20 ml of CH_2Cl_2, three washings with 20 ml of EtOH (absolute), and four washings with 20 ml of CH_2Cl_2; the trifluoroacetate salt was neutralized by the addition of 20 ml of 10% Et_3N in CH_2Cl_2 to the resin for a contact time of 10 min. This was followed by six washings with 20 ml of CH_2Cl_2; the coupling step was carried out by the addition of 4 equiv of the appropriate *tert*-butyloxycarbonylamino acid in 9 ml of CH_2Cl_2 to the resin, followed by 4 equiv of dicyclohexylcarbodiimide in 12 ml of CH_2Cl_2, and the mixture was shaken overnight. The resin was washed five times with 20 ml of DMF and then acetylated by the addition of 0.9 ml of Ac_2O and 0.6 ml of Et_3N in 20 ml of DMF and shaken for 20 min. This was followed by three washings with DMF, one washing with EtOH, three washings with 20 ml of AcOH, three washings with EtOH, and three washings with DMF. The amino acids with reactive side chains were used in the form of the following derivatives, Boc-Glu(Bzl) and Boc-Tyr(Bzl). After the last coupling the respective oligopeptides were cleaved from the resin and deprotected by suspending the resin in 40 ml of anhydrous CF_3COOH containing 3 ml of anisole. Through this was bubbled anhydrous hydrogen bromide for 90 min. The suspension was evaporated under reduced pressure and washed three times with 10 ml of EtOAc. The peptides were extracted into pyridine–AcOH buffer, pH 9, and lyophilized. The crude peptides were chromatographed on Dowex 1-X4 and also by Bio-Gel P6 columns. The main sharp fractions were collected for each oligopeptide. Amino acid analysis of an acid hyolrolysate of each of the oligopeptides indicated for the pentadecapeptide **3**, $Tyr_{2.9}Glu_{3.0}Ala_{4.0}Gly_{5.0}$; the hexadecapeptide **4**, $Tyr_{2.8}Glu_{4.1}Ala_{4.0}Gly_{5.1}$; the heptadecapeptide **5**, $Tyr_{3.7}Glu_{4.1}Ala_{4.1}Gly_{5.0}$. The final yields of the three oligopeptides, based on the amount of Boc-Gly initially esterified to the resin, were for the pentadecapeptide **3**, 35%; the hexadecapeptide **4**, 32%; and the heptadecapeptide **5**, 30%.

Immunochemical Procedures. To 1-ml aliquots of rabbit antisera to poly(Tyr-Glu-Ala-Gly)Gly-*l*-^{14}C ethyl ester (1) were added, in separate experiments, incremental amounts of up to 10,000 μg of each of the different molecular weight fractions of the polypeptide **2**, and also the oligopeptides **3–5**. No precipitation was observed with any of the fractions of the polypeptide **2** or the oligopeptides **3–5**.

To 1-ml aliquots of the rabbit antisera to poly(Tyr-Glu-Ala-Gly)Gly-*l*-^{14}C ethyl ester were added incremental amounts of up to 10,000 μg of each of the different molecular weight fractions of the polypeptide and also the oligopeptides **3–5**. To each tube was added the equivalence point amount (30 μg) of the homologous antigen **1**, and each tube was incubated at 37° for 1 hr. After standing at 4° for 48 hr, the precipitates were collected, washed twice with buffer (0.05 *M* K_2HPO_4–NaOH), pH 7.0, and collected by centrifugation. The total amount of protein precipitated was estimated by N analysis (Kjeldahl). No inhibition of the precipitin reaction was observed with the polypeptide **2** nor with the oligopeptide **3**. However, inhibition of the precipitin reaction was observed with the oligopeptides **4** and **5**, as shown in Table I.

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