Linear Polypeptides of a Known Primary Structure. Synthesis and Immunochemical Studies of Poly(L-glutamyl-L-tyrosyl-L-alanylglycyl)glycine-*1*-^{14}C Ethyl Ester†

BRIAN J. JOHNSON

Department of Chemistry, Tufts University, Medford, Massachusetts 02155

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The title compound was synthesized, and comparison of the antigenic properties of the title compound with those of poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester indicates that the primary structure of the antigen does at least partially control its immunochemical properties.

A recent investigation of the immunochemical properties of poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester[1,2] has shown that the polypeptide is antigenic, eliciting antibodies in rabbits.[3] In order to ascertain the role of the primary struture of this antigen on its immunochemical properties it was necessary to vary the order of the amino acids of the repeating sequence. To this end we wish to report the synthesis and immunochemical properties of poly(Glu-Tyr-Ala-Gly)-*1*-^{14}C Et ester(**1**).

Chemistry.—The polymerizing unit γ-*tert*-Bu-Glu-γ-*tert*-Bu-Tyr-Ala-Gly pentachlorophenyl ester·HCl (**5**) and the necessary intermediates for its preparation were synthesized as detailed in the Experimental Section. The polymerization was performed at a reagent concentration of 100 mmoles/l. in the presence of a preformed monomer since this has been shown to produce linear high molecular weight polypeptides.[1,2,4-8] Following this established procedure the insoluble polymer, poly(γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester was prepared; from which the protecting *tert*-Bu groups were removed by the use of 90% F_3CCO_2H to yield poly(Glu-Tyr-AlA-Gly)Gly-*1*-^{14}C Et ester (**1**). After extensive dialysis, the polymer was purified and fractionated by passage through calibrated columns of Sephadex G-100[9] and Corning CPG-10-240 glass granules. By this means the mol wt of the polypeptide was found to be at least 1×10^5.

Immunochemistry.—Two rabbits were immunized with **1** using the same protocol as that previously described.[3] To aliquots of the sera obtained from each rabbit was added up to 10,000 μg of the synthetic polypeptide **1**. No precipitin reaction was observed.

The possibility that the polypeptide **1** could be a hapten for the antigen poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester was also investigated. Incremental amounts of the polypeptide **1** were added to aliquots of antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester containing a known amount of the antigen. Mild inhibition of the precipitin reaction was observed such that 50% inhibition of precipitation was obtained in the presence of 6000 μg of the polypeptide **1**.

Conclusions

Rearrangement of the same amino acid moieties that constitute the repeating sequence of the antigenic polypeptide, poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester, to poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester **1** caused a loss in antigenicity, as shown by a negative precipitin reaction. Thus it has to be concluded that the primary structure of the antigenic polypeptide does at least partially control its immunochemical properties. However, the inhibition studies do indicate that the polymer **1** can cause coverage of part of the antigenic determinant of the antibodies to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester.

It is of interest that the polypeptide **1** contains the repeating unit in which the glutamyl residue is adjacent to a glycyl moiety. A similar situation occurs in the nonantigenic polypeptides poly(Tyr-Glu-Gly-Gly)Gly Me ester[4] and poly(Glu-Ala-Gly)Gly Me ester.[10]

Experimental Section

Melting points were taken with a Mel-Temp apparatus and are uncorrected. Optical rotations were taken with a Carl Zeiss precision polarimeter.

Z-*O*-*tert*-Bu-Tyr-Ala-Gly Me Ester (2).‡—To a mixt of 7.2 g (0.0366 mole) of Ala-Gly Me ester·HCl in 300 ml of CH_2Cl_2 was added 22.5 g (0.0364 mole) of Z-*O*-*tert*-Bu-Tyr pentachlorophenyl ester[1] and 3.7 g (0.0367 mole) of Et_3N. The mixt was stirred overnight at room temp and concd, and the product was dissolved in EtOAc, washed with 10% citric acid sol and H_2O, and then dried (Na_2SO_4) and concd *in vacuo* to give the product as an oil. This material was chromatographed on a column of Silicar CC7 using $CHCl_3$ as eluent, to give the fully blocked tripeptide; crystn from EtOAc-hexane yielded 13 g (70%): mp 122°, $[\alpha]^{27}D$ −7.6° (*c* 1.45, DMF). *Anal.* ($C_{37}H_{55}N_3O_7$) C, H, N.

Z-γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly Me Ester (3).—A suspension of 10% Pd/C in 5 ml of AcOH and 50 ml of MeOH contg 0.73 g (0.02 mole) of dry HCl was hydrogenated for 10 min. A soln of 10.3 g (0.02 mole) of Z-*O*-*tert*-Bu-Tyr-Ala-Gly Me ester was added in 100 ml of MeOH and hydrogenation was continued until there was no further evolution of CO_2. The reaction mixt was filtered and the filtrate was concd under reduced pressure to give the tripeptide·HCl as an oil; yield 8.15 g (98%). To a soln of this oil in 200 ml of CH_2Cl_2 was added 11.0 g (0.0188 mole) of Z-γ-*tert*-Bu-glutamic acid pentachlorophenyl ester and 2.0 g (0.0198 mole) of Et_3N. The mixt was stirred overnight and then concd under reduced pressure to yield a solid. This material was dissolved in EtOAc, washed with 10% citric acid soln and H_2O, dried (Na_2SO_4), concd, and chromatographed on a column of Silicar CC7 using EtOAc-$CHCl_3$ (1:1) as eluent. The tetrapeptide was crystd from EtOAc-hexane to yield 8.5 g (65%): mp 146°, $[\alpha]^{27}D$ −15.7° (*c* 1.85, DMF). *Anal.* ($C_{36}H_{50}N_4O_{10}$) C, H, N.

† All optically active amino acid residues are of the L variety.

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Z-γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly Pentachlorophenyl Ester (4).—To a soln of 9.0 g (0.0129 mole) of the fully blocked tetrapeptide **3** in 150 ml of MeOH was added 13.0 ml of 1 *N* NaOH and the soln was stirred for 90 min and then concd under reduced pressure. The residue was flooded with H_2O, acidified with 10% citric acid soln, and extd into EtOAc. The EtOAc soln was dried (Na_2SO_4) and concd under reduced pressure to give the tetrapeptide free acid as a solid; yield 8.0 g (91%). To this material in 200 ml of CH_2Cl_2 was added 3.1 g (0.0165 mole) of pentachlorophenol and 5.0 g (0.0118 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. The mixt was shaken for 48 hr at room temp. The solvent was removed *in vacuo* and the residue was washed with H_2O and crystd from MeOH to yield 5.1 g (47%): mp 187-188°, $[\alpha]^{27}D$ −12.9° (*c* 2.75, DMF). *Anal.* ($C_{41}H_{47}Cl_5N_4O_{10}$) C, H, N.

γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly Pentachlorophenyl Ester·HCl (5).—A fine suspension of 4.5 g (0.0048 mole) of the tetrapeptide active ester **4** and 0.5 g of 10% Pd/C in 150 ml of MeOH was treated with 0.173 g (0.0048 mole) of dry HCl in MeOH, and the suspension was hydrogenated for 2 hr. The reaction mixt was filtered and the filtrate was concd. The residue was crystd from MeOH-Et_2O to give 3.8 g (95%): mp 172°, $[\alpha]^{27}D$ +2.5° (*c* 4.2, DMF). *Anal.* ($C_{33}H_{42}Cl_6N_4O_8$) C, H, N.

Poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et Ester (1).—To a soln of 1.1 mg of glycine-*1*-^{14}C Et ester HCl (specific activity 3.15 mCi/mmole) and 1.82 g (0.018 mole) of Et_3N in 5 ml of DMSO was slowly added a soln of 3.0 g (0.00359 mole) of the polymerizing unit **5** in 25.0 ml of DMSO. The transfer vessels were washed with 5.5 ml of DMSO which was added to the reaction mixt giving a final concn of 100 mmoles/l. The reaction mixt was shaken for 6 days 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 0.95 g (49.5%) of the blocked polymer. The protected polypeptide was dissolved in 50 ml of 90% F_3CCO_2H and stirred for 50 min, and then concd under reduced pressure to yield the crude polypeptide 1. This material was washed with Et_2O, suspended in 20 ml of H_2O, and dissolved by the addition of 1 *N* NaOH to pH 7.5. The soln was dialyzed against distd H_2O for 2 days, then lyophilized to yield the Na salt of the polypeptide. This material was converted into its free acid by acidification with 1 *N* HCl to pH 2.5. The pptd polypeptide was collected by centrifugation and then lyophilized to yield 0.50 g (32%); radio assay, 33.3 nCi/mg indicates 73.7% incorporation of the radioactive label. *Anal.* ($C_{19}H_{24}N_4O_7 \cdot 1.5\ H_2O$) C, H, N.

Molecular Weight Determination.—Calibrated columns of Sephadex G-100 (2.5 × 38.5 cm) and of Corning CPG 10-240 glass granules (2.0 × 28 cm) were employed for the molecular weight determination. Using 0.1 *M* NaCl-0.05 *M* KH_2PO_4 corrected to pH 8.0 as eluent, 4 mg of poly(Gly-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester was passed through each of these columns. The polypeptide was eluted from each column in a vol equiv to that corresponding to a mol wt of at least 1 × 10^5.

Immunochemical Results.—Two rabbits were treated at weekly intervals with 500 μg of poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester **1**. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium and the 3rd week they were injected sc. The injection on the 4th week was done iv using buffered saline. Bleedings were conducted on the following week and the serum from each animal was not found to give a precipitin reaction with up to 10,000 μg of the polymer **1**.

Inhibition Studies.—To 1-ml aliquots of rabbit antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester was added incremental amounts of up to 7000 μg of the polypeptide **1**. To each tube was added the equiv point amount of the antigen (30 μg) and the tubes were then incubated at 37°. Each tube showed a precipitin reaction. After standing at 4° for 48 hr, the ppts were collected, washed twice with H_2O, and collected by centrifugation. The amount of protein pptd was estimated by the absorbancy at 280 mμ of a 0.1 *N* NaOH soln of the ppts. It was found that the precipitin reaction between poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester and its antisera was 50% inhibited by the addition of 6000 μg of poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester 1.

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Synthesis of [5-Valine,7-*N*-methylalanine]-angiotensin II, a Hypertensive Peptide[1-3]

RUDOLF H. ANDREATTA AND HAROLD A. SCHERAGA*

Department of Chemistry, Cornell University, Ithaca, New York 14850

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A synthesis of the octapeptide H-Asp-Arg-Val-Tyr-Val-His-Mal-Phe-OH ([Val5,Mal7]-angiotensin II) by the solid phase method is described. This peptide is an analog of [Val5]-angiotensin II, in which 7-proline is replaced by Mal. The observation that this peptide exhibits marked hypertensive and myotropic activities indicates that the proline residue is not essential for these pharmacological properties; instead the presence of an N-methylated amino acid in position 7 may be sufficient.

To date, more than 100 analogs[4] of angiotensin have been reported[5] in addition to the naturally occurring [Val5]- and [Ile5]-angiotensins I and II. Many of these analogs have been prepared and their biological activity evaluated for the purpose of elucidating the correlation between structural features and biological function. On the basis of these investigations, it has been concluded[5] that 4 of the 8 amino acids constituting these peptides are essential for biological activity. These are 4-Tyr, 6-His, 7-Pro, and 8-Phe. In addition, it has been asserted[5] that the C-terminal CO_2H must not be substituted. In a recent investigation,[6] it was shown that the imidazole portion of 6-His is not required for biological function. The conclusion that 7-Pro is essential for biological activity followed from an investigation of Page and Bumpus,[7] who found that the replacement of 7-Pro in [Ile5]-angiotensin II by Ala was accompanied by a total loss of hypertensive activity. Replacement of Pro in angiotensin has been the subject of

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(2) All amino acid residues are of the L variety. The following abbreviations are used: Boc = *t*-butoxycarbonyl; Narg = nitroarginine; Mal = *N*-methylalanine; TEA = Et_3N; TFA = F_3CCO_2H; AP-M = aminopeptidase *M*.[3]

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