Studies on the Conformation Dependency of Antibody to Poly(L-tyrosyl-L-glutamyl-L-alanyl-glycyl)glycine-*1*-^{14}C Ethyl Ester†

Brian J. Johnson

Laboratory of Molecular Biology, Department of Biochemistry and Department of Microbiology, Division of Clinical Immunology, University of Alabama Medical School, Birmingham, Alabama 35233.
Received September 21, 1971

It has recently been reported that the polypeptide poly(Tyr-Ala-Glu) possesses predominantly conformation-dependent antigenic determinants.[1] We wish to report that a similar situation exists for the antigen, poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester.[2-4] It has been inferred from previous work[5-7] that the conformation of the polymer poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester was important for describing the antigenic locus of this material. In order to investigate this point further, it was necessary to construct a polymer which possessed the same sequence of amino acids as the antigen poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester but which differed markedly from it in conformation. For this purpose we wish to report the synthesis and immunochemical properties of poly(Tyr-γ-Glu-Ala-Gly)Gly Me ester (**1**).

Chemistry. The polymerizing unit *0-tert*-Bu-Tyr-*tert*-Bu-γ-Glu-Ala-Gly pentachlorophenyl ester hydrochloride (**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.[2,3,5-12] Following this established procedure the insoluble polymer, poly(*0-tert*-Bu-Tyr-*tert*-Bu-γ-Glu-Ala-Gly)Gly Me ester was prepared; from which the protecting *tert*-Bu groups were removed by use of 90% F_3CCO_2H to yield poly(Tyr-γ-Glu-Ala-Gly)Gly Me ester (**1**). After extensive dialysis, the polymer was purified and fractionated by passage through calibrated columns of Sephadex G-50, and Corning CPG10-240 glass granules.

Immunochemistry. Incremental amounts of the polypeptide **1** were added to aliquots of antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester. No precipitin reaction was observed when up to 7000 μg of **1** was used. Inhibition studies were also performed in order to investigate the possibility that the polypeptide **1** could be a hapten for the antigen poly(Tyr-Gly-Ala-Gly)Gly-*1*-^{14}C Et ester. Incremental amounts of **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. No inhibition of the precipitin reaction was observed in the presence of up to 7000 μg of the polypeptide **1**.‡

Conclusions

Previous work had indicated that antibodies to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester are most probably dependent upon the conformation of the antigen. In order to investigate this point further poly(Tyr-γ-Glu-Ala-Gly)Gly Me ester was synthesized such that the same order of amino acid residues was maintained as those that constitute the antigen poly(Tyr-Glu-Ala-Gly)-Gly-*1*-^{14}C Et ester. However, due to the repeating γ-peptide linkage through the glutamyl residue the conformation of **1** should be considerably different from that of the antigen. It has been shown that the polypeptide **1** does not cross-react with antibodies produced by poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester nor does it inhibit the precipitin reaction between this antisera and the antigen. Thus it has been concluded that the antigenic locus of poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester is dependent on the conformation of the polypeptide.

Experimental Section

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

Z-O-*tert*-Bu-Tyr-*tert*-Bu-γ-Glu-Ala-Gly Me Ester (2).§ To a soln of 9.5 g (0.025 mole) of *tert*-Bu-γ-Glu-Ala-Gly Me ester hydrochloride and 2.5 g (0.0248 mole) of Et_3N in 200 ml of CH_2Cl_2 was added 15.5 g (0.025 mole) of Z-*0-tert*-Bu-Tyr pentachlorophenyl ester. The mixt was stirred overnight at room temp and concentrated, and the product was dissolved in ETOAc, washed with 10% citric acid soln 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 tetrapeptide: crystallization from EtOAc–hexane yielded 11.6 g (66%), mp 146° $[\alpha]^{29}D$ −18.5° (*c* 4.8, DMF). *Anal.* ($C_{36}H_{50}N_4O_{10}$) C, H. N.

Z-O-*tert*-Bu-Tyr-Bu-γ-Glu-Ala-Gly Pentachlorophenyl Ester (0.0165 mole) of **2** in 400 ml of MeOH was added 16.5 ml of 1 *N* KOH, and the soln was stirred for 90 min at room temp and then concentrated under reduced pressure. The residue was flooded with H_2O, acidified with 10% citric acid soln, and extracted with EtOAc. The EtOAc soln was dried (Na_2SO_4) and concentrated under reduced pressure to give the tetrapeptide free acid. Crystallization from EtOAc–hexane yielded 10.7 g (95%), mp 176°, $[\alpha]^{29}D$ −28.7° (*c* 2.05, DMF). *Anal.* ($C_{35}H_{48}N_4O_{10}$) C, H, N.

Z-O-*tert*-Bu-Tyr-*tert*-Bu-γ-Glu-Ala-Gly Pentachlorophenyl Ester (4). To a soln of 10.7 g (0.01565 mole) of 3 in 100 ml of DMF was added 4.4 g (0.0165 mole) of pentachlorophenol and 7.7 g (0.0181 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. The mixt was stirred at room temp overnight, added to 400 ml of H_2O, and the solid material was collected, washed with H_2O, and crystallized from MeOH to yield 7.0 g (47%), mp 172–173°, $[\alpha]^{29}D$ −21.4° (*c* 4.85, DMF). *Anal.* ($C_{41}H_{57}Cl_5N_4O_{10}$) C, H, N.

O-*tert*-Bu-Tyr-*tert*-Bu-γ-Glu-Ala-Gly Pentachlorophenyl Ester Hydrochloride (5). A fine suspension of 5.0 g (0.00535 mole) of **4** and 0.8 g of 10% Pd/C in 200 ml of MeOH was treated with 0.195 g (0.00535 mole) of dry HCl in MeOH, and the suspension was hydrogenated for 2 hr. The reaction mixt was filtered and the filtrate was concentrated. The residue was crystallized from MeOH–Et_2O to give 4.0 g (88%), mp 230°, $[\alpha]_4^{29}$ −22.1° (*c* 2.0, DMF). *Anal.* ($C_{33}H_{52}Cl_6N_4O_8$) C, H, N.

Poly(Tyr-γ-Glu-Ala-Gly)Gly Me Ester (1). To a soln of 1.5 g (0.01485 mole) of Et_3N and 1.1 mg of Gly Me ester hydrochloride in 10 ml of DMSO was added a soln of 4.0 g (0.00474 mole) of the polymerizing unit **5** in 37.4 ml of DMSO. The mixt 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 0.9 g (36%) of the blocked polymer. This material was treated with 50 ml of 90% F_3CCO_2H, stirred for 50 min, and then concentrated under reduced pressure to yield the crude polypeptide **1**. This material was suspended in 20 ml of H_2O, and dissolved by the addition of 1 *N* NaOH to pH 7.3. The soln was dialyzed against distilled H_2O for 24 hr and acidified with 6 *N* HCl to pH 2.5. The precipitated polypeptide **1** was collected by centrifugation and then lyophilized to yield 0.5 g (25%). *Anal.* ($C_{19}H_{24}N_4O_7$) C, H, N.

Molecular Weight Determination. Calibrated columns of Sephadex G-50 (2.5 × 38.5 cm) and of Corning CPG10-240 glass granules (2 × 24 cm) were employed for the molecular weight determination. Using 0.15 *M* NaCl as eluent, 4 mg of the Na salt of poly(Tyr-γ-Glu-Ala-Gly)Gly Me ester was passed through each of these columns. The polypeptide was eluted from each column in a volume equivalent to that corresponding to a molecular weight of at least 1.8×10^4.

Immunochemical Results. 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. Each tube was in-

†All optically active amino acids are of the L variety.

‡Studies with the small peptides (Tyr-Glu-Ala-Gly)$_n$Gly Me ester, where n = 1, 2, 3, 4 have shown neither cross reaction with the antisera nor inhibition of the precipitin reaction.

§ Z = Benzyloxycarbonyl.

cubated at 37° for 1 hr and then stood at 4° for 48 hr. No precipitate was observed in any tube.

Inhibition Studies. To 1-ml aliquots of rabbit antisera to poly(Tyr-Glu-Ala-Gly)Gly-*l*-^{14}C Et ester were added incremental amounts of up to 7000 μg of 1. To each tube was added the equiv point amount of the antigen (30 μg) and the tubes were then 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 in each tube was determined by N analysis by a micro-Kjeldahl method to give 105 μg of protein N for each tube. A control run simultaneously with the preceding experiment but not containing any added polypeptide 1 gave a protein N analysis of 105 μg. Therefore, the precipitin reaction between poly(Tyr-Glu-Ala-Gly)Gly-*l*-^{14}C Et ester and its antisera was not inhibited by the addition of up to 7000 μg of 1.

Acknowledgments. This work was supported by the National Science Foundation, the Hartford Foundation and the State of Alabama.

References

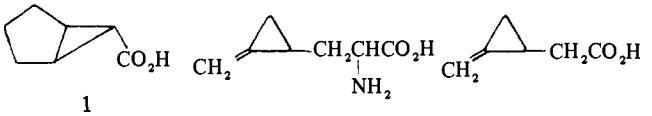
(1) B. Schechter, I. Schechter, J. Ramachandran, A. Conway Jacobs, M. Sela, E. Benjamini, and M. Shimizu, *Eur. J. Biochem.*, 20 (1971).
(2) B. J. Johnson and E. G. Trask, *J. Chem. Soc. C*, 2644 (1969).
(3) B. J. Johnson, *J. Pharm. Sci.*, 59, 1849 (1970).
(4) B. J. Johnson and E. G. Trask, *ibid.*, 59, 724 (1970).
(5) B. J. Johnson and E. G. Trask, *J. Med. Chem.*, 14, 251 (1971).
(6) B. J. Johnson, *ibid.*, 14, 488 (1971).
(7) B. J. Johnson, C. Cheng, and N. Tsang, *ibid.*, 15, 95 (1972).
(8) B. J. Johnson and F. Chen, *ibid.*, 14, 640 (1971).
(9) B. J. Johnson and C. Cheng, *ibid.*, 14, 1238 (1971).
(10) B. J. Johnson and D. S. Rea, *Can. J. Chem.*, 48, 2509 (1970).
(11) B. J. Johnson and E. G. Trask, *J. Chem. Soc. C*, 2247 (1970).
(12) B. J. Johnson, *ibid.*, *C*, 1412 (1969).

exo-Bicyclo[3.1.0]hexane-6-carboxylic Acid and Related Compounds, Oral Hypoglycemic Agents

Ronald H. Rynbrandt,* Fred E. Dutton, and Fredericka L. Schmidt

Diabetes Research, The Upjohn Company, Kalamazoo, Michigan 49001. *Received May 19, 1971*

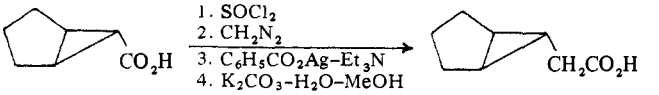
Screening for antidiabetic agents revealed that *exo*-bicyclo[3.1.0]hexane-6-carboxylic acid (**1**)† possessed good hypoglycemic activity in the glucose-primed, fasted, intact rat. This compound is structurally related to the hypoglycemic compounds, Hypoglycin A and its biological metabolite[2] (for a good review of Hypoglycin and Hypoglycin-like compounds see ref 3). However, although Hypoglycin-type



1 Hypoglycin A biological metabolite of Hypoglycin A

compounds produced an increase in plasma-free fatty acids (FFA's),[4] **1** caused a decrease in FFA's in the fasted rat.‡ This observation, coupled with the fact that **1** is relatively nontoxic (as opposed to Hypoglycin A),[5] suggested that these 2 types of compounds act by different mechanisms to lower blood sugar. A study aimed at obtaining insight into the various structural features necessary for hypoglycemic activity in this new class of hypoglycemic agents was made.

Chemistry. Acids **1**,[1] **2**,[6] **4**,[7] **7**,[1] **8**,[8] and **9**[9] were prepared by published procedures or slight modifications thereof. Compd **3** was obtained by the hydrolysis of the corresponding ethyl ester.[10] Compd **10** was formed by application of the Arndt-Eistert reaction sequence to acid **1**.



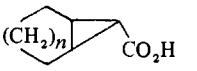
1 10

The esters in Table III and the amides in Table IV were prepared from the appropriate acid chlorides (see Experimental Section).

Structure-Activity Relationship Considerations. The bicyclic ring system in **1** appears to be necessary for activity in that cyclopropanecarboxylic acid (**5**) and cyclohexanecarboxylic acid (**6**) were both inactive at the highest doses tested. The exo configuration is required in that the corresponding endo isomer (**7**) and two of its amides, **15** and **16**, were inactive while the corresponding exo isomers (**1**, **17**, and **21**) were active. Variations in the size of the larger ring (see Table I) resulted in 2 which possessed substantially greater activity than the initial lead (**1**). A spirocyclopropane analog (**8**) and a tricyclic analog (**9**) possessed no or substantially reduced activity.

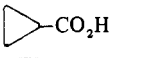
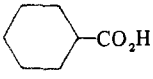
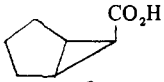
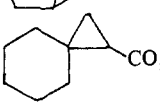
Analog **10**, in which CO_2H is separated from the cyclopropyl ring by CH_2 (as in the active metabolite of Hypoglycin A),[2] possessed good activity. This was surprising in

Table I

No.	*n*	Lowest dose with consistent activity, mg/kg[a]
		
1	1	6.25
2	2	2.0
3	3	100
4	4	>100

[a] Lowest dose of tolbutamide with consistent hypoglycemic activity = *ca.* 25 mg/kg.

Table II

No.	Structure	Lowest dose with consistent activity, mg/kg[a]
5[b]		>100
6[b]		>100
7		>100
8		>100
9		100
10		12.5

[a] Lowest dose of tolbutamide with consistent hypoglycemic activity = *ca.* 25 mg/kg. [b] Aldrich Chemical Company, Inc., Milwaukee, Wis.

†This compd was first prepd at The Upjohn Company by E. S. Cerda according to ref 1.

‡Unpublished work by G. C. Gerritsen, The Upjohn Company.