BIOORGANIC CHEMISTRY 8, 35–39 (1979)

Synthesis of the Facteur Thymique Serique and an Analog Also Related to Thymopoietin

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Received February 14, 1978

The known 9-amino-acid sequence of the facteur thymique serique (FTS) and the known 49-amino-acid sequence of thymopoietin, in conjunction with background peptide chemistry, raised the possibility that Gln[1] of FTS might be linked to Arg[49] of thymopoietin in a new 58-amino-acid peptide in tissue. A presumed cleavage between Gln[50] and Arg[49] adjacent to the Lys[48]-Arg[49] moiety would liberate [H-Gln[1]]-FTS and thymopoietin; the former fragment would cyclize to FTS by known chemistry. To study this possibility, FTS, and a new dodecapeptide consisting of Val-Lys-Arg linked to the N-terminal of [H-Gln[1]]-FTS were synthesized on a new solid-phase resin and were found to show comparable immune stimulating activity, *in vivo*. These data do not negate, but support, the concept of the 58-aminoacid peptide.

INTRODUCTION

The emerging chemistry of thymic peptides is of increasing importance in the elucidation of immune mechanisms.

Bach *et al.* (1) described, in 1977, the organic chemical and biological characterization of their facteur thymique serique (FTS) which was found in the serum of swine and other mammalian species. The presence of FTS in the serum of these species is thymus-dependent. FTS has the sequence of the nonapeptide: Glx-Ala-Lys-Ser-Glx-Gly-Gly-Ser-Asn. Glx[1] was presumed to be <Glu, "rather natively pyroglutamic acid or transformed *in vitro*." Glx[5] was presumed to be Gln, because of the isoelectric point of the peptide. Bach *et al.* acknowledged Hirschmann *et al.* of Merck Sharp Research Laboratories, West Point, Pa., for having synthesized <Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH which was found to behave chemically and biologically like FTS.

Goldstein (*2*) reported in 1974 two closely related polypeptides (thymin I and II) from bovine thymus which contain 49 amino acids and were renamed thymopoietin I and II. Thymopoietin I has Gly[1], Gln[2], and His[43]; thymopoietin II has Ser[1], Glu[2], and Thr[43]; otherwise, the two sequences are identical according to Schlesinger and Goldstein (1975) (*3*).

It seemed to Folkers and Wan (*4*) that FTS in the serum could be a fragment of a larger peptide of which had been cleaved, probably enzymatically, to yield the [H-Gln[1]]-nonapeptide which could then nonenzymatically cyclize to the [<Glu[1]]-nonapeptide which was the isolated FTS. Specifically, we considered a structural relationship between FTS of nine amino acids and thymopoietin (I or II) of 49 amino acids. The sequence of thymopoietin at the C-terminal end has Lys[48]-Arg[49]-OH. The N-terminal end of FTS is <Glu[1]. It is well known that H-Gln in position 1 of peptides is chemically

0045-2068/79/010035-05$02.00/0

unstable and readily cyclizes to <Glu. Examples are: H-Gln-His-Pro-NH_2 which readily cyclizes to TRH (*5*), and [H-Gln^1]-neurotensin [a tridecapeptide] which cyclizes to neurotensin (*6*).

In background peptide chemistry, it is known that certain peptides are cleaved, presumably enzymatically, at either side of a Lys-Arg moiety, and presumably because of the chemical uniqueness of this "double basic" moiety in a sequence. Four examples are as follows: Pro-glucagon having 37 amino acids cleaves between Thr^{29} and Lys^{30} of the moiety Thr^{29}-Lys^{30}-Arg^{31} to give glucagon having 29 amino acids (*7*). Bovine proinsulin, which cleaves between Arg^{60} and Gly^{61} of the moiety Lys^{59}-Arg^{60}-Gly^{61} gives bovine insulin (*8*). "Large gastrin" has been presumed to be cleaved between Lys^{17} and Gln^{18} of a Lys^{16}-Lys^{17}-Gln^{18} moiety to give [Gln^1]-gastrin which cyclized to [<Glu^1]-gastrin (*9*). Very recently, it was found that the cleavage of β-lipotropin to β-melanotropin and to β-endorphin involves two such cleavages; one is between Lys^{40} and Asp^{41} of Lys^{39}-Lys^{40}-Asp^{41}, and the other is between Arg^{60} and Tyr^{61} of Lys^{59}-Arg^{60}-Tyr^{61} (*10*). Other "double basic" moieties are Arg-Arg and Lys-Lys which, as reviewed by Steiner (*11*), may participate in such cleavages of peptide hormone precursors.

Consequently, it seemed very reasonable that in thymus, serum, or in other tissue there exists a peptide of 58 amino acids which has the moiety Lys^{48}-Arg^{49}-Gln^{50} and which is similarly cleaved enzymatically between Arg^{49} and Gln^{50} to give thymopoietin terminating in Arg^{49}-OH and the nonapeptide FTS with an unstable H-Gln^1 moiety which then cyclizes to give the [<Glu^1]-nonapeptide as isolated by Bach *et al.* (*1*). Our first approach in testing the possibility of the existence of a thymic peptide having 58 amino acids (which might also be a fragment of a larger peptide) was to synthesize FTS and a related dodecapeptide which "bridges into" the sequence of thymopoietin at the C-terminal end for potential cleavage. The dodecapeptide is H-Val-Lys-Arg-Gln-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-OH, which contains the critical Lys-Arg moiety. Initially, we thought that this moiety was more important than including Ala^{46}, Thr^{45}, etc., to lengthen the peptide beyond 12 amino acids.

EXPERIMENTAL

Synthesis

General procedure for solid-phase synthesis. Pure L-isomers of amino acid derivatives, purchased from Beckman Inc., Palo Alto, Calif., and Peninsula Laboratories, San Carlos, Calif. were used. The amino acid analyses were carried out on a Beckman amino acid analyzer, Model 119, after hydrolysis of the samples in 6 *N* HCl overnight in evacuated sealed tubes at 110°C.

The peptides were synthesized on the new PAM-resin (*12*, *13*), by a solid-phase methodology on a Beckman 990 peptide synthesizer. The α-amino groups were protected exclusively by the t-butoxycarbonyl group (Boc) with the exception of Arg (amyloxycarbonyl group, Aoc). The side-chain protecting groups which were used were 2-chlorobenzyl (2-Cl-Z) for Lys, *p*-toluenesulfonyl (Tos) for Arg, and benzyl (Bzl) for Ser, Glu, and Asp. <Glu was incorporated as Z-<Glu-OH in DMF–CH_2Cl_2.

Coupling reactions with a threefold excess of Boc- or Aoc-amino acid derivatives and dicyclohexylcarbodiimide (DCC) were generally performed in CH_2Cl_2 or DMF. The

coupling program used for peptide elongation involved the following successive operations (number of times each step performed, mixing time): (1) CH_2Cl_2 (three washes, 2 min); (2) 40% TFA in CH_2Cl_2 (v/v) (prewash, 2 min); (3) 40% TFA in CH_2Cl_2 (deprotection, 30 min); (4) CH_2Cl_2 (three washes, 2 min); (5) *i*-propanol (two washes, 2 min); (6) CH_2Cl_2 (three washes, 2 min); (7) 10% ET_3N (redistilled from NaOH pellets) in CH_2Cl_2 (v/v) (two prewashes, 2 min); (8) 10% ET_3N (neutralization, 10 min); (9) CH_2Cl_2 (four washes, 2 min); (10) amino acid derivative (one addition, 10 min); (11) 10% DCC in CH_2Cl_2 (v/v) (coupling); (12) CH_2Cl_2 (four washes, 2 min); (13) *i*-propanol (three washes, 2 min); (14) DMF (three washes, 2 min); (15) CH_2Cl_2 (three washes, 2 min). For the incorporation of Gln and Asn, an active ester coupling procedure was employed. A sixfold excess of the *p*-nitrophenyl ester derivative of Boc-Gln or Boc-Asn in purified DMF was used for the coupling reaction, and a longer coupling time was also required. The coupling reactions were monitored by the ninhydrin test. (*14*). A recoupling procedure was always employed if the ninhydrin test indicated that the coupling or recoupling was not completed. The following thin-layer chromatography systems were used (v/v): R_f^1, *n*-BuOH:HOAc:H_2O (1:1:1); R_f^2, ETOAc:Py:HOAc:H_2O (5:5:1:3); R_f^3, *n*-BuOH:Py:HOAc:H_2O (30:20:6:24). Thin-layer electrophoresis (tle) was run on cellulose plates at 900 V in the following two systems; pH 2.8, 1 *M* HOAc; pH 6.5, Py:HOAc:H_2O (30:1:270).

Boc-Asn-PAM-Resin. The procedure used to prepare the Boc-Asn-PAM-resin was similar to that described by Mitchell *et al.* (*12*) and Yamaguchi *et al.* (*13*). A solution of BocAsnOH (1.97 g; 8.48 m*M*) and carboxyldiimidazole (1.23 g; 7.59 m*M*) in a mixture of DMF and CH_2Cl_2 (40 ml each) was kept at −5°C for 30 min and added to a reaction vessel containing 4-(hydroxymethyl)-phenylacetamidomethyl resin (8.48 g). The residual solution was washed with CH_2Cl_2, and the suspension was shaken for 10 hr at room temperature. The resin was filtered, washed with CH_2Cl_2, DMF, CH_2Cl_2, EtOH, and CH_2Cl_2. The resin was then allowed to dry over vacuum overnight. The coupled resin was acetylated by being shaken with a mixture of pyridine and acetic anhydride [160 ml, 1:1 (v/v)] for 40 min. Subsequent filtration, washing with DMF, CH_2Cl_2, *i*-prOH, and CH_2Cl_2, and drying under vacuum gave the Boc-Asn-PAM-resin which contained 0.198 meq of Asn/g of substituted resin based on amino acid analysis.

Facteur thymique serique (*<Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-AsnOH*). Boc-Asn-PAM-Resin (9 g, 1.8 meq) was taken through four successive cycles of deprotection and coupling to give Boc-Gln-Gly-Gly-Ser(0-Bzl)-Asn-PAM-resin (10 g). A portion of this protected pentapeptide-PAM-resin (4 g) was extended to Boc-Ala-Lys(2-Cl-Z)-Ser(0-Bzl)/Gln-Gly-Gly-Ser(0-Bzl)-Asn-PAM-resin by sequentially coupling with Boc-Ser(0-Bzl), Boc-Lys(2-Cl-Z), and Boc-Ala. The protected octapeptide-PAM-resin was then divided into two portions. One portion was incorporated with Z-<Glu to give the projected protected nonapeptide resin (2.2 g).

The protected nonapeptide-PAM-resin was treated with anhydrous HF(CoF_3) in the presence of ca. 10% anisole for 1 hr at 0°C. The excess HF was then quickly removed at reduced pressure. The dried reaction mixture was first washed with Et_2O and ETOAc before extracting the free peptide acid with 10% HOAc. The combined extracts were lyophilized and gave 215 mg of crude product as a white powder. The lyophilized product was purified over a Sephadex G-15 gel-filtration column (2.5 × 110 cm) by elution with 1.3% HOAc. The major fraction (ea. 120 mg) was further purified over

another Sephadex G-15 column to give the desired product (54 mg; yield 18%). Amino acid analyses gave the following ratios: Glu, 2 × 1.04; Ala, 0.90; Lys, 0.91; Ser, 2 × 1.04; Gly, 2 × 1.08; Asp, 0.89; NH_3, 2 × 0.98. The product appears to be homogenous on tlc with two different buffer systems (pH 2.8, pH 6.5). The tlc data are as follows: $R_f^1 = 0.41$, $R_f^2 = 0.21$, $R_f^3 = 0.18$.

H-Val-Lys-Arg-Gln-Ala-Lys-Ser-Gln-Gly-Gly-Ser-AsnOH. The second portion of the protected octapeptide PAM-resin, Boc-Ala-Lys(2-Cl-Z)-Ser(0-Bzl)-Gln-Gly-Gly-Ser(0-Bzl)-Asn-PAM-resin (2.1 g) was taken through four successive cycles of deprotection and coupling to give the protected dodecapeptide-PAM-resin, Boc-Val-Lys(2-Cl-Z)-Arg(Tos)-Gln-Ala-Lys(2-Cl-Z)-Ser(0-Bzl)-Gln-Gly-Gly-Ser(0-Bzl)-Asn-PAM-resin (2.25 g).

The protected dodecapeptide-PAM-resin was cleaved and deprotected by anhydrous HF. The crude lyophilized product (280 mg) was purified on a Sephadex G-25 column (2.5 × 110 cm) by elution with 1.3% HOAc. The major fraction (ca. 200 mg) was rechromatographed on another Sephadex G-25 column to give 98.5 mg of peptide (yield, 22.4%). Amino acid analyses gave the following ratios: Val, 0.89; Lys, 2 × 0.99; Arg, 0.91; Glu, 2 × 0.99; Ala, 1.00; Ser, 2 × 1.01; Gly, 2 × 1.10; Asp, 1.04; NH_3, 4 × 0.92. The product is homogenous on tlc with two different buffer systems (pH 2.8, pH 6.5). The tlc data are as follows: $R_f^1 = 0.03$, $R_f^2 = 0.11$, $R_f^3 = 0$.

RESULTS AND DISCUSSION

The nonapeptide, facteur thymique serique, and the dodecapeptide which is structurally related to both FTS and thymopoietin were synthesized by a solid-phase methodology and purified without difficulty. The peptide chains were constructed on the relatively new PAM-resin of Mitchell *et al.* (*12*) and synthesized differently by Yamaguchi *et al.* (*13*).

From the viewpoint of the biological activity of a peptide of the molecular weight of FTS, the addition of the two basic amino acids, Lys-Arg-, to FTS may be considered a substantial chemical change; one of the amino acids could significantly alter the biological activity of FTS, particularly to decrease activity.

In our ongoing cooperative studies with Dr. Emile G. Bliznakov of the New England Institute, Ridgefield, Conn., the synthetic FTS and the related dodecapeptide were compared for reactivation of the impaired immune competence in aged mice. The data of Bliznakov *et al.* (*14*) unambiguously showed the profound impairment of a humoral, hemolytic, primary, immune response in mice aged 22 months when compared to this response in young 10-week-old mice. The sc administration of these two synthetic peptides caused a partial and significant reactivation of the age-determined impairment of the immunological responses. The synthetic FTS and the dodecapeptide showed comparable activity at levels of 10 μg/mouse/injection.

Although we have not yet had the opportunity to maximize the immunological activities of the two synthetic peptides, it is significant that the addition of the tripeptide moiety, Val-Lys-Arg- to the N-terminal end of FTS maintained the immune stimulating activity rather than having significantly decreased it.

These results do not negate, but rather support the concept of a new peptide of 58 amino acids consisting of FTS linked through Gln^1 to Arg^{49} of thymopoietin.

ACKNOWLEDGMENT

Appreciation is expressed to the Robert A. Welch Foundation for support of this research.

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