SYNTHESIS OF A PROPOSED GROWTH HORMONE RELEASING FACTOR.

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SUMMARY

The synthesis of Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala, isolated by Schally, et al. from porcine hypothalamus and reported by him to possess growth hormone releasing activity, is described. Similarities between this decapeptide and the amino-terminal sequence of the β-chain of porcine hemoglobin are pointed out. The syntheses of two analogs including the amino-terminal decapeptide of the β-chain of human hemoglobin are also described.

INTRODUCTION

In pioneering studies, Schally and his associates reported that the release of growth hormone (GH) by the pituitary gland is mediated by a hypothalamic substance named growth hormone-releasing hormone (GH-RH)[1]. The isolation of a peptide with GH-RH activity from porcine hypothalamic extracts was also described by him[2], and its structure was reported to be H•Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala•OH (I)[3].

It was observed by one of us (C.B.) that a striking similarity exists between I and the proposed amino-terminal sequence of the β-chain of porcine hemoglobin[4], viz. (Val.His.Leu.Ser.Ala.Glx.Glx)Lys(Ala,Glx,Val . . .).[a] Edman dansyl degradation studies showed the amino-terminal sequence of the

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[a] Parentheses enclose a region, the composition but not the complete sequence of which has been determined experimentally. A period between amino acids within parentheses indicates that the amino acid to its left has been placed with at least 90% confidence by homology with known sequences, whereas a comma within parentheses indicates that the amino acid to its left could not be positioned with confidence by homology.

β-chain of porcine hemoglobin to be indeed Val-His-Leu-Ser-Ala-Glu-Glu-Lys, suggesting that I may be derived from hemoglobin as angiotensin I arises from an α_2-globulin. Examination of the known β-chain sequences of other species[4] showed that the amino acid in position 9 was always neutral and was Asn in one instance. Moreover, we found an N-terminal Gln and no Glu on a total tryptic digest of porcine β-chain hemoglobin. These observations suggested that Gln-9 might have been converted to Glu during isolation of GH-RH and were a primary reason for undertaking the synthesis of II, the 9-Gln analog of I. Schally, et al. have also obtained evidence for some Gln in position 9 of the isolated hypothalamic peptide[3]. If the isolated[2] peptide is indeed derived from porcine hemoglobin and if I or II should prove to be a regulator of the release of GH rather than an artifact, the N-terminal decapeptide III of the β-chain of human hemoglobin[4], Val-His-Leu-Thr-Pro-Glu-Glu-Lys-Ser-Ala, would be of considerable interest.

SYNTHESIS

We describe herewith the synthesis of I, II and III. The carboxy-terminal pentapeptide H·Glu-Glu-(N^{ε}-benzyloxycarbonyl-Lys)-Glu-Ala·OH (IV) was prepared as follows. Ala was allowed to react with the α-amino acid N-carboxyanhydride (NCA) of Glu[5,6] and the crude reaction mixture, after decarboxylation, was diluted with ethanol and allowed to react with N-hydroxysuccinimido-N^{α}-tert-butoxycarbonyl-N^{ε}-benzyloxycarbonyllysinate. After extraction purification, the tripeptide was converted into the free α-amino peptide with trifluoroacetic acid (TFA). This crystalline tripeptide gave [b] $Lys_{0.99}$ $Glu_{1.00}$ $Ala_{1.00}$. The glutamic acid residues 6 and 7 were introduced as NCA's without isolation of the tetrapeptide intermediate and the resulting pentapeptide IV was purified by preparative free flowing electrophoresis to afford a specimen $[\alpha]_{589}^{25}$ -28.2° (c 1.08, 50% acetic acid) which gave [b] $Glu_{3.04}$ $Lys_{1.00}$ $Ala_{0.98}$.

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[b] Amino acid analysis after 20 hr acid hydrolysis, except Val-His values (70 hr).

The amino-terminal pentapeptide ester Boc•Val-His-Leu-Ser-Ala•OMe (V) was prepared *via* the azide coupling of Boc•Val-His-Leu•N_3 with Ser-Ala•OMe. The former was prepared from Leu•OMe using the α-amino acid N-thiocarboxy-anhydride (NTA)[7,8] of His and the hydroxysuccinimide ester of Boc•Val. The resulting crystalline V, (dec pt 183°, $[\alpha]_{589}^{25}$ -67.7° [c 1, 50% acetic acid], Anal. Found: C, 50.04; H, 7.52; N, 13.95) gave [b] $Val_{1.00}$ $His_{1.00}$ $Leu_{1.00}$ $Ser_{1.00}$ $Ala_{1.00}$. The ester V was converted to the crystalline hydrazide VI (dec pt 230°, $[\alpha]_{589}^{25}$ -66.7° [c 0.9, 50% acetic acid], Anal. Found: C, 50.82; H, 7.79) using previously described conditions[9]. After azide coupling of VI with IV, the crude protected decapeptide was purified by gel filtration to afford Boc•Val-His-Leu-Ser-Ala-Glu-Glu-(N^{ε}-benzyloxycarbonyl-Lys)-Glu-Ala which gave [b] $Val_{1.00}$ $His_{0.99}$ $Leu_{0.99}$ $Ser_{0.97}$ $Ala_{2.01}$ $Glu_{3.03}$ $Lys_{0.99}$. The two N-blocking groups were removed with anhydrous HF at 0° (45 min) in the presence of anisole and the product was purified by chromatography on Sephadex G-50 to give an amorphous solid I (purity > 99% [c]) ($[\alpha]_{589}^{25}$ -67.7°, 50% acetic acid) which gave $Val_{0.98}$ $His_{0.98}$ $Leu_{1.00}$ $Ser_{1.00}$ $Ala_{1.98}$ $Glu_{3.03}$ $Lys_{0.99}$. After digestion with aminopeptidase M, an amino acid analysis gave $Val_{1.01}$ $His_{0.98}$ $Leu_{1.06}$ $Ser_{0.93}$ $Ala_{1.96}$ $Glu_{3.01}$. This material could be crystallized from aqueous ethanol.

The O-benzyl ether of V was smoothly synthesized on solid support[10] and cleaved from the resin by hydrazinolysis. The resulting O-benzyl ether afforded I, indistinguishable from the decapeptide described above. The pentapeptide prepared by this procedure was used for the synthesis of the 9-Gln analog II described below.

Synthetic and natural I were indistinguishable by tlc in three systems

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[c] All peptides were evaluated in several tlc systems. To be judged to be ≥ 99% pure, a peptide had to move as a single component in these systems when spotted in an amount sufficiently large that a one-hundred-fold dilution still gave a detectable product spot after development of the plate with the hypochlorite-potassium iodide sprays. This method, therefore, does not take into account such nonpeptidal impurities as solvents.

and by paper electrophoresis at pH 1.8, 4.3 and 6.4. Moreover, the amino-terminal octapeptides obtained by cleavage with trypsin were identical as were the heptapeptides derived from synthetic and natural I after digestion with carboxypeptidase A followed by carboxypeptidase B. II ($[\alpha]_{589}^{25}$ -64.1° [c 0.34, 50% acetic acid]) was synthesized as described above, using the NCA of $Gln^{5,6}$ to prepare Gln-Ala. The C-terminal pentapeptide required for the synthesis of III was prepared *via* Boc•(N^{ϵ}-benzyloxycarbonyl-Lys)-Ser-Ala•OMe (prepared with hydroxysuccinimide esters) which was saponified and treated sequentially with TFA and twice with the NCA of Glu. The N-terminal pentapeptide hydrazide was synthesized by coupling Boc•Val-His-Leu•N_3 with Thr-Pro•OMe. An azide coupling followed by treatment with HF led to III, $[\alpha]_{589}^{25}$ -89.2° (c 0.31, 50% acetic acid) which gave $Val_{1.01}$ $His_{0.99}$ $Leu_{1.02}$ $Thr_{1.00}$ $Pro_{0.99}$ $Glu_{1.99}$ $Lys_{1.03}$ $Ser_{0.98}$ $Ala_{0.97}$.

Schally and his associates are reporting[3,11] their biological studies with synthetic I and II by the tibia test method[12] and by the sulfation factor assay[13]. They found neither natural nor synthetic I active by the radioimmuno assay for rat GH. Since they conclude that further biological studies are required, we have synthesized sufficient amounts of I, II and III to make these compounds available to investigators for continuing biological studies.

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REFERENCES

1. A. V. Schally, A. Arimura, C. Y. Bowers, A. J. Kastin, S. Sawano and T. W. Redding, Recent Progress in Hormone Research 24, 497 (1968).

2. A. V. Schally, S. Sawano, A. Arimura, J. F. Barrett, I. Wakabayashi and C. Y. Bowers, Endoc. 84, 1493 (1969).
3. A. V. Schally, Y. Baba, R. M. G. Nair and C. D. Bennett, J. Biol. Chem., In the Press.
4. M. O. Dayhoff, Atlas of Protein Sequence and Structure 4, D-49 (1969).
5. R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Veber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob and R. Hirschmann, J. Amer. Chem. Soc. 88, 3163 (1966).
6. R. Hirschmann, H. Schwam, R. G. Strachan, E. F. Schoenewaldt, H. Barkemeyer, S. M. Miller, J. B. Conn, V. Garsky, D. F. Veber and R. G. Denkewalter, ibid. 93, 2746 (1971).
7. R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arrison, D. F. Veber, R. G. Denkewalter and R. Hirschmann, ibid. 90, 3254 (1968).
8. R. S. Dewey, E. F. Schoenewaldt, H. Joshua, W. J. Paleveda, Jr., H. Schwam, H. Barkemeyer, B. H. Arison, D. F. Veber, R. G. Strachan, J. Milkowski, R. G. Denkewalter and R. Hirschmann, J. Org. Chem. 36, 49 (1971).
9. R. G. Strachan, W. J. Paleveda, Jr., R. F. Nutt, R. A. Vitali, D. F. Veber, M. J. Dickinson, V. Garsky, J. E. Deak, E. Walton, S. R. Jenkins, F. W. Holly and R. Hirschmann, J. Amer. Chem. Soc. 91, 503 (1969).
10. R. B. Merrifield, Advan. Enzymol. 32, 221 (1969).
11. A. V. Schally, A. Arimura, I. Wakabayashi, T. W. Redding, E. Dickerman and J. Meites, J. of Clin. Endocrinol. Metab., In the Press.
12. F. S. Greenspan, C. H. Li, M. E. Simpson and H. M. Evans, Endoc. 45, 455 (1949)
13. W. D. Salmon, W. H. Danghady, J. Lab. and Clin. Med. 49, 825 (1957); E. J. Collins, V. F. Baker, Metabolism 9, 556 (1960).