SYNTHESIS OF HUMAN CONNECTING PEPTIDE DERIVATIVES AND THEIR IMMUNOLOGICAL PROPERTIES

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SUMMARY Synthesis of human C-peptide (human proinsulin$_{33-63}$) (I), [64-formyllysine]-human proinsulin$_{31-65}$ (II) and [41-glutamic acid, 43-glutamine, 64-formyllysine]-human proinsulin$_{31-65}$ (III) are described. Immunological reactivities of these synthetic polypeptides were examined using a guinea pig antiserum to the natural human C-peptide conjugated with rabbit serum albumin. I, II and the natural human C-peptide displaced ^{125}I-tyrosylated natural human C-peptide from the antibody almost identically, while III failed to completely displace the labeled peptide.

The isolation and structural determination of the human C-peptide (1, 2, 3) led us to undertake systematic studies on the characterization of its immunological features by means of highly purified synthetic polypeptides related to the connecting peptide segment. The polypeptide chain of human proinsulin was proposed to be ordered as: NH_2-B chain·Arg·Arg· C-peptide· Lys·Arg·A chain-COOH (1).

Amino acid sequence
of the connecting peptide segment of human proinsulin

31	32	33	34	35	36	37	38	39	40
B chain-Arg-	Arg-	Glu-	Ala-	Glu-	Asp-	Leu-	Gln-	Val-	Gly-

41	42	43	44	45	46	47	48	49	50	51	52	53	54
Gln-	Val-	Glu-	Leu-	Gly-	Gly-	Gly-	Pro-	Gly-	Ala-	Gly-	Ser-	Leu-	Gln-

55	56	57	58	59	60	61	62	63	64	65	
Pro-	Leu-	Ala-	Leu-	Glu-	Gly-	Ser-	Leu-	Gln-	Lys-	Arg-	A chain

This communication provides the syntheses of the human C-peptide (human proinsulin$_{33-63}$) (I) and [64-formyllysine]-human proinsulin$_{31-65}$

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(II), based on the amino acid sequence of the human connecting peptide segment proposed by Oyer et al. (2). Synthesis of [41-glutamic acid, 43-glutamine, 64-formyllysine]-human proinsulin$_{31-65}$ (III), having the sequence based on an earlier publication (1), was also accomplished. The immunological properties of these synthetic polypeptides were investigated using a guinea pig antiserum directed against the natural human C-peptide rendered antigenic by conjugation with rabbit serum albumin (4). Some details of the synthetic procedures used have been presented elsewhere (5, 6). Recently, Naithani (7) and Geiger et al. (8) have also reported the syntheses of the human C-peptide and of its analog.

The synthetic strategy employed in the present investigation was similar to that successfully used in our earlier syntheses of the porcine (9) and bovine (10) connecting peptide derivatives. The construction of I, II and III was conducted exclusively by the azide fragment condensation method starting from the C-terminal tripeptide, H-Ser-Leu-Gln-OH, or pentapeptide, H-Ser-Leu-Gln-Lys(F)-Arg-OH. Protected peptide fragments used for these syntheses are listed in Chart 1, and these peptide derivatives were prepared mainly by stepwise chain elongation according to the conventional method for peptide synthesis. The tert-butyloxycarbonyl group on the hydrazide function of protected peptide intermediates was removed with anhydrous trifluoroacetic acid prior to fragment condensation. The tert-butyl ester of glutamic acid or aspartic acid was also cleaved by this procedure. Benzyloxycarbonyl and nitro protecting groups were removed by hydrogenolysis. The formyl group, which was used for protection of ε-amino group of the lysine residue in position 64, was intentionally not removed, since this protection on the penultimate lysine residue of porcine (9) or bovine (10) connecting peptide segment was shown not to disturb the

Chart 1 Synthetic Protected Peptide Fragments

1. Z-Arg(NO$_2$)^{31}-Arg-Glu-Ala-Glu(OBu^t)-Asp(OBu^t)-Leu-Gln^{38}-NHNH-Boc
2. Z-Arg(NO$_2$)^{31}-Arg-Glu-Ala-Glu(OBu^t)-Asp(OBu^t)-Leu-Gln-Val-Gly^{40}-NHNH-Boc
3. Z-Glu-Ala-Glu(OBu^t)^{33}-Asp(OBu^t)-Leu-Gln^{38}-NHNH-Boc
4. Z-Val-Gly-Gln-Val-Glu(OBu^t)^{39}-Leu-Gly^{45}-NHNH-Boc
5. Z-Glu(OBu^t)^{41}-Val-Gln-Leu-Gly-Gly-Gly-Pro-Gly-Ala-Gly^{51}-NHNH-Boc
6. Z-Gly-Gly-Pro-Gly-Ala-Gly^{51}-NHNH-Boc^{46}
7. Z-Ser-Leu-Gln-Pro-Leu-Ala-Leu-Glu(OBu^t)^{52}-Gly^{60}-NHNH-Boc
8. Z-Ser-Leu-Gln-Lys(F)^{61}-Arg^{65}-OH
9. Z-Ser-Leu-Gln^{63}-OH^{61}

F = formyl

immuno-reactivity. The final products were purified extensively by CM-Sephadex chromatography and gel filtration on Sephadex G-25. Each of the products behaved as a homogeneous component on tlc when detected by the chlorine-tolidine, Sakaguchi or ninhydrin reagent as well as on polyacrylamide gel electrophoresis. Their acid hydrolysates contained the constituent amino acids in theoretical ratios. Table I indicates the chemical and physical properties of I, II, III and other fragments prepared in the present investigation. Their detailed syntheses will be described elsewhere.

Immunoassay of the synthetic polypeptides was carried out according to the method of Melani et al. (4). The double-antibody method was employed

Table I Characterization of Synthetic Polypeptides Related to Human Connecting Peptide Segment

Human proinsulin$_{33-63}$ (Human C-peptide) (I)

$[\alpha]_D^{20}$ -103.2° (c 1.00, 50% AcOH); Rf^I 0.14, Rf^{II} 0.46. a)

Amino acid composition of acid hydrolysate b): $Asp_{0.92}Ser_{1.80}Glu_{7.90}Pro_{2.01}Gly_{7.04}Ala_{3.05}Val_{2.03}Leu_{6.05}NH_3{}_{4.52}$.

[64-Formyl-Lys]-Human proinsulin$_{31-65}$ (II)

$[\alpha]_D^{29}$ -96.8° (c 0.58, 10% AcOH); Rf^I 0.11, Rf^{II} 0.35.

Amino acid composition of acid hydrolysate: $Asp_{1.04}Ser_{1.78}Glu_{7.88}Pro_{2.05}Gly_{6.84}Ala_{3.07}Val_{2.06}Leu_{6.04}Lys_{0.98}Arg_{3.03}NH_3{}_{4.28}$.

[41-Glu, 43-Gln, 64-formyl-Lys]-Human proinsulin$_{31-65}$ (III)

$[\alpha]_D^{20}$ -86.0° (c 1.07, 10% AcOH); Rf^I 0.11, Rf^{II} 0.37.

Amino acid composition of acid hydrolysate: $Asp_{1.01}Ser_{1.75}Glu_{8.11}Pro_{2.13}Gly_{6.86}Ala_{3.02}Val_{1.95}Leu_{5.92}Lys_{0.95}Arg_{2.80}NH_3{}_{4.38}$.

Human Proinsulin$_{52-63}$

$[\alpha]_D^{30}$ -85.3° (c 0.50, 10% AcOH); Rf^I 0.34, Rf^{II} 0.62.

Amino acid composition of acid hydrolysate: $Ser_{1.81}Glu_{3.02}Pro_{1.04}Gly_{0.97}Ala_{0.99}Leu_{3.98}NH_3{}_{2.21}$.

[64-Formyl-Lys]-Human proinsulin$_{52-65}$

$[\alpha]_D^{29}$ -86.7° (c 1.08, 10% AcOH); Rf^I 0.15, Rf^{II} 0.51.

Amino acid composition of acid hydrolysate: $Ser_{1.79}Glu_{3.05}Pro_{0.91}Gly_{0.94}Ala_{0.97}Leu_{4.06}Lys_{1.03}Arg_{1.05}NH_3{}_{2.41}$.

a) Rf^I and Rf^{II} values refer to the solvent systems 1-BuOH-AcOH-H_2O (4 : 1 : 5) (upper layer) and 1-BuOH-pyridine-AcOH-H_2O (30 : 20 : 6 : 24), respectively.

b) Acid hydrolysis was performed at 110° for 48 hr in a sealed tube.

using ^{125}I-tyrosylated natural human C-peptide as tracer and a guinea pig antiserum to natural C-peptide conjugated with rabbit serum albumin. As shown in Fig. 1, the cross-reactivities of I and II were approximately

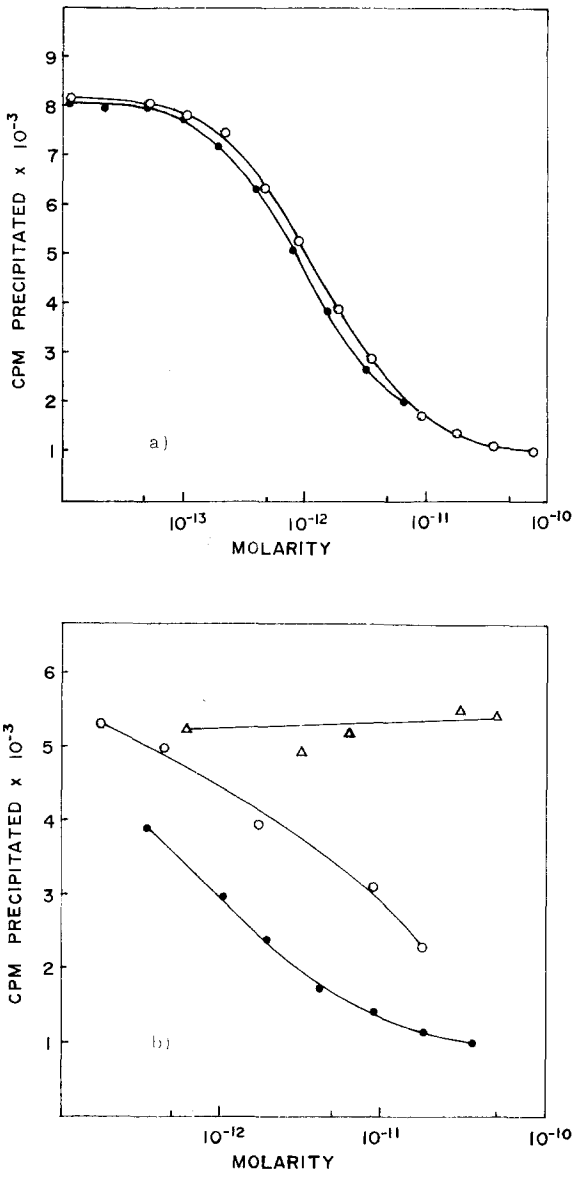

Fig. 1. Double-antibody immunoassays using ^{125}I-tyrosylated natural human C-peptide as tracer and guinea pig antiserum to natural C-peptide conjugated with rabbit serum albumin. a) (•) natural human C-peptide: (o) synthetic [64-formyl-Lys]-human proinsulin$_{31-65}$ (II). b) (•) natural human C-peptide: (o) synthetic [41-Glu, 43-Gln, 64-formyl-Lys]-human proinsulin$_{31-65}$ (III): (▲) synthetic [64-formyl-Lys]-human proinsulin$_{52-65}$.

85-90% of that of the natural C-peptide when expressed on molar basis, and parallel displacement curves were obtained. II was incubated with TPCK-treated trypsin for 24 hr at 37° to remove the N-terminal Arg-Arg

sequence. The resulting polypeptide ([64-formyllysine]-human proinsulin$_{33-65}$) also showed a displacement curve identical to that of II. On the other hand, III failed to completely displace the tracer. Because of non-parallelism of the displacement curve of III to that of the natural C-peptide, the cross-reactivity of this peptide could not be evaluated accurately. The C-terminal tetradecapeptide, [64-formyllysine]-human proinsulin$_{52-65}$, did not displace the standard at all.

These findings indicate that the synthetic human C-peptide has nearly identical immuno-reactivity to the natural peptide and that the four basic residues at the amino and carboxyl termini in the connecting peptide segment do not disturb the cross-reaction with human C-peptide antiserum. The distinct decrease in immunological reactivity of III seems to indicate that the $\overset{41}{\text{Gln}}$-Val-$\overset{43}{\text{Glu}}$ sequence contributes to the interaction between the antigen and the particular antiserum used. In addition, the lack of immunological activity of the C-terminal tetradecapeptide demonstrates that the major antigenic determinants are not located in this region of the connecting peptide.

Tager et al. (11) have recently reported that 15 to 25% of their rat C-peptide preparation appears as the amino terminal 22-residue fragment of the C-peptide. Although similar heterogeneity has not yet been demonstrated in the case of the natural human C-peptide, the slight discrepancy observed in the immuno-reactivity of synthetic polypeptides I and II from that of the natural C-peptide could possibly arise, at least in part, from the presence of small amounts of immunologically active fragment(s) of the C-peptide in the natural human C-peptide preparations. Further investigation of the antigenic properties of the human C-peptide by means of the synthetic approach is under way.

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