THE AMINO ACID SEQUENCE OF HUMAN GLUCAGON

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1. Introduction

The amino acid sequence of bovine glucagon has been found to be the same as that reported for porcine glucagon [1, 2]. Human glucagon has been isolated and crystallized [3]. The crystal form and the amino acid composition of human glucagon proved to be identical with those of porcine and bovine glucagon.

The present study reports on the amino acid sequence determination of human glucagon and on abnormal cleavage of histidine observed during Edman degradation.

2. Experimental

2.1. Glucagon

The crystalline human glucagon was prepared by the method previously described for rat glucagon [3], and was shown by polyacrylamide electrophoresis at pH 4.5 and 8.7 to be homogeneous. Porcine glucagon (Novo Research Institute, Copenhagen, Denmark) was used for preliminary experiments.

2.2. Amino acid analysis

Amino acid analysis of the human glucagon was carried out after hydrolysis for 24 hr in 6 N HCl at 110° in evacuated and sealed vials.

2.3 Edman degradation

The automatic Edman degradations were carried out in a Beckman sequencer, Model 890B. The reagents and solvents used for the sequencing were as follows: phenyl isothiocyanate, Quadrol-trifluoroacetic acid buffer, *N,N*-dimethylallylamine-trifluoroacetic acid buffer in pyridine/water, heptafluorobutyric acid, heptane, benzene, ethyl acetate, and 1-chlorobutane (sequencer grade, Beckman Instruments, Palo Alto, California). *N,N*-dimethylallylamine (DMAA) and 1-propanol (sequanal grade, Pierce, Rockford, Illinois). *N*-methylmorpholine (for synthesis, Merck, Darmstadt, W. Germany) was distilled over ninhydrin before use.

1,4-Butanedithiol (pract. grade, Fluka, Buchs, Switzerland) was added without further purification to the 1-chlorobutane in a concentration of 50 µl per 950 ml in order to improve the yield of serine [4]. Before use, the Quadrol-trifluoroacetic acid buffer was diluted 5 times with 1-propanol/glass distilled water 3:2(v/v) in order to obtain a 0.2 M Quadrol buffer [5]. A 1 M solution of DMAA in 1-propanol/glass distilled water 3:2(v/v) was used without pH adjustment [5]. A 1 M solution of *N*-methylmorpholine in 1-propanol/glass distilled water 3:2(v/v) was also used without pH adjustment.

2.4 Conversion and identification

Conversions of the thiazolinones to phenylthio-

hydantoins (PTH) and their subsequent extraction were carried out according to Edman and Begg [6]. Most of the PTH-amino acids were identified by gas chromatography. For detection of PTH-arginine the phenanthrenequinone test [7] was used, and amino acid analysis after hydrolysis in 6 N HCl at 130° for 24 hr in evacuated and sealed vials. The identity of some of the other PTH-amino acids was confirmed by amino acid analysis. PTH-tryptophan was detected by thin-layer chromatography in the Edman H-system [8] followed by spraying with Ehrlich's reagent. The C-terminal amino acid was identified as the free amino acid remaining in the reaction cell by amino acid analysis without previous hydrolysis.

2.5 Enzymatic digestion

Digestion with carboxypeptidase-A (Sigma, St. Louis, Missouri) was performed in 0.2 M *N*-methylmorpholine-acetic acid buffer, pH 8.0, at 37° for 20 min. The enzyme/substrate ratio was approx. 1:14 (mole/mole).

2.6. Cleavage

Cleavage with *N*-bromosuccinimide [9] was carried out after acetylation in acetic anhydride/methanol 1:4 (v/v).

Table 1

Amino acid analysis of human glucagon, calculated relative to Asp which is set to 4.0. The values in brackets are the nearest integer. Besides the tabulated amino acids 4.7 moles of ammonia, 0.15 moles of isoleucine, and traces of proline, cysteine and cystine were found.

	Amino acid residues per molecule		
Amino acid	Human glucagon		Porcine glucagon *
Trp	–	(–) **	1
Lys	1.1	(1)	1
His	0.9	(1)	1
Arg	2.2	(2)	2
Asp	4.0	(4)	4
Thr	2.9	(3)	3
Ser	3.7	(4)	4
Glu	3.3	(3)	3
Gly	1.2	(1)	1
Ala	1.2	(1)	1
Val	1.2	(1)	1
Met	0.9	(1)	1
Leu	2.2	(2)	2
Tyr	2.0	(2)	2
Phe	1.9	(2)	2

* According to Bromer et al. [2].

** Only traces, but typical degradation products of tryptophan.

3. Results and discussion

3.1 Amino acid analysis

The results of the amino acid analysis of the human glucagon are given in table 1. Tryptophan was destroyed during the hydrolysis and appeared only in traces; however, typical tryptophan degradation products were seen. The values for threonine and serine are corrected for destruction during the hydrolysis according to results obtained in experiments with hen egg-white lysozyme. The value for ammonia is corrected for the amount of ammonia present in the hydrochloric acid and the buffers used. It appears that the amino acid composition of human glucagon is identical with that of porcine glucagon.

3.2. Abnormal cleavage of histidine during Edman degradation

When Edman degradation of porcine glucagon was carried out using the volatile DMAA buffer and procedures for reducing the loss of peptide [10], both residue no.1, histidine, as well as no.2, serine, were found after the first degradation cycle. In the following cycle the remainder of residue no. 2 was found together with a portion of no.3, and so on, with an overlap of 30–50%.

An abnormal degradation pattern of histidine has also been reported by Blombäck et al. [11] and Schroeder [12] while it has not been observed by Edman [8]. As the two former authors used volatile buffers and the latter used the non-volatile Quadrol buffer, this would indicate that there is a relation between the abnormal degradation of the histidine residue and the use of a volatile buffer. Experiments showed that "pH" decreased to about 6 during the drying of the DMAA buffer at the end of the coupling stage, and that a histidine derivative could be extracted from the reaction cell before the cleavage with heptafluorobutyric acid. After conversion of the derivative, PTH-histidine could be identified.

It was shown that the overlap caused by the abnormal cleavage of histidine may be reduced by an extraction with benzene immediately after the end of the coupling stage, before the drying takes place, but this extraction will spread the film more than acceptable. The overlap could be completely prevented if the coupling was performed in a 1 M solution of *N*-methylmorpholine in 1-propanol/water 3:2 (v/v) and followed by a slight evaporation. The *N*-methylmorpholine has a boiling point at 115–116°. The "pH" of the solution was about 9.5 and was not lower than 9.2 during the drying. It was shown that the abnormal cleavage of histidine could also be completely prevented by using a 0.2 M Quadrol buffer. This result is in agreement with the observations of Edman [6, 8]. It was not possible to bring about the abnormal cleavage by lowering the "pH" in the Quadrol buffer. This might indicate that the decrease in "pH" is not the only factor responsible for the abnormal cleavage, although experiments with volatile buffers seemed to indicate a certain correlation between the magnitude of the "pH" decrease and the abnormal cleavage.

It was shown that 1.5–2 μmole of porcine glucagon could be degraded completely in the sequencer by using the 0.2 M Quadrol buffer and double cleavage for the first 17 degradation steps, until the second arginine. The DMAA buffer was used and single cleavage carried out for the remaining degradation steps. Overlap due to incomplete cleavage was thereby kept at a minimal level for the first 20 steps. Later on it was found that double cleavage, if performed carefully, could be used in some steps between step 18 and 25. This strategy was used for the sequence determination of the human glucagon.

3.3. Sequence determination of human glucagon

The complete sequence of human glucagon is shown in fig. 1 and also the strategy used and the methods of identification. 5.1 mg of human glucagon, equivalent to 1.5 μmole, was used for the sequencing. After completion of step 17 the peptide film was dried overnight in order to ensure better adsorption to the reaction cell. The program was then changed to the use of DMAA buffer. The film was dried overnight after steps 21 and 25. After step 23 the DMAA buffer was changed to a1M DMAA solution in 1-propanol/water because the former gave rise to extraneous peaks which interfered with the gas chromatographic identification. In the 28th cycle the benzene wash was omitted. The degradation was stopped at the end of the 28th cycle and the C-terminal was identified from an aliquot of the remaining material in the reaction cell.

The gas chromatographic identification failed at steps 22 and 28 due to interfering peaks and at step 25 because there was no distinct peak. The phenylalanine at step 22, however, was clearly identified by amino acid analysis.

The residue at step 25 which, by analogy with porcine glucagon, was expected to be tryptophan, was possibly almost destroyed during the degradation. Thin-layer chromatography of step 25 followed by spraying with Ehrlich's reagent revealed a faint violet spot at the position of tryptophan. Additional information was obtained by carboxypeptidase-A digestion of another aliquot of the remaining material after the 28th degradation cycle. The sample residue was present in quantities indicating that some blocking had occurred during the degradation, for which reason it was expected to contain not only the free C-terminal amino acid but also some peptide. Amino acid analysis of the digest revealed the last 5 amino acids from the C-terminal (concentrations in nmoles): Trp(2.0), Leu(2.1), Met(7.7), Asn/Gln or Ser(11.8), and Thr(12.3). The relative concentrations indicate this sequence as the amino acid found in the highest amount is the C-terminal residue. Asn, Gln, and Ser are not separated by the technique used. The presence of tryptophan was confirmed by this experiment, which also indicated its position relative to the C-terminal. The final part of the residue from the reaction cell was cleaved by treatment with *N*-bromosuccinimide after previous acetylation and applied to the sequencer again. After the first degradation cycle, leucine was identified, which lends support to the finding that tryptophan was present at no. 25. Two more degradation cycles were performed but no futher residues could be identified.

The identity of residue no.28 could be deduced from the carboxypeptidase-A experiment as being asparagine. The amino acid analysis of the digest indicated one or more residues of asparagine, glutamine, or serine. All of the serines and glutamines, however, had already been accounted for. The last

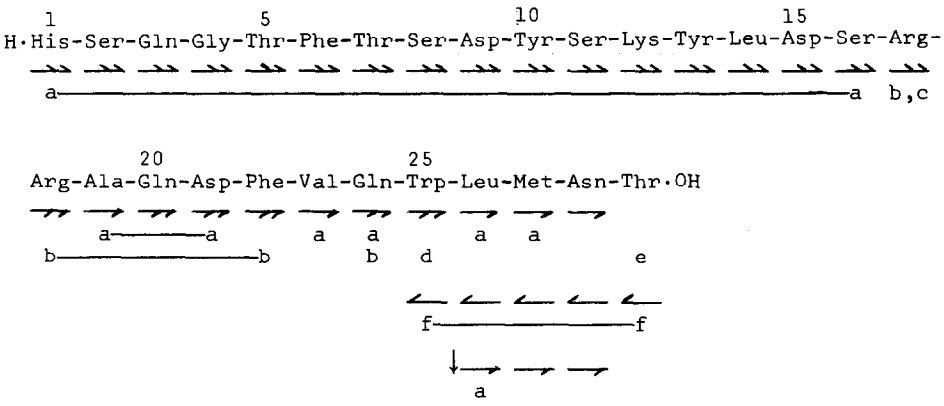

Fig. 1. The complete amino acid sequence of human glucagon.

→ Edman degradation, Quadrol program, double cleavage.
→ Edman degradation, DMAA program, double cleavage.
→ Edman degradation, DMAA program, single cleavage.
← Carboxypeptidase-A digestion.
↓ Cleavage by N-bromosuccinimide.
a : The PTH-derivative identified by gas chromatography.
b : The PTH-derivative hydrolyzed and identified by amino acid analysis, Gln is identified as Glu.
c : Identified by the phenanthrenequinone test.
d : Indicated by thin-layer chromatography and Ehrlich's reagent.
e : The free C-terminal amino acid remaining in the reaction cell identified by amino acid analysis.
f : Identified by amino acid analysis of the carboxypeptidase-A digest.

of the 4 serine residues was identified at step 16 and consequently could not interfere. The last of the 3 glutamine residues was found at step 24 and would at the most be expected to contribute to the peak of Asn/Gln with some 2 nmoles. Thus the remaining part of the peak could only be asparagine. The 3 glutamines and 1 asparagine found, giving 4 amide groups, agree well with the 4.7 mole of ammonia shown by the amino acid analysis.

The amino acid sequence found for human glucagon thus proved to be identical with that reported for porcine and bovine glucagon [1, 2]. So far, no variations have been found in the amino acid sequence of mammalian glucagons. This may indicate that preservation of the primary structure is essential for the biological activities of glucagon. The fact that the crystal form and the amino acid composition of rat and rabbit glucagon [3, 13] are found to be identical with those of bovine, human, and porcine glucagon supports this assumption.

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References

[1] W.W. Bromer, M.E. Boucher, and J.E. Koffenberger, Jr., J. Biol. Chem. 246 (1971) 2822.
[2] W.W. Bromer, L.G. Sinn, and O.K. Behrens, J.Am. Chem. Soc. 79 (1957) 2807.
[3] F. Sundby and J. Markussen, Horm. Metab. Res. 3 (1971) 184.
[4] M.J. Gordon, personal communication (1970).
[5] H.D. Niall, J. Agr. Food Chem. 19 (1971) 638.
[6] P. Edman and G. Begg, European J. Biochem. 1 (1967) 80.

[7] S. Yamada and H.A. Itano, Biochim. Biophys. Acta 130 (1966) 538.
[8] P. Edman in : Protein Sequence Determination, ed S.B. Needleman (Springer-Verlag, Berlin, Heidelberg, New York, 1970) p. 211.
[9] L.K. Ramachandran and B. Witkop in: Methods in Enzymology Vol. XI, ed. C.H.W. Hirs (Academic Press, New York and London, 1967) p. 283.
[10] R. Sauer, H.D. Niall and J.T. Potts, Jr., Federation Proc. 29 (1970) 728.
[11] B. Blombäck, M. Blombäck, B. Hessel and S. Iwanaga, Nature 215 (1967) 1445.
[12] W.A. Schroeder, in: Methods in Enzymology, Vol. XI, ed. C.H.W. Hirs (Academic Press, New York and London, 1967) p. 445.
[13] F. Sundby and J. Markussen, Horm. Metab. Res., in press.