CRYSTALLIZATION* AND AMINO ACID SEQUENCE** OF DUCK GLUCAGON

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1. Introduction

The amino acid sequence of glucagon from pig, ox and man was found to be identical [1, 2]. Glucagon has recently been isolated from turkey pancreas and crystallized. The only difference compared to sequenced glucagon from mammalian species seemed to be in position 28 where a serine residue is present instead of an asparagine residue. The immunological properties were found to differ markedly from those of pig glucagon [3].

The present study reports on the isolation, crystallization and amino acid sequence determination of duck glucagon.

2. Experimental

2.1. *Isolation and crystallization*

The ventral, dorsal and splenic portions of pancreases from Peking ducks were removed within 10–15 min after the birds were killed. The pancreases were frozen immediately and stored on dry ice until used. Extraction, preparation of crude glucagon and localization of the glucagon-containing fractions during purification were performed as previously described for turkey glucagon [3], while the method of purification and crystallization was that described for rat glucagon [4].

2.2. *Electrophoresis*

Polyacrylamide gel electrophoresis of the glucagon was performed at two pH values using 0.5 × 7 cm tubes. At pH 8.7 the buffer system was that of Ornstein [5] using 7.5% acrylamide in the lower gel. At pH 4.5 the method of Reisfeld [6] was used with 10% acrylamide in the lower gel. In both cases 8 M urea was added to the solutions to avoid precipitation of proteins during the run. Staining was carried out with 1% amido black in 7% acetic acid, and the gels were destained by washing with 7% acetic acid.

2.3. *Amino acid analysis*

Amino acid analysis of the glucagon was carried out after hydrolysis according to Moore and Stein [7] and Matsubara and Sasaki [8].

2.4. *Edman degradation*

Edman degradation was carried out in a Beckman sequencer, Model 890D, using procedures earlier described [2]. Reagents and solvents used were: phenyl isothiocyanate, M Quadrol-trifluoroacetic acid buffer in 1-propanol/water, heptafluorobutyric acid, heptane, benzene, ethyl acetate, and 1-chlorobutane (sequencer grade, Beckman Instruments, Palo Alto, Calif.); 1-propanol (sequanal grade, Pierce, Rockford, Ill.); *N*-methylmorpholine (for synthesis, Merck, Darmstadt, W. Germany), distilled over ninhydrin before use, and trifluoroacetic acid (analytical grade, Merck, Darmstadt, W. Germany).

1,4-Butanedithiol (pract. grade, Flucka, Buchs, Switzerland) was added to the 1-chlorobutane at a concentration of 50 μl per 950 ml.

0.2 M Quadrol buffer was prepared from the M buffer by 5-fold dilution with 1-propanol/glass distilled water 3:2 (v/v) [9]. A molar solution of *N*-methylmorpholine in 1-propanol/glass distilled water 3:2 (v/v) adjusted to pH 9.4 (glass electrode) with trifluoroacetic acid served as volatile buffer.

2.5. *Identification*

The phenylthiohydantoin (PTH)-amino acids were identified by gas chromatography except for PTH-arginine, which was hydrolyzed in 6 N HCl at 130° for 24 hr in evacuated and sealed vials and identified as arginine by amino acid analysis. PTH-tryptophan was indicated by thin-layer chromatography in the Edman H-system [10], followed by spraying with Ehrlich's reagent. The free C-terminal amino acid remaining in the reaction cell after completion of the 28th degradation cycle was identified by amino acid analysis.

2.6. *Digestion with carboxypeptidase-A*

1.10 mg of the intact duck glucagon was digested with 0.10 mg of carboxypeptidase-A at 37° in 500 μl of 0.2 M *N*-methylmorpholine–acetic acid buffer, pH 8.0. 100 μl aliquots were withdrawn after 3, 6, 9, 12 and 20 min of digestion and added to 1.00 ml of 0.2 M sodium citrate buffer, pH 2.2.

One-half of the material remaining in the reaction cell after the 28th degradation cycle was digested with 0.10 mg of carboxypeptidase-A at 37° in 430 μl of *N*-methylmorpholine buffer for 10 min. This digestion was terminated by addition of 670 μl of 0.1 N HCl.

500 μl aliquots of the resulting solutions were used for amino acid analysis. Neutral and acidic amino acids were analyzed using lithium citrate buffers [11]. Tryptophan was determined using sodium citrate buffer on the 5 cm column.

3. Results and discussion

3.1. *Crystallization*

Just like pig, ox, rat, human, rabbit and turkey glucagon, duck glucagon crystallizes as rhombedodecahedrons. The yield of crystalline glucagon was 3.74 mg per kg of pancreas.

3.2. *Electrophoresis*

Disc electrophoresis showed identical mobilities for duck and pork glucagon at both pH values. In addition to the main component, the electrophoresis at pH 8.7 showed the duck glucagon to contain two trace components moving slightly slower towards the anode than glucagon. At pH 4.5 only one (unidentified) trace component was detected in the duck glucagon, moving a little slower towards the cathode than the main component.

3.3. *Amino acid analysis*

The results of the amino acid analysis of duck glucagon are given in table 1 along with the amino acids present in human glucagon. It appears that the amino acid compositions of the two glucagons differ with respect to aspartic acid and threonine. Furthermore, the contents of ammonia indicate that only three amide groups are present in the duck glucagon.

3.4. *Sequence determination*

The complete amino acid sequence of duck glucagon is shown in fig. 1. 4.6 mg, equivalent to 1.3 μmole, was used for the sequencing. The strategy used was mainly that reported earlier [2]. Diluted Quadrol buffer was used in the first 13 steps, and *N*-methylmorpholine buffer in steps 14–28. Double cleavage was performed in steps 1–13, 15 and 22, and single cleavage in the remaining steps. The peptide film was dried overnight after steps 7, 13, 16 and 22 in order to ensure better adsorption to the reaction cell, and to facilitate conversion and identification of the labile derivatives as shortly as possible after their delivery into the fraction collector of the sequencer. The benzene wash after the coupling stage in step 28 was omitted.

Fig. 2 shows the yield at each step. The average repetitive yield is calculated to be approx. 92% based on a regression line of y on x, where y = log yield, and x = degradation cycle. Only the yields indicated by the solid circles were used for the calculation. As expected, the yield of PTH-serine was low, but the serine residues were identified unequivocally, except for the serine residue in position 28. Due to deamidation during the conversion, the sum of PTH-glutamine and PTH-glutamic acid is taken as the yield of PTH-glutamine. The yield of arginine is not corrected for losses in the hydrolytic procedure. In position 25,

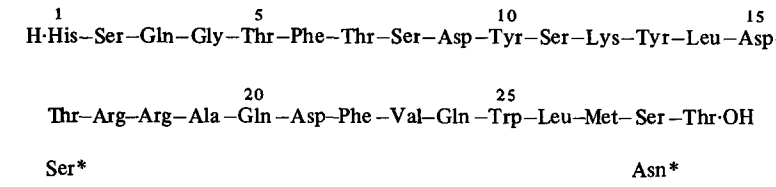

Fig. 1. The complete amino acid sequence of duck glucagon. The sequence was found to differ from that of human glucagon only at positions 16 and 28. The amino acid residues found at these positions in human glucagon are indicated with an *.

Table 1
Amino acid analysis of duck glucagon.

Amino acid	Duck glucagon (a)*	(b)*	(c)	Human glucagon [2]
Trp	0.37	0.93	1	1
Lys	1.01	1.02	1	1
His	0.93	0.94	1	1
NH_4^+	3.99(d)			4.7(d)
Arg	1.98	1.96	2	2
Asp	3.00		3	4
Thr	4.03(e)		4	3
Ser	4.00(e)		4	4
Glu	3.07		3	3
Gly	1.01		1	1
Ala	0.99		1	1
Val	0.99		1	1
Met	0.97		1	1
Leu	1.98		2	2
Tyr	1.96		2	2
Phe	1.93		2	2

(a) Hydrolyzed in 6 N HCl at 110° for 24 hr [7].
(b) Hydrolyzed in 6 N HCl containing 4% mercaptoacetic acid at 110° for 24 hr [8].
(c) Nearest integer.
(d) Corrected for ammonia present in the hydrochloric acid and the buffers used.
(e) Corrected for destruction during hydrolysis according to experiments with hen egg-white lysozyme. Thr + 10%, Ser + 20%.

* Calculated relative to Asp which is set to 3.0. Besides the tabulated amino acids, traces of Pro and Ile were found.

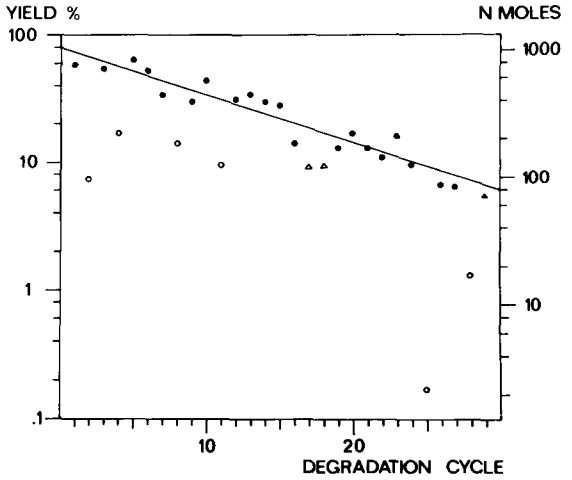

Fig. 2. Yield of PTH-amino acids in each degradation cycle. (•) Determined by gas chromatography. (○) Determined by gas chromatography but obtained in particularly low yield (PTH-Ser, -Gly, -Trp). (▲) Determined by amino acid analysis. (△) The PTH-derivative hydrolyzed and determined by amino acid analysis.

PTH-tryptophan was identified by gas chromatography, though in low yield. Thin-layer chromatography of steps 23–28 followed by spraying with Ehrlich's reagent also indicated a tryptophan residue at position 25. Apparently, the tryptophan residue was destroyed to a great extent during the degradation. This was also observed during the degradation of human glucagon [2], and similar observations have been reported (see e.g. [12,13]).

Figs. 3 and 4 show gas chromatograms from steps 26 and 27. Besides the overlap from the preceding amino acid, which does not disturb the sequential interpretation, the chromatograms are almost free of background. Due to the omitted benzene wash, the background was so pronounced in the 28th degradation cycle that, unfortunately, an unequivocal identification by gas chromatography was not possible. The C-terminal amino acid was, however, unambiguously identified as threonine. The analysis showed the presence of 74 nmoles of threonine. Except from a

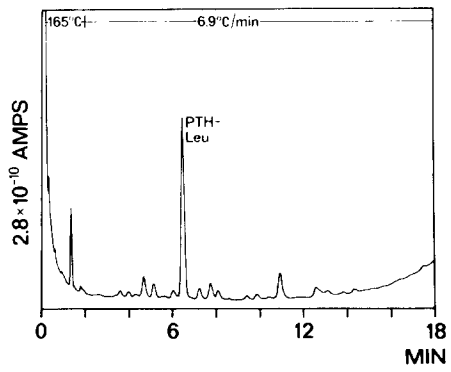

Fig. 3. Gas chromatogram from step 26. Conditions for the gas chromatography: Glass columns 4 feet × 2 mm i.d. packed with "SP 400" (Beckman Instruments). Helium flow 80 ccm/min. Flame ionization detector. Beckman gas chromatograph, Model GC-45. Aliquot of 4% of the sample was injected.

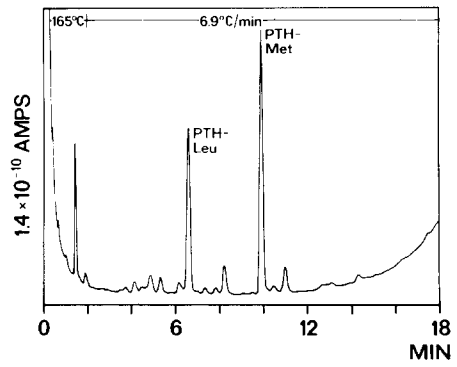

Fig. 4. Gas chromatogram from step 27. Conditions as decribed for step 26.

trace of serine no other free amino acid could be detected. Consequently, a serine residue must occupy position 28.

The sequence near the C-terminal was verified by the carboxypeptidase digestion experiments. Digestion of the intact molecule allowed deduction of the following C-terminal sequence: –Trp–Leu–(Met,Ser)–Thr·OH.

The result obtained after the digestion of residual material from the reaction cell, was even more favourable since the decrease in yield in this experiment was much more pronounced due to partial blocking and/or asynchronism. The amounts found were, in nmoles: Gln (1.5), Trp (1), Leu (5), Met (16), Ser (26), Thr (67), originating from approx. 1/4 of the total amount remaining in the reaction cell. Due to the above mentioned destruction of tryptophyl during the Edman degradation, the sequence could not be deduced from these figures beyond position 26. The same technique was used in the sequence determination of human glucagon [2], and it seems to be a most useful method for confirming the sequence near the C-terminal if the amino acids are released by the carboxypeptidase used.

The sequence was found to be identical with that of human glucagon up to residue no. 16. At this position, duck glucagon contains a threonine residue instead of the serine residue present in human glucagon. At position 28, a serine residue was found instead of an asparagine residue. Three glutamine residues were identified in accordance with the three amide groups indicated by the amino acid analysis.

The immunological properties of duck and turkey glucagon were identical but differed significantly from those of pork glucagon when analyzed by radioimmunoassay using two different anti-pig glucagon rabbit sera [3].

The conservation of the primary structure of glucagon so far observed in mammalians is not found among birds. Turkey glucagon differs from human glucagon at position 28, where an asparagine residue has been substituted by a serine residue. Duck glucagon has the same sequence change at position 28 as well as a substitution at position 16 where a threonine residue has replaced a serine residue.

It remains to be established whether these substitutions influence the biological properties of the avian glucagons relative to each other and relative to mammalian glucagons.

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