RADIATION STABILITY OF ANGIOTENSIN II-AMIDE AND OF ITS TRITIATED DERIVATIVES.

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Summary

The gamma-radiolysis of aqueous solutions of the peptide hormone angiotensin II-amide and the beta-autoradiolysis of its tritiated preparations were studied. The radiation loss and the decrease of biological activity of angiotensin II-amide was evaluated. The main fractions of radiation products were separated and compared with the products formed during the storage of tritiated angiotensin II-amide. The principal radiation processes taking place during the irradiation of angiotensin II-amide were discussed.

Key Words: angiotensin II-amide, aqueous solution, gamma-irradiation, separation, [^{3}H] angiotensin II-amide, autoradiolysis

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INTRODUCTION

During the irradiation of some biologically active oligopeptides[1-3] it was found that their biological activity decreased in consequence of the changes taking place in their primary and secondary structure, especially if the radiolysis was carried out in aqueous solutions.

For a more detailed study of the radiation changes of peptides we used in this investigation the hormone angiotensin II-amide as a model. This octapeptide, prepared synthetically as H-Asn-Arg-Val-Tyr-Val-His-Pro-Phe-OH, distinctly increases blood pressure. The study of its metabolism has recently been the subject of research in a number of biological laboratories. At the same time preparations of angiotensin labelled with radioactive isotopes are widely used today and applied in medical diagnosis. Therefore we considered that information obtained on the radiation stability of this peptide might contribute not only to the extension of present knowledge on the radiolysis of higher peptides, but also to the elucidation of some problems connected with autoradiolysis of labelled preparations of angiotensin during their storage and application.

EXPERIMENTAL

For the external irradiation with gamma-rays a synthetic preparation of hypertensin CIBA (Val^5-angiotensin II-asp-beta-amide) was used from which 0.5% solutions were prepared in redistilled water ($4.24.10^{-3}$M). Before irradiation the solutions were degassed throughly with oxygen-free nitrogen.

For irradiations, a ^{60}Co source of an intensity of 46.8 krad/h was used. The doses applied ranged from 0.3 to 4 Mrad. The decrease in biological activity of the irradiated samples of angiotensin II was evaluated from the difference in pressor response elicited

in rats by intravenous administration of an equal volume of the non-irradiated angiotensin II-amide.

The separation of the radiation-transformed peptides and their characterization was carried out by the means of vertical electrophoresis on Whatman No.3 paper in pyridine-acetate buffer of pH 5.6 at 35 V/cm. The products formed were detected with ninhydrin, Sakaguchi reagent, 2,4-dinitrophenylhydrazine and Pauly´s reagent, which was also used for quantitative determination of the original angiotensin II-amide after its elution from the electrophrograms with 5% Tris-buffer.

A partial separation of angiotensin II-amide from its recombination products with a higher molecular weight and from the deamidated derivatives takes place on a column of Sephadex G 15 / 52 cm long and 1.1 cm diameter/eluted with 0.05 M phosphate buffer of pH 7.4. The fractions eluted from the column were measured with a Specord-UV-VIS spectrophotometer at 215 nm.

The enzymatic hydrolysis was carried out using pronase(Serva) and leucyl-aminopeptidase(Koch-Light) and the modification of the method elaborated by Arakawa[4] and Pfeifer[5]. The qualitative composition of amino acids was determined by uni- and bi-dimensional thin-layer chromatography on cellulose (Lucefol, Kavalier) in phenol-ethanol-water-25% NH_4OH (50:35:22:3) and n-butanol-acetic acid-water (4:1:1).

The determination of the ammonia was carried out by a microdiffusion method according to Conway[6]. The carbonyl products were determined according to Lappin[7].

When autoradiolysis was investigated,tritiated preparations of $[Tyr^{3}H]^{4}$-angiotensin II-amide of specific activity 5.24 Ci/mmol and $[Phe^{3}H]^{8}$-angiotensin II-amide of specific activity 2.75 Ci-mmol were used, synthetized by Dr.Mezö[8]. The estimation of the radioactivity on the electropherograms was carried out using the scintillation method.

RESULTS AND DISCUSSION

The decrease in the content of angiotensin II-amide and in its biological activity, the formation of carbonyl products and the liberation of ammonia in dependence on the dose of gamma-irradiation are given in Table 1.

TABLE 1

Dependence of the radiation loss and the decrease in biological activity of angiotensin II-amide, the formation of ammonia and of carbonyl substances on the irradiation dose

Dose	Decrease of biological activity	Loss of the original peptide		NH_3	>C=O products
Mrad	%	%	Number of molecules.10^{16}		
0.65	22	21	56	37	10
1.3	28	38	97	60	18
2.7	50	60	153	85	48
4.0	60	70	180	110	144

From this dependence the D/37 dose was estimated, at which the content of irradiated substance decreases to 37%; it corresponds to the value 3.1 Mrad. The initial radiation loss of angiotensin II-amide G_i/-M/, calculated therefrom is 1.3.

Two main basic fractions (I,II) and a neutral fraction (III) were isolated from the irradiated solution by paper-electrophoresis:

I. The first basic fraction with the mobility 6.5 cm corresponds to the original angiotensin II-amide.

II. The second basic fraction with the mobility 2.7 cm contains - according to the results of a separation on Sephadex G 15 and according to the chromatographic analysis of enzymatic hydroly-

zates - the following components:

asp-derivative of angiotensin II formed on radiation hydrolysis of the amide group of asparagine

derivatives of angiotensin II-amide with a decreased basic nature which are formed by radiation demage of the amino acid residues, especially histidyl residues where a cleavage of the imidazole ring takes place. The decrease of histidine content and the presence of glu, ser, gly and ala found in the hydrolyzates of this fraction correspond to the results described in the radiolysis of the free histidine and its simple peptides[9]

products with a higher molecular weight, formed by recombination of angiotensin II-amide molecules with its radiation--transformed products

III. Neutral fractions containing:

radiation products with a carbonyl group. In addition to deaminated angiotensin II-amide the another radiation products in which the formation of dialdehydes took place in consequence of the radiation cleavage of the aromatic rings in the tyrosine and phenylalanine residues also belong to this group. A similar result was observed in the case of the irradiation of free tyrosine[10] or phenylalanine and its dipeptides[11]

products of recombination of transformed molecules of angiotensin II-amide among themselves.

The presence of the mentioned transformed products of angiotensin II-amide was also detected in its tritiated preparations [Tyr^{3}H]^{4} and [Phe^{3}H]^{8} stored for a long time. Table 2 presents the results obtained on electrophoresis of these substances stored for $\geq$11 months under various conditions and at various radiochemical concentrations. Even though it is necessary to reckon with the

TABLE 2

Stability of tritiated preparations of angiotensin II-amide in dependence on the conditions of storage

Conditions	Prep.	Spec. act. Ci/mmol	Rad. conc. mCi/ml	Time months	Content of the original peptide /I/ and the fractions of radiation products in % of total radioactivity of irradiated solution			
					I	II	III	IV
H_2O+2% EtOH at 4°C	[Tyr ^{3}H] ^{4}	5.24	0.49	12	34	38	15	12
H_2O+2% EtOH +1% AcOH at 4°C	[Phe ^{3}H] ^{8}	2.75	0.33	11	26	28	10	30
50% EtOH at -20°C	[Phe ^{3}H] ^{8}	2.75	0.33	11	22	51	13	6.7
90% MeOH at -20°C	[Phe ^{3}H] ^{8}	2.75	0.002	11	50	not evaluated		
90% MeOH at -20°C	[Phe ^{3}H] ^{8}	2.75	0.003	11	48	20	12	-
90% MeOH at -20°C	[Phe ^{3}H] ^{8}	2.75	0.005	11	50	22	9	-

The aliquots of fractions I,II and III were evaluated by measuring the radioactivity of the electrophorograms of the ^{3}H-preparations. The numbering of the fractions is similar to that of the inactive preparation of angiotensin II-amide irradiated with an external source (see text). Fraction IV was calculated from the difference of radioactivity of the evaporated and non-evaporated samples of ^{3}H angiotensin II-amide preparations; it corresponds to the volatile tritiated products which are formed on storage of these ^{3}H preparations.

inherent high chemical instability of the solutions of angiotensin II-amide[5] when the mentioned results are evaluated, the considerable differences in its losses observed in stored ^{3}H preparations indicate that the share of autoradiolysis is distinct especially in samples with a higher radiochemical concentration.

The solutions stored in methanol at considerable dilution display a higher radiation stability. With these extremely diluted solutions complications arise, of course, connected with the absorption of matter on the walls of the vials in which the samples were stored. It was found that individual fractions of the radiation products, which are formed in the labelled preparations, are absorbed on the vial walls non-uniformly. This was shown on the basis of the results of the electrophoresis of the original solution in which the preparation was stored (90% MeOH) and dilute acetic acid used for rinsing the vial. In the first case the ratio of the radioactivity measured in fractions I, II and III corresponds to 48, 20 and 12%, while in the second case to 52, 13 and 10%, respectively. Hence, the absorption of the original angiotensin II-amide is higher than the absorption of fraction II. Therefore this circumstance must be reckoned with during the performance and evaluation of the control analyses of labelled preparations.

From the content of free NH_3 and >C=O groups in the irradiated solutions of angiotensin II-amide (Table 1) and from the results of the electrophoretic and chromatographic (Sephadex) results it may be concluded that in the solutions of this peptide, both irradiated with an external gamma-radiation source and with internal sources of beta-radiation, the following five radiation processes are the principal ones:

1. Deamination of the NH_2-terminal group.

2. Radiation hydrolysis of the amide group of asparagine at which the formation of asp-derivative of angiotensin II takes place.
3. Radiation transformation of amino acid residues, especially of histidyl residues.

All these reactions take place with the liberation of ammonia and participate in the total loss of original molecules of angiotensin II-amide by about 60%.

The high increase in the yield of the >C=O groups, observed in the solution of the angiotensin II-amide that was irradiated with the highest dose, indicates that at these irradiation dosed the formation of dialdehydes evidently takes place in consequence of the radiation cleavage of the aromatic rings in tyrosine and phenylalanine residues.

4. Radiation recombinations of the molecules of angiotensin II--amide at which recombination products with a higher molecular weight are formed. The total proportion of the recombination products increases with the radiation dose. At the highest dose used in this study the fraction of the radiation recombination products is about 18% of the original amount of the polypeptide. According to the electrophoretic properties of the recombinates formed it may be judged that the recombination reactions occur mainly either between molecules of angiotensin II-amide and its radiation-transformed derivatives, or between these derivatives among themselves.

5. The radiation cleavage of the peptide bond according to the reaction of Garrison[12] with formation of lower molecular weight products, containing partly amide and partly carbonyl groups, takes place in the oxygen-free solutions of angiotensin II-amide only to a very limited extent. This was indicated by the results of the separation of the irradiated solution on a Sephadex G 15 column; only a very negligible increase in absorption with the elution volume corresponding to the products of lower molecular weight was

observed. The nature of this product was not investigated.

The difference between the decrease in the amount of angiotensin II-amide and the decrease in the biological activity of its irradiated solutions is about 10% after irradiation with higher doses, apparently corresponding to its asp-derivative which is formed in irradiated solutions and which has according to Juliano and co-workers[13] an approximately equal biological activity as angiotensin II-amide. From this it would then follow that other radiation products are biologically inactive. The radiation changes taking place in the molecules of angiotensin II-amide, especially the radiation transformations in its his, tyr and phe residues lead not only to a change in the conformation of the irradiated peptide, but - judging on the basis of the high yield of free ammonia and >C=O groups - to profound structural changes as well. From this a considerable variability of the radiation products follows in spite of the fact that the radiation loss of the irradiated peptide G_i/-M/ is relatively low (1,3).

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