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ENHANCED INCORPORATION OF NONHYDROLYZABLE TRITIUM IN GNRH AND TRF BY CATALYTIC EXCHANGE LABELING

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Summary

Gonadotropin releasing hormone (GnRH), D-Phe$_6$-GnRH and thyrotropin releasing factor (TRF) were tritiated by direct catalytic exchange using Rh/Al_2O_3 + HT under conditions which lead in model deuterations of N_{α}-acetylhistidine amide to a high incorporation of deuterium into position 5 of the histidine ring. Specific activities up to a range of 400 GBq/mmol in form of nonhydrolyzable tritium are attainable after removal of the label incorporated into position 2 of the histidine ring. A crucial reason for diminished specific activities was found to be a catalystmediated hydrogen transfer between the peptides and traces of water, contained in the reaction mixture, competing with the tritiation.

Key words: Exchange labeling, tritiation, ^{3}H-histidine peptides

Introduction

As a simple method to label peptides containing aromatic amino acids by circumventing the syntheses of precursors, the direct catalytic exchange labeling has frequently been studied by several groups /1 - 6/. In histidine containing peptides specific activities up to 500 GBq/mmol could be achieved, but the main part of the radioactivity was introduced into position 2 of the histidine ring /2,5/. From this position the label can be exchanged by hydrolysis unter physiological conditions /2,5/.

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In a previous work /7/ we studied the deuteration of N_{α}-acetyl-histidine amide ($AcHisNH_2$) by direct catalytic exchange to find conditions for a higher incorporation of the label into position 5 of the histidine ring. In this position, the hydrogen isotope is stable under physiological conditions against hydrolytic exchange /7,8/. The best conditions for a high incorporation of deuterium both in position 2 and in position 5 of the histidine ring were a solvent free of labile or activated hydrogen, enhanced temperature (40 - 60 °C), and a relatively high quantity of an appropiate catalyst /7/.
In continuation of earlier investigations in our group on direct catalytic exchange labeling of GnRH /3,4/ we wanted to find out in the present work whether the results of the model deuterations are applicable to the tritiation of GnRH. Therefore GnRH and, for comparison, D-Phe_6-GnRH and TRF were tritiated in dimethyl-acetamide (DMA) at elevated temperature using a catalyst that proved to be sufficiently active in the model deuteration of $AcHisNH_2$ according to ref. /7/.

Results and discussion

In agreement with the results of the model investigations /7/ a higher content of nonhydrolyzable tritium was found in the labeled products (table 1) in comparison with nearly 20% in earlier reports /1-6/.
For an explanation of the low yields especially of hydrolyzable tritium (table 1) the dilution of the reacting gas by hydrogen and a lowered exchange reactivity of the peptides compared to $AcHisNH_2$ do not seem to be sufficient.
If the dilution of the tritium gas would be mainly responsible for the achieved low specific activities, a high isotopic effect would be needed. But according to informational deuterations of $AcHisNH_2$ using different D_2/H_2 mixtures the value of the isotopic effect should not be higher than 1.5 for the reaction both at position 2 and at position 5 of the histidine ring.
Although the exchange reactivity of the peptides was lower than that of $AcHisNH_2$, comparable rates of exchange can be achieved using a nearly tenfold amount of the catalyst for the peptides as needed for $AcHisNH_2$. So using the relations mol Rh/mol substrate of 10/1 and 1/1 for the catalytic deuteration of the tritiated peptides and $AcHisNH_2$ respectively (see Experimental) the exchange of the hydrogen isotope from position 2 of the histidine ring was nearly quantitative in all cases, and the exchange at 40 °C of the

nonhydrolyzable hydrogen isotope was in a range of 40% for the peptides compared to 60% for $AcHisNH_2$. Interestingly, no significant difference between the reactivities of the GnRH-peptides and TRF could be found.

Table 1: Specific radioactivities of ^{3}H-GnRH, ^{3}H-D-Phe$_6$-GnRH and ^{3}H-TRF after direct catalytic exchange labeling in DMA in the presence of Rh/Al_2O_3 5%.

Peptide	mol Rh/ mol peptide	min.	temperature (°C)	^{3}H-content in the tritium gas (%)	specific activity (GBq/mmol) nonhydrolyzable	hydrolyzable +)
GnRH	10	15	40	ca. 90	100	122
"	10	60	40	20-25	85	82
"	10	60	60	ca. 90	296	74
D-Phe$_6$-GnRH	2	60	40	80-85	127	183
"	10	15	40	ca. 90	113	127
"	10	60	40	30-35	132	108
TRF	10	60	40	ca. 70	270	40

+) part of the label (incorporated into position 2 of the histidine ring) which can be exchanged by hydrolysis at pH 7.4 and 37 °C with a half-life time of 90 h

At least the same inportance for the labeling result as the dilution of the reacting gas by hydrogen or the lowered exchange reactivity should have a catalyst-mediated reaction directly between the substrate and traces of water contained in the reaction mixture. So after 1 h deuteration of the tritiated GnRH-peptides and TRF both at 40 °C and at 60 °C (mol Rh/mol peptide - 10/1) 20-30 % of the starting peptide-bound radioactivity were found in the solvent in form of tritiated water (see Experimental). This amount of radioactivity can only be transferred to the solvent by a catalyst-mediated reaction between the peptide and water in agreement with findings of Evans et al. /10/ and own model investigations in D_2O /7/.
Taking into account the amount of radioactivity transferred in this way during the model reaction, such sidereaction, competing

with the tritiation, should be a main reason for lowered apecific activities after catalytic exchange tritiation. As a support for this conclusion can be regarded the important amount of ca. 200 GBq solvent radioactivity found after tritiation of GnRH, which could be assigned nearly quantitatively to tritiated water by fractional distillation and treatment with molecular sieve. Correspondingly, after tritiation of TRF a decrease in the tritium content of the reacting gas from ca. 70% to ca. 30% was found.

On the other hand, the tritiation results (table 1) show that competing hydrogen incorporations are of remarkably lower importance for the tritiation of the 5-position of the histidine ring than for the more reactive 2-position. Thus, using pure tritium gas specific activities in form of nonhydrolyzable tritium in the range of 400 GBq/mmol should be attainable even without additional attempts to reduce the amount of water in the reaction mixture. The higher reactivity of position 2 of the histidine ring allows the removal of the label incorporated into this position by hydrogenation under mild reaction conditions without important losses of nonhydrolyzable tritium. Advantageously, this removal could be achieved quantitatively for the GnRH-peptides and TRF by simple 1 h refluxing of an aqueous solution at pH 7-8. Subsequent HPLC gave no indications of remarkable damage of the peptides. According to an Arrhenius-plot using the exchange half-life time for the hydrolyzable tritium at pH 7.4 and 37 °C and 60 °C, (see Experimental), the half-life at 100 °C is in the range of 10 minutes, which confirms the practical finding that 1 h refluxing is sufficient for quantitative removal of the hydrolyzable tritium.

In the case of the GnRH-peptides, expecially after removal of the hydrolyzable tritium, considerable amounts of the label were found also in tyrosine and phenylalanine (table 2). According to the investigations of Evans et al. /10/ the benzylic hydrogens of tyrosine and phenylalanine should have been exchanged. A labeling of the alkyl chain of histidine seems to be not of importance, because after deuteration of $AcHisNH_2$ no evidence for an incorporation into the alkyl chain higher than 0.05 D/mol could be found. In the same way more than 90% of the radioactivity incorporated into TRF could be assigned to the histidine ring by reaction with diazotized sulfanilic acid. Using a tenfold excess of diazotized sulfanilic acid the reaction was still incomplete yet according to the spectrum, but nevertheless 90% of the tritium was transferred into the solvent.

Table 2: Percentage of incorporated tritium associated with amino acid residues after acidic hydrolysis. (labeling conditions: 1 h at 60 °C for GnRH and at 40 °C for D-Phe$_6$-GnRH and TRF; mol Rh/mol peptide - 10/1 for GnRH and TRF, 2/1 for D-Phe$_6$-GnRH).

Peptide	% nonhydrolyzable tritium	His	Tyr	Phe
GnRH	80	83.7	4.0	-
D-Phe$_6$-GnRH	41	90.6	0.3	3.7
D-Phe$_6$-GnRH	100	74.6	1.3	14.7
TRF	87	94.8	-	-

Not more than about 1% of the starting radioactivity associated with other amino acid residue than histidine, tyrosine or phenylalanine could be found after amino acid analysis. Outside the elution fractions of these three amino acids, a total of not more than ca. 10% of the starting radioactivity was measured in the elution liquid of the amino acid analyzer. These results in connection with the results of HPLC, tlc, paper electrophoresis and uv- measurement allow the conclusion that the purity of the labeled products was higher than 90% after HPLC-isolation. The yields after HPLC-isolation were in the range of 15-50% related to the peptide amount used in the tritiation reaction. In agreement with these yields an amount of radioactivity corresponding to 30-60% of the peptide amount could be removed from the catalyst only by refluxation with hot acid after deuteration of the tritiated peptides (see Experimental). In contrast, only 5-10% of the starting amount of radioactivity was bound on the presaturated catalyst before the reaction. An analoguos amount of radioactivity corresponding to 5-10% of the peptide could be removed from the catalyst by addition of an excess of nonlabeled peptide after the reaction, indicating a reversible absorption only in the 10% range.

Experimental

Materials and methods

TLC was performed on silicagel-60 plates (Merck, FRG) with n-butanol/acetic acid/water/ethyl acetate 1/1/1/1 and n-butanol/acetic acid/pyridine/water 21/12/2/14 and 15/3/10/12. Electropho-

resis was carried out on paper type FN 7 (VEB Papierfabrik Niederschlag, GDR) at 25 V/cm in 7% acetic acid or 0.01 m ammonium acetate pH 7.4. For HPLC a LiChrosorb RP 18 (10 µm) column was used with the elution solvents acetonitrile/0.07 m phosphate buffer, pH 7.0 23/77 for GnRH; 0.25 m TEAP buffer, pH 4.5/ methanol 4/6 for D-Phe$_6$-GnRH and acetonitrile/0.01 m ammonium acetate, pH 4.0 1.5/98.5 for TRF.
Rhodium on alumina 5% catalyst was purchased from Fluka (Switzerland). Dimethylacetamide (Merck-Schuchart, FRG) was distilled and stored over molecular sieve 10X before use.
GnRH and D-Phe$_6$-GnRH were gifts from Berlin-Chemie (GDR). TRF was prepared according to /9/. Tritium gas was purchased from Techsnabexport (UdSSR) and stored in the form of uranium tritide.

General tritiation procedure

2 µmol of the peptide (5 µmol in the case of TRF) were dissolved in 0.5 ml DMA. To the solution were added a magnetic stirrer rod. and the Rh/Al_2O_3 5% catalyst, placed in a cartridge of aluminium foil in such a manner that contact between the catalyst and the solution was avoided. The reaction vessel was connected to the tritiation manifold, cooled by liquid nitrogen and evacuated (p=0.1 Pa). After tritium gas (0.2-0.3 mmol; 20-90%) had been introduced, the reaction temperature was adjusted by means of a water bath and after liberation of the catalyst by manipulating the magnetic stirrer rod. the reaction mixture was agitated at a tritium pressure of ca. 70 kPa. Reaction conditions are given in table 1. After stopping the reaction the catalyst was removed by membrane filtration or centrifugation and washed with 10 ml 2% acetic acid. The solution was evaporated to dryness and 4 times reevaporated using 2% aqueous acetic acid to remove labile tritium. The remaining solid was dissolved in 2% acetic acid/ ethanol 1/1 to give a radioactive concentration of about 50 MBq/ ml, and was stored at -20 °C.
Removal of instably bound tritium from position 2 of the histidine ring, if required, was performed by 1 h refluxing of the peptide solution in 0.07 m phosphate buffer of pH 7.4 or in 0.01 n ammonia.
Purifications were carried out by HPLC, completed by TLC and paper electrophoresis.

Estimation of the specific radioactivity

The amount of the labeled GnRH peptides was determined at 278 nm (2% acetic acid; = 6800) and with the same results after dia-

zo coupling at 500 nm against reference values. The second method was used also for TRF and was performed in the following manner: To 5-50 nmol peptide, dissolved in 1.8 ml water, 0.2 ml 1 m Na_2CO_3 and 0.2 ml of a diazotized sulfanilic acid solution were added (to 17 mg sulfanilic acid in 2 ml 0.1n HCl, 7 mg $NaNO_2$ in 0.04 ml water were given and the reaction mixture was stored for 5 min. at ambient temperature before use). After 30 sec, 1 ml of 1n NaOH was added and 15-30 min later on the solution was measured at 500 nm. Using the same reagent and with nonradioactive peptide the same procedure was followed for obtaining the reference values.

Analysis of the distribution of tritium

About 0.5 MBq of the labeled peptide was hydrolyzed together with 200 nm of unlabeled peptide with 20% hydrochloric acid, containing 2% thioglycolic acid in evacuated sealed tubes at 110 °C for 20 h, and was analyzed by amino acid analyser AAA 331 (Microtechna, Prague, Czechoslovakia). The fractions of the individual amino acids were collected and measured by liquid scintillation counting.

Estimation of the amount and the hydrolytic exchange half-life time of the label, exchangeable at pH 7.4

0.5-2 MBq of the labeled peptide was dissolved in 5 ml 0.07 m phosphate buffer, pH 7.4 containing 0.1% NaN_3, and this solution was stored in test tubes at 60 °C and 37 °C, respectively. After appropriate time intervals microtubes for centrifugation filled with dry ice were put on the test tubes, and the water condensing during 30-60 min on the microtubes was collected and measured by liquid scintillation counting.

After about 30 h at 60 °C the radioactivity in the condensed water remained constant (tested during 5 days) so that this value could serve as a reference also for the estimation at 37 °C and represented at the same time the amount of the hydrolyzable tritium (incorporated into position 2 of the histidine ring). Plotting of the logarithms of the difference between the reference value and the single value, obtained after a certain time of storage, against the incubation time gave a straight line according to a first-order reaction. From these plots half-lifes of about 90 h (± ca. 10%) at 37 °C and 5.5 h (± ca. 10%) at 60 °C were deducible. No clear differences could be found between the values for the three investigated peptides.

Model deuterations

The deuterations were performed in rectangular 20-ml Warburg vessels, equipped with an additional side-bulb, under normal pressure and with a constant flow of deuterium (ca. 100 ml/h). The deuterium was produced by electrolyzing D_2O (98%) containing 5% H_2SO_4 on Pt-electrodes.

The substrate (10 µmol $AcHisNH_2$ or ca. 0.5 MBq of tritiated peptide filled up to 0.3 µmol with nonradioactive peptide), dissolved in 1.4 ml DMA and the catalyst were introduced separately into the reaction vessel and combined after 1 h presaturation at ambient temperature. The reaction was then performed by shaking 1 h at the given temperature.
The deuterium content of the deuterated $AcHisNH_2$ was estimated by ir- and ms-measurements as described previously /7/.
The results after the deuteration of the tritiated peptides were analyzed in the following manner:
Measurement of the radioactivity in solution immediately after combining with the catalyst allowed an estimation of the peptide absorption to the presaturated catalyst. It was in the range of 5% for the GnRH-peptides and of 10% for TRF.
After reaction, a portion of 0.5 ml of the solution was decanted from the catalyst, counted and introduced into a test tube containing 1 ml water. To the residual reaction mixture 0.5 ml water was added, and 0.5 ml of the solution was decanted an treated as above. To the remaining reaction mixture 0.1 mg nonradioactive peptide dissolved in 0.1 ml water was added to obtain information about the reversible peptide absorption to the catalyst.
Finally, the reaction mixture was boiled shortly after addition of 0.2 ml conc. HCl and 0.2 ml conc. HNO_3 and also counted after cooling to get information about irreversible peptide absorption to the catalyst or about the absorption of radioactive splitting products, respectively.
The values of radioactivity which are in the reaction mixture in the form of tritiated water and in form of tritium bound instably in position 2 of the histidine ring were received by counting the radioactvities in the condensed water obtained after 0.5 h and 30 h storage at 60 °C (as described above) from the 0.5 ml samples of the reaction solution filled up with 1 ml water. Quantitative removal of the solvent by vacuum evaporation resulted in the same value for the radioactivity contained in the solvent as estimated in the condensed water.

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