# COUPLING OF CARBOXYMETHYLATED (His)$^2$ LUTEINIZING HORMONE RELEASING HORMONE TO POLY-DL-ALANYL CHAINS (SPACER GROUPS) POLYMERISED ON HUMAN LACTOFERRIN

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## 1. Introduction

Different conjugates of LH-RH and LH-RH derivatives were used to elicit antibodies anti LH-RH [1–9]. This work describes a new conjugate which is formed by the reaction of carboxymethylated (His)$^2$-LH-RH prepared as reported in a previous paper [10] with poly-Dl-alanyl chains polymerised on human lactoferrin. This new conjugate presents 3 advantages:

(i) The terminal groups and the tyrosine residue of the carboxymethylated (His)$^2$-LH-RH are respected [10].

(ii) The carboxymethylated (His)$^2$-LH-RH will be spaced from a powerfully antigenic protein matrice by flexible non antigenic poly-Dl-alanyl chains.

(iii) All the new bonds introduced by the reaction are the common protein amide bonds.

## 2. Materials and methods

### 2.1. *Polymerization procedure*

The general polymerisation procedure described by Anfinsen et al. [11] was followed to obtain poly-Dl-alanyl–Fe-lactoferrin.

*N*-carboxy-Dl-alanine anhydride (65.3 mg Miles) was dissolved in 5 ml of dioxan (Merck) and slowly added to 10 ml of a solution of 50 mg of Fe-lactoferrin dissolved in 0.1 M phosphate buffer pH 7.

After 16 hr of reaction at room temperature, the products were dialysed against distilled water at 4°C.

### 2.2. *Coupling*

Coupling of [$^{14}$C]carboxymethylated (His)$^2$-LH-RH to poly-Dl-alanyl–Fe-lactoferrin was conducted in one- and two-step procedures.

#### 2.2.1. One-step procedure [12]

Radioactive [$^{14}$C]carboxymethylated (His)$^2$-LH-RH (2.25 $\mu$M) [10] and 0.025 $\mu$M of poly-Dl-alanyl–Fe-lactoferrin were dissolved in 1.4 ml of 0.07 M phosphate buffer at pH 7.0.

To this solution was added 0.6 ml of an aqueous solution containing 4 $\mu$M of 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (Sigma). The solution was stirred magnetically at room temperature. The reaction was allowed to proceed for 16 h in the dark under a stream of nitrogen and was terminated by dialysis against distilled water at 4°C.

Aliquots of 20 $\mu$l were taken to check the radioactivity before and after dialysis.

#### 2.2.2. Two-step procedure [13]

A volume of 0.6 ml of pyridine was added to 7 $\mu$M of radioactive [$^{14}$C]carboxymethylated (His)$^2$-LH-RH dissolved in 0.6 ml of distilled water. Then 14 $\mu$M of carbodiimide dissolved in 20 $\mu$l of water/pyridine (1:1, v/v) was added.

After 0.5 h, 0.4 ml of an aqueous solution of poly-Dl-alanyl–Fe-lactoferrin (3.8 mg/ml) was added and the reaction was left to proceed for 2 h.

The solution was then dialysed against distilled water at 4°C and lyophilized after aliquots of the

dialysate were taken for bio-fiber dialysis and deamination reaction.

2.3. *Bio-fiber dialysis*

The sample was passed by means of an LKB peristaltic pump through the fibers of a bio-fiber 80 minibeaker (Bio-Rad) then through an automatic ultraviolet recorder (LKB uvicord II).

The bottom-cap of the minibeaker was removed and the beaker was plunged into one liter of the dialysis solution which was magnetically stirred. Urea was added to samples up to 6.0 M and left for 3 days at room temperature. Afterwards the samples were dialysed against 6.0 M urea for 2 h.

2.4. *Lysine residue deamination*

Deamination of the lysine residues of Fe-lactoferrin and of poly-Dl-alanyl–Fe-lactoferrin was performed as follows: An aliquot of 0.5 ml of dialysate was mixed with 1.5 ml of an $NaNO_2$ saturated aqueous solution and 0.5 ml of glacial acetic acid [11]. After 16 h of reaction at room temperature, the products were dialysed against distilled water at 4°C.

Aliquots were subjected to amino acid analysis performed after acid hydrolysis in 6.0 M HCl at 110°C in evacuated sealed tubes for 24 h.

2.5. *Immunization*

Seven rabbits were given an initial s.c. injection of an emulsion of 0.6 ml of Freund's complete adjuvant and 0.6 ml of distilled water containing 200 $\mu$g of the antigen, and for each of the five following months, 60 $\mu$g in the same emulsion with the incomplete adjuvant, then after a rest of 3 months, 700 $\mu$g in this emulsion. Animals were bled every three to four weeks and serum titered.

2.6. *Radioimmunoassay*

The general method of Arimura [14] for the labelling was adopted with the following modifications:

(a) A solution of 500 ng/500 $\mu$l of lactoperoxidase (Boehringer) instead of 100 mg/500 $\mu$l solution.

(b) An ammonium acetate gradiant (0.2–0.5 M, pH 4.6) was used to elute the radioactive LH-RH instead of the 0.1 M, pH 4.6 solution.

(c) Unused fractions from the CMC column were kept at −20°C in solution instead in the lyophilised state.

To each disposable polyethylene tube (1 × 6 cm) was added 0.1 ml of EWPBS buffer [14], 0.1 ml of antisera at 1/5000 dilution, 0.1 ml of LH-RH at various concentrations (2.5–100 pg) and 0.1 ml labelled hormone (approximately 2500 cpm). Final dilution was 1/20 000.

All the solutions were made up in EWPBS. The reaction mixture was incubated at 4°C during 24 h. then 1 ml of ice-cold dextran-coated charcoal suspension was added, mixed and left at 4°C for 30 min. The charcoal suspension was made up by adding 100 mg activated charcoal (Norit A) to 100 ml dextran solution (25 mg dextran T-70 in 100 ml of 0.01 M phosphate buffered saline). The tubes were then centrifuged as described [14].

## 3. Results and discussion

3.1. *Polymerisation of Dl-alanine with Fe-lactoferrin*

The product of the reaction of Fe-lactoferrin with N-carboxy-Dl-alanine was characterised by the determination of the number of added alanine residues, the number and the mean length of the poly-Dl-alanyl chains. The number of alanine residues added by the reaction was determined by amino acid analysis of the poly-Dl-alanyl–Fe-lactoferrin (table 1, first column).

The number of poly-Dl-alanyl chains polymerised on the $\epsilon$-$NH_2$ groups of lysine in Fe-lactoferrin was determined by comparative amino acid analysis of deaminated poly-Dl-alanyl–Fe-lactoferrin and deaminated Fe-lactoferrin.

By this reaction, only lysine residues with free $\epsilon$-$NH_2$ groups were deaminated while those with $\epsilon$-$NH_2$ amide bond, which carried the poly-Dl-alanyl chains, were left intact.

It is thus easy to calculate the number of the chains polymerised which are equal to the number of intact lysine residues given by the amino acid analysis (table 1). The mean number of alanine residues per chain was calculated by dividing the number of added alanine residues by the number of chains.

The derivative with the mean length of 5 (experiment 2) was chosen for the conjugation with [$^{14}$C]carboxymethylated (His)$^2$-LH-RH.

Table 1
Number of alanine and lysine residues in deaminated and non-deaminated poly-Dl-alanyl–Fe-lactoferrin and Fe-lactoferrin

| | Experiment | Alanine residues | | No. of chains after deamination | Mean number of alanine residues per chain |
|---|---|---|---|---|---|
| | | Total | Added | | |
| poly-Dl-alanyl–Fe-lactoferrin | 1 | 112 | 56 | 20 | 2.8 |
| | 2 | 145 | 89 | 18 | 4.9 |
| | 3 | 101 | 45 | 13 | 3.5 |
| Fe-lactoferrin | 1,2,3 | 56 | 0 | 0[a] | – |

[a]All the 39 lysine residues of Fe-lactoferrin were deaminated

3.2. *Coupling*

Coupling of [$^{14}$C]carboxymethylated (His)$^2$-LH-RH to poly-Dl-alanyl–Fe-lactoferrin results as follows:

The one-step reaction – the most commonly used – had not been successful as no radioactivity was detectable in the solution of the dialysis bag. On the other hand the conjugation has been successful in the two-step reaction. Radioactivity was measured to calculate the number of LH-RH molecules in one molecule of lactoferrin (table 2). It is worth mentioning that in both, one- and two-step reactions, Fe-lactoferrin lost the iron as the characteristic coloration vanished, indicating some structural change in the protein [15]. This observation could explain the failure of the conjugation in one-step reaction. Probably the modification of structure has favoured the intra reaction between carboxyl and amino groups in the protein compared to the inter reaction between carboxyl groups of the [$^{14}$C]carboxymethyl (His)$^2$-LH-RH and terminal amino groups of the poly-Dl-alanyl chains of the poly-Dl-alanyl–Fe-lactoferrin.

In the two-step reaction, carbodiimide reaction gave an active mixed anhydride derivative of [$^{14}$C]-carboxymethylated (His)$^2$-LH-RH which in turn substituted the terminal -$NH_2$ groups of poly-Dl-alanyl chains. To remove the possibility of adsorption of [$^{14}$C]-carboxymethylated (His)$^2$-LH-RH on

Table 2
Number of LH-RH molecules per molecule of lactoferrin

| Solutes (in 1.5 ml 6.0 M urea) | Recovery after 2 h dialysis | No. of LH-RH molecules in one molecule of lactoferrin |
|---|---|---|
| LH-RH 0.104 μM | 0.00026 μM (0.25%) | – |
| Lactoferrin 0.033 μM | 0.033 μM (100%) | – |
| Synthetic antigen 0.084 μM of LH-RH | 0.069 μM (82%) | 16.7 |

The synthetic antigen was left in 6.0 M urea for 3 days before being dialysed against 6.0 M urea for 2 h in the bio-fiber system.

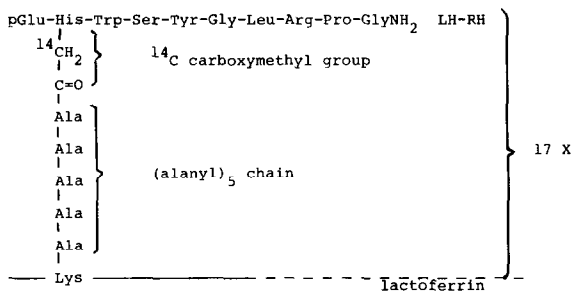


Fig.1. Schematic representation of the synthetic antigen.

poly-Dl-alanyl lactoferrin instead of covalent binding, the products were left 3 days in 6.0 M urea prior to being dialysed against the same solution in the biofiber system. To check the efficiency of the dialysis, LH-RH and lactoferrin were dialysed separately as well as mixed in the same conditions. More than 99% of LH-RH was dialysed away while during that time no lactoferrin was lost (table 2).

The synthetic antigen retained 82% of LH-RH after 2 h of dialysis while only 0.25% of LH-RH remained when dialysed in the same conditions (table 2). The synthetic antigen contained 16.7 molecules of LH-RH for one molecule of lactoferrin. This number of 16.7 was derived from the fraction [$^{14}$C]carboxymethylated (His)$^2$-LH-RH/lactoferrin and agrees very well with the number of 18 polyalanyl chains calculated from the amino acid compositions (experiment 2, table 1). The schematic representation of the synthetic antigen is shown in fig.1. This synthetic antigen of which the rough formula:

$$[CM(His)^2\text{-LH-RH (Dl-Alanyl)}_5]_{17}Lf$$

was used to elicit the production of antibodies in rabbits.

### 3.3. *Standard curve*

The fraction of the CMC chromatography which gave the highest radioactive binding was used to make the standard curve (fig.2.) This curve gives mean value and S.D. of four standard assays up to 30 pg and three from 40–90 pg. The antiserum at a final dilution of 1/20 000 bound 25–30% of tracer in the absence of unlabelled LH-RH.

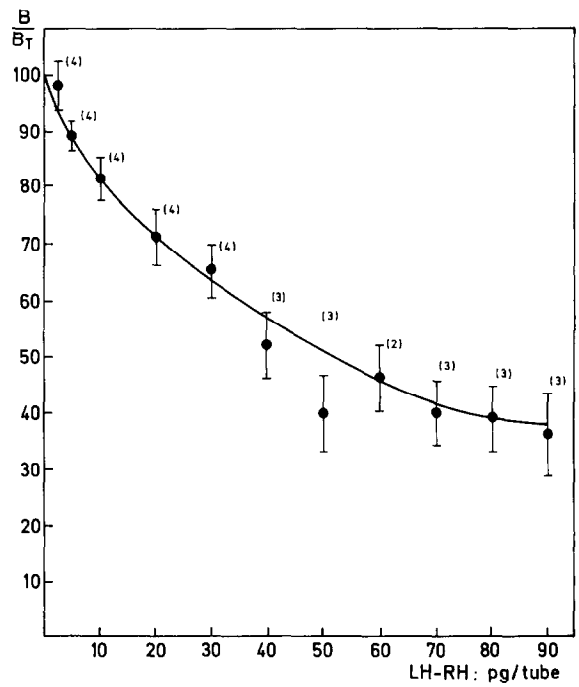


Fig.2. Composite standard curve for LH-RH radioimmunoassay. In brackets are indicated the number of standard assays. $B/B_T$ relates percentage of binding (B) to the percentage of tracer bound in the absence of antigen ($B_T$).

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