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SUBSTRATE CONFORMATION DIRECTS SELECTIVE ENZYMIC CLEAVAGE OF β-LIPOTROPIN INTO β-ENDORPHIN

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SUMMARY: It is shown that whilst the Arg^{51}-Trp^{52} and Arg^{60}-Tyr^{61} peptide bonds of β-lipotropin are preferentially split by trypsin-Sepharose in aqueous solution, in a secondary structure promoting environment the Arg^{60}-Tyr^{61} peptide bond is almost exclusively cleaved. It is suggested that the specificity of intracellular precursor processing may be directed by the conformation of the substrate.

It is now generally accepted that the small peptide hormones are formed by a common biosynthetic process that involves the synthesis of relatively large precursors on membrane-bound polysomes and subsequent proteolytic cleavages of these precursors during their transport within the cell (1-3). Sequence comparisons of known precursors and their products indicate that precursor processing is mediated by trypsin-like proteinases which, however, do not split all the theoretically trypsin-sensitive peptide bonds in the precursors (3). Geisow (3) and Loh and Gainer (4) have suggested that the specificity of proteolytic processing is directed by the conformation of the precursors stored in the granules, rather than by the unique substrate specificities of the individual converting enzymes.

In an attempt to provide experimental support for the above hypothesis, we have studied how the conformation of β-lipotropin (β-LPH*), the direct biological precursor of β-endorphin [LPH-(61-91)] (2), affects the action of trypsin on β-LPH.

MATERIALS AND METHODS

Lipotropins: Porcine β-LPH and γ-LPH [LPH-(1-58)] were prepared by procedures described previously (5, 6).

Circular dichroism measurements: Circular dichroism (CD) spectra of β-LPH and γ-LPH were obtained on a Jobin – Yvon – Roussell – Jouan model III dichrograph at room temperature. Path length of the cell used was 0.01 cm. Concentration of the samples ranged between 0.3-0.6 mg/ml. No concentration-dependence of the CD spectra was observed in this concentration range. The percentage content of α-helix in the structure of β- and γ-LPH was calculated from the CD spectra by the method of Greenfield and Fasman (7). Though the values obtained are considered as estimates rather than reliable absolute α-helix contents (see refs 8, 9), they may certainly serve to measure conformational changes of the peptides in response to changes of solvent composition (8).

Preparation of trypsin-Sepharose 4B: 0.8 mg trypsin (Calbiochem) was coupled to 0.6 g pre-swollen CNBr-activated Sepharose 4B (Pharmacia) in 0.1 M $NaHCO_3$ buffer of pH 7.5 containing 0.5 M NaCl at room temperature. The excess active groups of the gel were blocked by treatment with 1 M ethanolamine at pH 8, and the product was alternately washed with the coupling buffer and 0.1 M sodium acetate buffer of pH 4 containing 0.5 M NaCl. Trypsin-Sepharose 4B was kept in the pH 4 buffer solution at 4 °C for later use.

* Abbreviations: β-LPH, β-lipotropin; LPH, lipotropin; CD, circular dichroism; TFE, trifluoroethanol.

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Enzyme activity measurements with synthetic substrate: The specific activity of trypsin and trypsin-Sepharose was assayed with a highly sensitive trypsin substrate, Z-Lys-Pro-Arg-*p*-nitroanilide (10). In a typical experiment 0.15 μg soluble or 1 μg immobilized trypsin was incubated with 5×10^{-4} M substrate in 0.04 M tris buffer of pH 8.2 containing 0-30 % (v/v) trifluoroethanol (TFE) (Fluka) at 37 °C for 2-15 min. The reaction was terminated by adding 100 μl of glacial acetic acid to 1 ml of the incubation mixture. The amount of *p*-nitroaniline formed was determined spectrophotometrically at 410 nm.

Digestion of β-LPH with trypsin-Sepharose: Digestion of porcine β-LPH with trypsin-Sepharose was carried out in 0.1 M ammonium bicarbonate buffer of pH 8.2 in the absence or presence of 20 % TFE at a protein ratio 35 : 1 of substrate-enzyme at 37 °C. Aliquots were taken at 0, 10, 30 and 60 min and the reaction was terminated by adding a potent serine proteinase inhibitor, Boc-D-Phe-Pro-Arg-aldehyde (10) to each aliquot in a concentration of 3×10^{-3} M. The samples were lyophilized for disc electrophoresis in polyacrylamide gel (8 %) at pH 9 (11) and at pH 4 (12). The isolation and identification of the cleavage products were performed by techniques described previously (13).

Table 1

Percentage of α-helix[+] of β- and γ-LPH in TFE/water Mixtures Estimated by the Method of Greenfield and Fasman (7)

TFE concentration (%)	β-LPH	γ-LPH
0	12.5 (3)	10.5 (2)
20	28.0 (3)	30.0 (2)
35	29.5 (2)	32.5 (2)
50	31.5 (2)	34.5 (2)
65	33.0 (2)	38.5 (2)
80	41.0 (2)	–[++]
100	47.0 (2)	–[++]

[+] Average values are given. The number of measurements performed with different solutions is in parentheses.

[++] γ-LPH was not soluble in these TFE/water mixtures.

Table 2

Specific Activities[+] of Trypsin and Trypsin-Sepharose Measured on Z-Lys-Pro-Arg-*p*-nitroanilide

TFE concentration (%)	Trypsin	Trypsin-Sepharose
0	54.2 ± 1.1 (4)[§]	13.4 ± 0.6 (5)
10	47.4 ± 0.4 (4)	14.5 ± 0.3 (5)
20	0 (3)	16.3 ± 0.3 (5)
30	0 (2)	1.9 ± 0.1 (5)

[+] Expressed as μmoles of *p*-nitroaniline released per min per mg protein.

[§] Mean ± SEM (number of experiments).

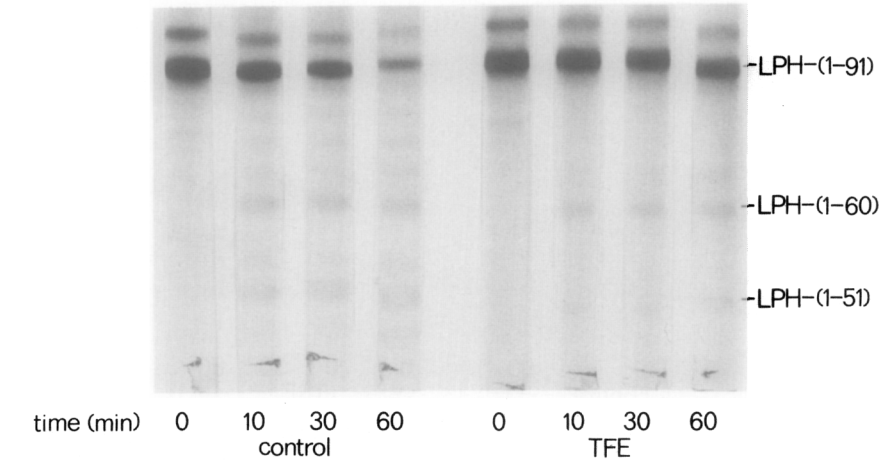

Figure 1. Time-course of the digestion of β-LPH with trypsin-Sepharose in the absence (control) and presence of 20 % TFE (TFE). The samples contained 200 μg of β-LPH and the gel electrophoresis was carried out at pH 9. The discs of β-LPH [LPH-(1-91)] and two N-terminal fragments identified are marked.

RESULTS

As Table 1 shows, the α-helix content of β- and γ-LPH both increased in a similar fashion in response to increases of TFE concentration of the solutions. The largest increment of α-helix content for both peptides was achieved by the addition of 20 % TFE to the aqueous solutions. Further increase of TFE concentration lead to less pronounced increases of the α-helix contents.

Data in Table 2 indicate that soluble trypsin is void of enzyme activity in 20 % TFE in assays with synthetic substrate, whereas immobilized trypsin displays even higher activity in the latter environment than in aqueous solution. Thus, our comparative studies on the trypsin cleavage of β-LPH in aqueous and TFE containing solutions were performed with trypsin-Sepharose. Disc electrophoresis at pH 9 of the trypsin-Sepharose digests of β-LPH revealed a marked difference between the conversion processes in the absence and presence of 20 % TFE (Fig. 1). The main cleavage products of β-LPH were isolated from the 1-hour digests and identified[§] as LPH-(1-51), LPH-(1-60) and LPH-(61-91), resp. (see Figs 1 and 2). In aqueous solution the formation of LPH-(1-51) was faster than that of LPH-(1-60) (Fig. 1), and in addition to these two main cleavage products several unidentified fragments were generated from β-LPH. In the TFE containing solution, however, the cleavage of β-LPH to LPH-(1-60) (Fig. 1) and LPH-(61-91) = β-endorphin (Fig. 2) was almost exclusive. Chromatographic separation of the 1-hour tryptic digest (prepared in 20 % TFE) on Sephadex G-50 and carboxymethyl-cellulose columns (13) indicated that about 15 % of β-LPH was converted to LPH-(1-60) and β-endorphin, and the amount of other fragments was below 1 %.

§ No details of the preparative work are given in this paper. The techniques used for the isolation and identification of the fragments were described in a previous paper (13).

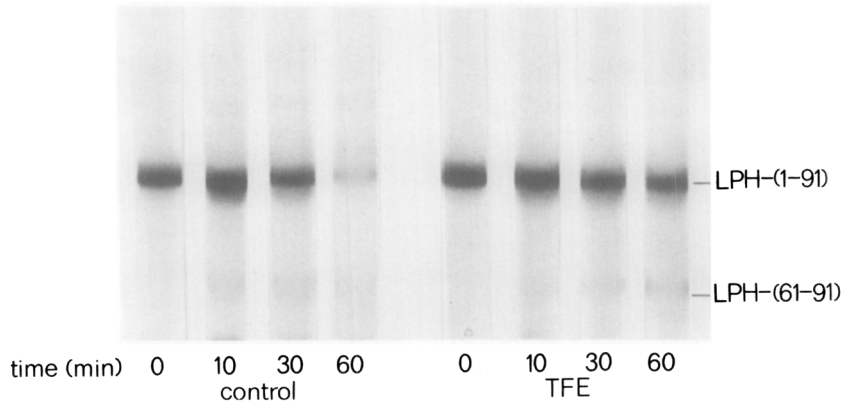

Figure 2. Time-course of the digestion of β-LPH with trypsin-Sepharose in the absence (control) and presence of 20 % TFE (TFE). The samples contained 200 μg of β-LPH and the gel electrophoresis was carried out at pH 4. The discs of β-LPH [LPH-(1-91)] and β-endorphin [LPH-(61-91)] are marked.

DISCUSSION

Since the addition of 20 % TFE to the reaction mixture increased rather than decreased the catalytic activity of trypsin-Sepharose towards synthetic substrate (Table 2), the inhibitory effect of TFE on the proteolysis of β-LPH (Figs 1, 2) is clearly due to a conformational change of this peptide. Indeed, CD spectroscopy indicated a conformational transition of β-LPH on addition of 20 % TFE, and this is interpreted as an increase in its α-helix content (Table 1). Similar effect of different secondary structure promoting solvents on the β-LPH conformation has been reported from other laboratories (14, 15, 9).

By applying the predictive rules of Chou and Fasman (16) to locate the potentially ordered (α-helix, β-turn, β-sheet) regions in porcine β-LPH structure (Fig. 3), we found that one of the

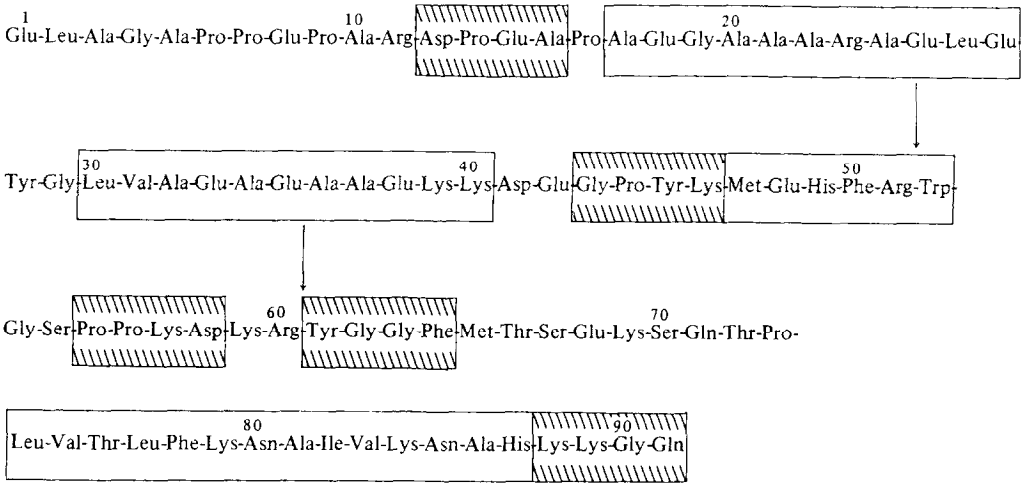

Figure 3. α-helix (empty rectangles) and β-turn (hatched rectangles) regions predicted for the β-LPH structure. Arrows mark the most accessible peptide bonds to trypsin-Sepharose in aqueous solution.

two peptide bonds most susceptible to trypsin in aqueous solution, Arg^{51}-Trp^{52} occurs in a region with a high α-helix potential. The comparison of the CD data on β-LPH and γ-LPH in Table 1 suggests that the conformation of the γ-LPH portion (residues 1-58) of β-LPH may be more affected by the addition of 20 % TFE than that of the C-terminal region, residues 61-91 (see also ref. 8). Thus, it is reasonable to assume that the rigid conformation of the Arg^{51}-Trp^{52} containing region of the molecule, induced by 20 % TFE, may protect this bond from proteolysis. Alternately, the two β-turns predicted for residues 55-58 and 61-64 may render the Arg^{60}-Tyr^{61} peptide bond to be sterically exposed for enzymic attack (Fig. 3).

The above data clearly show that considerable specificity of the proteolysis can be achieved by effects influencing the substrate conformation, and these data together with those of Loh and Gainer (4) strongly support Geisow's view (3) that the post-translational proteolytic processing may be controlled through such conformational effects.

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