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# C-TERMINAL GROUPS IN CHYMOTRYPSINOGEN AND DFP-α-CHYMOTRYPSIN IN RELATION TO THE ACTIVATION PROCESS

by

JULES A. GLADNER* AND HANS NEURATH
*Department of Biochemistry, University of Washington, Seattle, Washington (U.S.A.)*

Although the action of carboxypeptidase has been generally related to the hydrolysis of simple peptides, evidence has come to fore that this peptidase is also capable of liberating amino acids from protein substrates[1,2], presumably from the carboxyl (C-terminal) end of polypeptide chains. This enzymatic reaction is of interest since it permits the controlled, stepwise degradation of proteins and the identification of C-terminal amino acids of the original and residual protein. However, the success of this method as an analytical tool depends on the removal of the last traces of active endopeptidases (chymotrypsin and trypsin) and free amino acids from the preparations of enzyme and substrate. In applying this method to chymotrypsinogen and DFP-α-chymotrypsin as substrates, purified crystalline preparations of these proteins were subjected to additional crystallizations until chymotrypsin and trypsin contents (as determined by hydrolysis of synthetic substrates) were reduced to less than 0.2%. Last traces of these proteinases were inactivated by di*iso*propylfluorophosphate (DFP)[3] which does not significantly inactivate carboxypeptidase, and free amino acids and peptides were removed by exhaustive dialysis against 0.001 *M* HCl. Carboxypeptidase, dissolved in lithium chloride, and substrates were incubated for varying lengths of time at 25° and pH 7.8 and the liberated amino acids were determined with a modified colorimetric ninhydrin method[4] after precipitation of proteins by trichloracetic acid.

With chymotrypsinogen as substrate (substrate/enzyme mole ratios of 17 and 50) not more than 0.2 to 0.3 moles of amino acids per mole of chymotrypsinogen (molecular weight 22,500) were maximally found in less than 30 minutes of hydrolysis. However, when crystalline DFP-α-chymotrypsin was used as substrate (substrate/enzyme mole ratio of 22 and 32), 2 moles of amino acids per mole of substrate were maximally liberated after 2 to 3 hours of incubation. Carboxypeptidase remained fully active even after 6 hours of incubation.

In order to identify the amino acids, one- and two dimensional paper chromatography was used with phenol—water, and *n*-butanol—acetic acid—water (200:30:75), respectively, as solvent systems. The enzymatic reaction was terminated either by the addition of trichloracetic acid or by acidification with HCl to pH 3.0. No supporting buffers were employed but lithium chloride (0.5%)

* Predoctorate Research Fellow of the Public Health Service.

and trichloracetic acid were removed prior to chromatography by a modification of the ion exchange method of PIEZ *et al.*[5] When the appearance of amino acids with time was determined, the "molecular sieve" method of PARTRIDGE[6] and THOMPSON[7] was used* and the enzymatic reaction was merely halted by acidification with hydrochloric acid, Dowex -50 ($H^+$ form, 15–20 mesh) serving as ion exchange resin.

With chymotrypsinogen as substrate, faint traces of several amino acids could be detected on the paper chromatograms. With DFP-$\alpha$-chymotrypsin as substrate, 6 spots were apparent after 30 seconds of hydrolysis, the most intense ones being leucine and tyrosine. These reached maximum intensity after not more than 30 minutes of hydrolysis, whereas the originally much fainter spots, corresponding to glycine, serine, alanine, and presumably lysine (traces) continued to increase in intensity up to 2 hours of hydrolysis but never reached the full intensity of the leucine and tyrosine spots**.

These results suggest that in DFP-$\alpha$-chymotrypsin, leucine (or isoleucine) and tyrosine occupy C-terminal positions whereas the other amino acids which have been identified occupy adjacent, internal positions, and are liberated in turn. In contrast, chymotrypsinogen seems to be composed of cyclic polypeptide chains or else the C-terminal amino acids do not conform to the structural requirements of carboxypeptidase. The former explanation is in qualitative accord with the report of DESNUELLE *et al.*[8] who failed to detect any dinitrofluorobenzene-reactive N-terminal groups in chymotrypsinogen, in contrast to 2 terminal groups in $\alpha$-chymotrypsin.

According to these results, the tryptic activation of chymotrypsinogen involves an opening of the cyclic polypeptide chain(s) of chymotrypsinogen. However, if the specificity of trypsin in this process is the same as in its action toward synthetic substrates, lysine or arginine would result as C-terminal groups in chymotrypsin, and since these do not conform to the specificity requirements of carboxypeptidase[9], no hydrolysis by the latter enzyme would be expected. It appears, therefore, that the activation of chymotrypsinogen involves a second phase in which lysine- or arginine-containing peptides are split off from the intermediate, presumably by an autolytic process involving chymotrypsin, resulting in the formation of C-terminal leucine (or isoleucine) and tyrosine. This conclusion, which is in qualitative accord with JACOBSEN's interpretations of the activation process[10] is being subjected to further experimental tests. The results of this and similar studies on the formation of $\beta$ and $\gamma$ chymotrypsin and on the activation of trypsinogen will be published at a later time.

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*** Quantitative analysis of each amino acid by column chromatography remains to be carried out to substantiate our present assumption that the amino acids which produce these fainter spots on paper do not contribute significantly to the color yield in bulk.