# ON THE QUESTION OF TRANSESTERIFICATION IN CARBOXYPEPTIDASE-A CATALYZED HYDROLYSES

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For a number of the proteolytic enzymes (chymotrypsin, trypsin, papain, etc.) it has been demonstrated by a variety of experimental approaches that the mechanism for the catalysis of peptide or ester hydrolysis includes the formation of a covalent acyl-enzyme intermediate in addition to the usual non-covalent or "Michaelis" complex (Bender and Kezdy, 1965; Kirsch and Igelström, 1966). This mechanism is schematically represented in eq. 1 where

$$E + S \underset{}{\overset{K_1}{\rightleftharpoons}} ES \overset{K_2}{\rightleftharpoons} ES' \xrightarrow{k_3} E + P_2 \qquad (1)$$

$$ES' \rightarrow + P_1$$

ES' is the acyl-enzyme intermediate. $K_1$ and $K_2$ are equilibrium constants and $k_3$ is the rate constant for the step regenerating the free enzyme and the final product of hydrolytic cleavage. Although several attempts have been made to test the applicability of such a scheme to hydrolyses catalyzed by the metalloenzyme carboxypeptidase (McClure and Neurath, 1966; Ginodman, Mal'tzev and Orekhovich, 1966; Carson and Kaiser, 1965), all have failed to produce any evidence for the existence of an acyl-enzyme intermediate, ES'.

With the above observations in mind we have carried out some experiments with carboxypeptidase designed to test a very basic consequence of the mechanism implied by eq. 1. If indeed this mechanism applies to reactions catalyzed by carboxypeptidase and if steps 1 and 2 of the mechanism are reversible, then it should be possible, by incubating the enzyme and a suitable substrate with an excess of isotopically labeled product, $P_1$, to detect exchange of the label into the substrate.

Our substrate in this work was O-cinnamoyl-L-β-phenyllactate prepared from cinnamoyl chloride and L-β-phenyllactic acid (Ash Stevens, Inc., Detroit, Mich.) by the method of McClure and Neurath (1966). We were unable to crystallize the optically active acid, but we did succeed in obtaining its sodium salt in solid form by careful NaOH titration of a suspension of the oily acid in water, evaporation of the water, and washing of the semi-solid residue with hot acetone. The product was optically active ($[\alpha]_D^{27^\circ}$ = $-67.2^\circ$, observed for a 0.32% solution of the salt in water), and its UV spectrum in water was identical to that of the sodium salt of the well characterized racemate (Awazu, Carson, Hall and Kaiser, 1967). The product appeared to be optically pure since it was found to be 100% hydrolyzed in the presence of carboxypeptidase A, whereas the racemate was exactly 50% hydrolyzed under the same conditions. This substrate is an excellent one for carboxypeptidase A with $K_m = 1.55 \times 10^{-4}$ M and $k_{cat} = 4.6 \times 10^3$ $min^{-1}$ at $25^\circ$ at pH 7.5 in an 0.5 ionic strength aqueous medium (Awazu, Carson, Hall and Kaiser, 1967).

The products of the enzyme catalyzed hydrolysis of this substrate at pH 7.5 are cinnamate and β-phenyllactate. Although β-phenyllactate is a strong inhibitor of the reaction, the detailed kinetics of inhibition have not yet been described. If the postu-

lated acyl-enzyme mechanism shown in eq. 1 were operating, one would expect in our case to get cinnamoyl-carboxypeptidase as ES' and β-phenyllactate as $P_1$. However, exchange experiments were conducted not only with initially added radioactive β-phenyllactate but also with initially added radioactive cinnamate, and finally with an initial excess of both radioactive cinnamate and stable β-phenyllactate. Radioactive β-phenyllactic acid was made from DL-β-phenylalanine-3-$C^{14}$ (purchased from International Chemical and Nuclear Corporation) by nitrous acid deamination according to the method of Wright, Brown and Neish (1958). Cinnamic acid-2-$C^{14}$ was purchased from Volk Radiochemical Co., Burbank, Calif.

All of the experiments were conducted as follows. Appropriate amounts of stock solutions of the substrate and the labelled product (or products) were mixed at 25° and the pH adjusted to 7.5. (No hydrolysis takes place under these conditions in the absence of carboxypeptidase.) An appropriate amount of enzyme stock solution was then added to the mixture and the reaction monitored through one half-life on an automatic Radiometer pH stat titrator at pH 7.5. At one half-life enough 5 N HCl was added to take the pH below 1, whereupon the reaction immediately is quenched and the enzyme is irreversibly denatured. The acidified suspension of precipitated acids was then extracted with $CHCl_3$. The extract was dried over $Na_2SO_4$, the $CHCl_3$ removed, and the residue taken up in ether. The ether solution was then treated with $CH_2N_2$ in ether to make the methyl esters of all the carboxylic acids present. These were then separated on silica gel TLC (thin layer chromatography) plates developed with $CCl_4/CHCl_3$ (60:40). The spots (visualized under UV illumination) were scraped off the plates (equal areas scraped off for every spot) and counted in a Packard scintillation counter. Control runs with no enzyme added were

carried out simultaneously with each enzyme run.

The results are given in Table I. It can be seen that although the separation of the methyl esters on TLC is not quite complete (i.e., some activity is found in the methyl-(cinnamoyl-L-β-phenyllactate) spot on the chromatogram), the agreement between

TABLE I[a]

| | % recovered CPM in origin and Me(βφL) area on TLC plate[b] | % recovered CPM in Me(CinβφL) area on TLC plate[b] | % recovered CPM in Me(Cin)area on TLC plate[b] |
|---|---|---|---|
| βφL* runs[c] | | | |
| a) with(CPD)A | 98.1 ± 0.8 | 1.75 ± .75 | 0.0 ± 0.2 |
| b) without (CPD)A | 98.1 ± 0.4 | 1.86 ± .44 | 0.0 ± 0.1 |
| Cin* runs[d] | | | |
| a) with (CPD)A | 8.6 ± 4.1[f] | 6.0 ± 0.5 | 85.4 ± 4.2 |
| b) without (CPD)A | 13.3 ± 3.1[f] | 6.1 ± 0.1 | 80.5 ± 3.0 |
| Cin* + βφL Runs[e] | | | |
| a) with (CPD)A | 8.4 ± 0.5[f] | 6.4 ± 0.3 | 85.2 ± 0.2 |
| b) controls not run | - | - | - |

[a]Abbreviations are as follows: CPM - counts per minute. Me(βφL) - methyl β-phenyllactate. Me(CinβφL) - methyl O-cinnamoyl-β-phenyllactate. Me(Cin) - methyl cinnamate. βφL-β-phenyllactate. Cin - cinnamate. (CPD)A - carboxypeptidase A. CPM - counts per minute. * - labelled material.

[b]All entries are averages from several runs. The enzyme concentrations used varied from $2 \times 10^{-8}$ to $3 \times 10^{-6}$ M.

[c]At pH 7.50, 25° in 0.5 M NaCl with $[\beta\varphi L^*]_o = 0.005$ M, $[Cin\beta\varphi L]_o = 0.005$ M.

[d]At pH 7.50, 25° in 0.5 M NaCl with $[Cin^*]_o = 0.05$ M, $[Cin\beta\varphi L]_o = 0.005$ M.

[e]At pH 7.50, 25° in 0.5 M NaCl with $[Cin^*]_o = 0.03$ M, $[\beta\varphi L]_o = 0.03$ M, $[Cin\beta\varphi L]_o = 0.0043$ M.

[f]Reflects non-quantitative conversion of acids to methyl esters upon treatment with $CH_2N_2$ in ether. Unesterified acids remain at origin on TLC plate when developed with ($CCl_4/CHCl_3$) solvent system... see text.

enzymatic and non-enzymatic control runs in all cases is quite close, certainly within experimental error. In addition, we have found upon analyzing TLC chromatograms for the isotopically labelled esters by themselves that radioactivity is smeared out enough upon TLC development to account entirely for the activity found in the methyl-O-cinnamoyl-L-β-phenyllactate spots.

These results indicate quite clearly that within a fraction of 1%, no return of either product to the substrate is found in the carboxypeptidase A-catalyzed hydrolysis of O-cinnamoyl-L-β-phenyllactate. We cannot of course absolutely rule out the mechanism indicated in eq. 1 on the basis of these results since the nature of the observations made depends upon the relative magnitude of the forward and reverse rate constants implied in $K_1$ and $K_2$ and upon their relationship to the catalytic rate constant, $k_3$. It is entirely conceivable that this mechanism could be correct and that the net forward reaction is so highly favored over the reverse that what little exchange does occur escapes detection in our experiments. Nevertheless, we feel that our findings are quite meaningful and important in that they make it possible to rule out any mechanism which calls for transesterification of really major proportions under our experimental conditions. We are also led to believe that our results, coupled with the failure of previous investigations to demonstrate an acyl-carboxypeptidase intermediate, suggests the importance of exploring the possibility that catalysis by carboxypeptidase may not be mechanistically analogous to the catalytic action of some of the other better understood proteolytic enzymes. Experiments designed to test alternative mechanistic hypotheses are currently underway in our laboratories.

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