REACTION OF p-NITROPHENYL TRIMETHYLACETATE WITH YEAST CARBOXYPEPTIDASE. EVIDENCE FOR AN ACYL-ENZYME INTERMEDIATE

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Summary: The kinetics of the hydrolysis of p-nitrophenyl trimethylacetate catalyzed by yeast carboxypeptidase have been measured under conditions of substrate in excess and indicate that the release of p-nitrophenol in two discrete stages can be observed. A fast release of p-nitrophenol in a concentration approximating that of the enzyme is seen initially, followed by a slow release, corresponding to the "turnover" reaction of the ester. These observations provide strong support for the postulation of a three step reaction sequence including the formation and decomposition of not only a Michaelis complex but also an acyl-enzyme species.

Despite the likelihood that acyl-enzyme species may be involved in the catalytic action of the pancreatic metalloenzyme carboxypeptidase A on ester and peptide substrates (1-3), the direct detection of such intermediates has not as yet been accomplished. We wish to report now that we have been able to obtain evidence for the stepwise reaction of an ester with yeast carboxypeptidase (4-7) which strongly supports the postulation of an acyl-enzyme as an intermediate in the reaction sequence.

When yeast carboxypeptidase, an enzyme which does not have a metal ion requirement, was purified by the method of Hayashi et al. (6) and was reacted at 25° with excess p-nitrophenyl trimethylacetate in sodium phosphate buffer, pH 7.04, μ = 0.1, containing 2.4% acetonitrile, a rapid burst of p-

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nitrophenol could be observed by measurement at 400 nm on a Cary 15 spectrophotometer. The burst was followed by a slower zero-order release of p-nitrophenol, corresponding to a "turnover" reaction. Typically, when 1.03×10^{-4} M p-nitrophenyl trimethylacetate was reacted with 3.59×10^{-6} M yeast carboxypeptidase (concentration calculated on the basis of an assumed molecular weight of 61,000 (6)), an absorbance burst at 400 nm which indicated the rapid release of 3.59×10^{-6} M p-nitrophenol was seen. From the subsequent slow zero-order release of p-nitrophenol a k_{cat} value of 1.92×10^{-2} sec^{-1} was calculated. The kinetic behavior seen is highly reminiscent of that for the reaction of p-nitrophenyl trimethylacetate with α-chymotrypsin (8). In the α-chymotrypsin case, just as we have seen for yeast carboxypeptidase, a burst of p-nitrophenol approximating the enzyme concentration was observed, followed by a slower zero-order release of p-nitrophenol. By analogy with the arguments presented for the α-chymotrypsin reaction, the pathway shown in equation 1 below seems to account most reasonably for our observations on the reaction of p-nitrophenyl trimethylacetate with yeast carboxypeptidase. According to this scheme, a Michaelis complex, ES, is first formed between the enzyme and substrate which then decomposes to give an acyl-enzyme, ES' with release of p-nitrophenol, P_1. Subsequently, the acyl-enzyme decomposes, releasing the carboxylic acid product, P_2, and the free enzyme which then can react further with the p-nitrophenyl ester. As already demonstrated, the scheme of equation 1 gives rise to the relationships in equation 2.

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$+ P_1$$

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \quad \text{and} \quad K_{m(app)} = \frac{K_s k_3}{k_2 + k_3} \quad (2)$$

To analyze the rate behavior in the burst portion of the reaction between p-nitrophenyl trimethylacetate and yeast carboxypeptidase and to provide further support for the postulation of the sequence in equation 1, kinetic measurements were performed using a Durrum-Gibson stopped-flow spectrophotometer at a wavelength of 400 nm under conditions of substrate in excess. After correction for the slow zero-order reaction, the initial release of p-nitrophenol was found to follow pseudo first-order kinetics, and a plot of the reciprocals of the observed rate constants $1/k_{obs}$ vs $1/S$ gave the values of k_2 and K_s shown in Table 1. Using these values, together with that for k_{cat}, the k_3 and $K_{m(app)}$ values given in Table 1 were computed by means of the relationships of equation 2.

Considering the difference in the pH values at which the measurements

TABLE 1

COMPARISON OF HYDROLYTIC ACTION OF YEAST CARBOXYPEPTIDASE WITH THAT OF α-CHYMOTRYPSIN ON p-NITROPHENYL TRIMETHYLACETATE

Parameter	Yeast Carboxypeptidase[a]	α-Chymotrypsin[b]
k_2	$0.58\ sec^{-1}$	$0.37 \pm 0.11\ sec^{-1}$
k_3	$1.99 \times 10^{-2}\ sec^{-1}$	$(1.3 \pm 0.03) \times 10^{-4}\ sec^{-1}$
K_s	1.96×10^{-5} M	$(1.6 \pm 0.5) \times 10^{-3}$ M
k_{cat}	$1.92 \times 10^{-2}\ sec^{-1}$	$1.3 \times 10^{-4}\ sec^{-1}$
$K_{m(app)}$	6.5×10^{-7} M	5.6×10^{-7} M

[a] Conditions: sodium phosphate buffer, pH 7.04, $\mu = 0.1$, 2.4% CH_3CN, 25.0°

[b] Conditions: Tris-HCl buffer, pH 8.17, $\mu = 0.06$, 1.8% CH_3CN, 25.0°.

See reference 8.

have been made, Table 1 shows that the k_2 step proceeds approximately equally rapidly in the reaction of p-nitrophenyl trimethylacetate with α-chymotrypsin as it does for the corresponding reaction with yeast carboxypeptidase and, similarly, the $K_{M\,(app)}$ values are very close. The principal differences in the kinetic behavior of p-nitrophenyl trimethylacetate with the two enzymes are that the K_s value for the binding of the ester to yeast carboxypeptidase is considerably smaller than that for its binding to α-chymotrypsin, and the k_3 value for the deacylation of the intermediate acyl-enzymes is considerably larger in the case of yeast carboxypeptidase than it is for α-chymotrypsin.

We believe that the results described here are the first ones for either metallo- or nonmetallo-carboxypeptidases in which strong direct evidence has been obtained for the formation of covalent enzyme-substrate intermediates. The site of acylation of yeast carboxypeptidase by p-nitrophenyl trimethylacetate has not yet been established firmly. However, the esterase activity of yeast carboxypeptidase can be blocked by reaction of the enzyme with α-toluenesulfonyl fluoride, a reagent which is known to modify a serine residue in the enzyme, and in view of this observation and the other similarities with α-chymotrypsin, it appears likely that the acyl-enzyme formed is produced at a serine residue.

At this point, the relationship between the esterase and exopeptidase activities of yeast carboxypeptidase has not been fully elucidated. Therefore, whether the exopeptidase action of the enzyme proceeds by a route similar to that which we have established for the reaction of p-nitrophenyl trimethylacetate remains an open question to which we are currently addressing ourselves.

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