ON THE ACTION OF CARBOXYPEPTIDASE A ON ESTER SUBSTRATES IN ALKALINE SOLUTION

J. Glovsky,* P. L. Hall,** and E. T. Kaiser***
Departments of Chemistry and Biochemistry
University of Chicago
Chicago, Illinois 60637

Received February 24, 1972

Summary: Kinetic measurements on the pH dependency of the rate parameter k_{cat}/K_M for the Mn^{++}-carboxypeptidase A_δ-catalyzed hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate have shown that an ionizing function in the enzyme with a pK_a value of 9.3 is involved in this reaction. Since experiments on the acetylation of the native γ-form of the enzyme appear to indicate that the phenolic hydroxyl groups of tyrosine residues in carboxypeptidase do not play a crucial role in the enzymatic catalysis of the hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate, it is suggested that the group of pK_a = 9.3 is probably the active site Mn^{++}-H_2O complex.

The determination of the pH dependency of the carboxypeptidase A-catalyzed hydrolysis of the ester O-(trans-cinnamoyl)-L-β-phenyllactate has shown that the kinetic parameter k_{cat}/K_M is affected by the ionization of two functional groups in the enzyme, one with pK_1 = 6.5, and the other with pK_2 = 9.4 (1). The assignment of pK_1 to the ionization of the carboxyl group of Glu 270 at the active site of the enzyme has received considerable support recently

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*National Institutes of Health Predoctoral Trainee.

**National Institutes of Health Predoctoral Fellow.

***Fellow of the Alfred P. Sloan Foundation, 1968-70. Partial support of this work by a grant from the National Institute of Arthritis and Metabolic Diseases is gratefully acknowledged.

from chemical modification studies (2-5). The present communication deals with experiments which bear upon the identification of the ionizing group with $pK_a = 9.4$.

On the basis of the X-ray structural studies (6) on carboxypeptidase A the two most likely groups which could be responsible for the pK_2 ionization appear to be Tyr 248 or the active site zinc-water complex (7). It has been reported that acetylation of tyrosine residues in carboxypeptidase A eliminates enzymatic activity toward the ester O-acetyl-L-mandelate (8). However, the pK_2 value seen in the k_{cat}/K_M-pH profile for the hydrolysis of this compound by the γ-enzyme is 7.5 (9), a value very significantly below that found for O-(trans-cinnamoyl)-L-β-phenyllactate. It has been suggested that the pK_2 value observed in the hydrolysis of O-acetyl-L-mandelate may be due to the ionization of the phenolic hydroxyl group of Tyr 248 (7). If this suggestion is valid, then presumably the pK_2 value observed for O-(trans-cinnamoyl)-L-β-phenyllactate should be assigned to the ionization of another group, probably the active site zinc-water complex, and the possibility must be considered that Tyr 248 may not play a significant role in the hydrolysis of this substrate.

In line with such a hypothesis, we have found that the acetylation of carboxypeptidase A has little effect on the enzymatic hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate, a compound which shows neither substrate activation or inhibition (1), anomalies commonly seen in the hydrolysis of synthetic substrates. This provides strong evidence that the presence of a proton in the phenolic group of Tyr 248 is not required for the action of carboxypeptidase on this substrate. Acetylation of carboxypeptidase A_γ was carried out at room temperature with acetyl imidazole in 0.05 M Tris buffer containing 2 M NaCl at pH 7.5 (10). Spectral evidence (11) indicated

that three tyrosine residues were acetylated and the kinetic properties of the acetylated enzyme obtained corresponded to those described in the literature for enzyme acetylated near the active site (10, 11). At pH 7.5 in 0.005 M Tris buffer at 25°, $K_M = 1.87 \times 10^{-4}$ M and $k_{cat} = 67$ sec^{-1} for native carboxypeptidase A_γ. Under the same conditions, we have found that $K_M = 1.44 \times 10^{-4}$ M and $k_{cat} = 35$ sec^{-1} in the case of our acetylated enzyme. Thus the only appreciable effects of acetylation were an approximately two-fold decrease in the k_{cat} value and a very slight decrease in the K_M value.

Support for the assignment of the pK_2 value > 9 to the ionization of the active site metal ion-water complex has been obtained from an examination of the pH dependency of the hydrolysis of O-(trans-cinnamoyl)-L-β-phenyllactate catalyzed by the Mn^{++} form of carboxypeptidase A_δ. The native form of the latter enzyme species was prepared from an acetone powder of beef pancreas by the procedure of Allan et al. (12), and the active site zinc ion was replaced by Mn^{++} using the method of Coleman and Vallee (13).

Analysis (1) of the pH dependency of k_{cat}/K_M for the manganese enzyme revealed that the pK_2 value was 9.3. The value of $(k_{cat}/K_M)_{lim}$ was 1.7×10^5 M^{-1} sec^{-1}. For comparison, similar measurements were carried out with the zinc form of carboxypeptidase A_δ, and the pK_2 value obtained was 9.2 and that of $(k_{cat}/K_M)_{lim}$ was 3.7×10^5 M^{-1} sec^{-1}. In agreement with the hypothesis that the pK_2 value we have found for the action of Mn^{++}-carboxypeptidase A_δ on O-(trans-cinnamoyl)-L-β-phenyllactate is due to the ionization of the active site metal ion-water complex, nmr dispersion experiments indicate that there is no proton ionization from the hydration shell of manganese carboxypeptidase A in the pH range from 8 to 9 (14). It appears from the latter experiments that the pK_a value for the ionization of

a water ligand bound to Mn^{++} in the enzyme must be above 9.

While the implication of the present findings that the ionization constants for active site Zn^{++} and Mn^{++}-water complexes in the metallocarboxypeptidases are very similar may be somewhat surprising, there are no adequate inorganic models with which to compare our results. With the data presently in hand, the pK_2 assignments we have made seem most reasonable.

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