MODIFICATION OF THE ACETYL AND NITRO DERIVATIVES OF CARBOXYPEPTIDASE A BY *N*-BROMOACETYL-*N*-METHYL-L-PHENYLALANINE

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1. Introduction

Glutamic acid 270 and tyrosine 248 have been implicated in the catalytic mechanism of carboxypeptidase A. The γ-carboxylate of glutamic acid 270 is believed to function as nucleophile in the hydrolysis of peptide and ester substrates [1]. Modification of glutamic acid 270 either by Woodward reagent K [2,3] or by the affinity label, *N*-bromoacetyl-*N*-methyl-L-phenylalanine (BrAcN(Me)Phe) [4,5] produces loss of enzymatic activity. The reaction of carboxypeptidase A with BrAcN(Me)Phe obeys saturation kinetics allowing separate estimation of the contributions of binding and reactivity (i.e. nucleophilicity and orientation) to the observed rate of inactivation.

Modification of tyrosine 248 by several reagents, including *N*-acetylimidazole [7] produces enzyme derivatives with greatly reduced peptidase activity. Lipscomb has proposed that the phenolic hydroxyl of tyrosine 248 serves as proton donor in the hydrolysis of peptide substrates based both on the effect of chemical modification of tyrosine 248 and upon the location of this residue relative to the susceptible peptide bond as determined by X-ray crystallographic analysis [1]. Alternatively, tyrosine 248 could function by orienting the substrate for nucleophilic attack by glutamic acid 270 rather than by direct participation in catalysis.

The affinity labeling of the nitro and acetyl derivatives of carboxypeptidase A by BrAcN(Me)Phe was undertaken to further elucidate the role of tyrosine 248.

2. Experimental

2.1. *Materials*

Carboxypeptidase A_γ^{Leu} was prepared from commercial, crystalline enzyme (Worthington Biochemical Corp.) by ion exchange chromatography in the presence of 3-phenylpropionate [8].

Nitro-carboxypeptidase was prepared by treating the enzyme with tetranitromethane (Aldrich Chemical Co.) in 1 M NaCl–0.05 M Tris–HCl, pH 8.0 for 45 min at 25°C [6]. Enzyme derivatives containing 1.4 and 3.6 residues of 3-NO_2-tyrosine per molecule were prepared by employing eight-fold and sixty-fold molar excesses of reagent, respectively. Nitro-carboxypeptidases A_γ^{Leu} was separated from a small amount of cross-linked species and from excess reagents by chromatography on a column of Sephadex G-100 (Pharmacia).

Acetyl-carboxypeptidase A_γ^{Leu} was prepared using a sixty-four fold molar excess of *N*-acetylimidazole as described previously [7]. De-acetylation of the modified tyrosine residues was effected by incubating acetyl-carboxypeptidase with 1.18 M hydroxylamine-HCl at pH 7.5 [7].

N-Bromoacetyl-*N*-methyl-L-phenylalanine was prepared as described previously [4].

2.2. *Methods*

Protein concentrations of stock solutions of native acetyl- and nitro-carboxypeptidase A_γ^{Leu} were deter-

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mined by amino acid analysis after acid hydrolysis of appropriate aliquots. Amino acid analysis was also employed to assess the extent of modification of tyrosine by tetranitromethane.

Peptidase activity of carboxypeptidase and its derivatives was determined at pH 7.5 and 25°C using 1.18 mM carbobenzoxyglycyl-L-phenylalanine (Fox Chemical Co.) as substrate [9]. Esterase activity toward 10 mM hippuryl-D-L-β-phenyllactic acid (Cyclo Chemical Corp.) was determined at 25°C by pH titration using a pH-stat and toward 1 mM substrate by monitoring the increase in absorbance at 254 mM [10].

The rates of inactivation of carboxypeptidase and its derivatives by various concentrations of BrAcN-(Me)Phe were determined in 1 M NaCl–0.01 M Tris–HCl, pH 7.5 at 25°C [4]. The half-times of inactivation ($t_{1/2}$) were determined from semilogarithmic plots of remaining esterase activity (determined with 1 mM BzGlyOPhe) against time. The constants K_{inact}, the concentration of BrAcN(Me)Phe producing half-maximal rate of inactivation, and T, the half-time of inactivation extrapolated to saturating levels of the reagent, were calculated by plotting $t_{1/2}$ against the reciprocal reagent concentration according to eq. 1 [4,11].

$$t_{1/2} = \frac{K_{inact}\, T}{[\text{BrAcN(Me)Phe}]} + T \qquad (1)$$

3. Results

3.1. *Nitro-carboxypeptidase A*

Treatment of carboxypeptidase with low concentrations of tetranitromethane resulted in preferential modification of a catalytically functional tyrosine residue [6]. In analogy with the nitration of the homologous enzyme, carboxypeptidase B [12], this residue has been tentatively identified as tyrosine 248. Enzyme derivatives containing on the average 1.4 and 3.6 3-NO_2-tyrosine residues per molecule exhibited decreased peptidase activity toward Z–Gly–Phe (10% of original) and slightly increased esterase activity (120% of original) using 10 mM Bz–Gly–OPhe as substrate. These results are in agreement with previous observations [6].

Inactivation of nitro-carboxypeptidase by BrAcN-(Me)Phe followed first order kinetics with respect to remaining active enzyme suggesting that the various forms of nitro-carboxypeptidase B present in each preparation of modified enzyme all react at approximately the same rate.

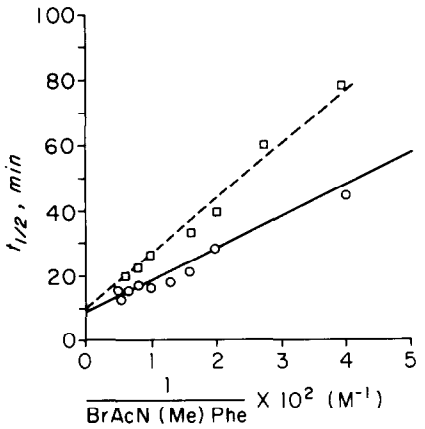

Fig. 1. Half-time of inactivation of nitro-carboxypeptidase A as a function of the reciprocal concentration of BrAcN(Me) Phe. Enzyme derivatives contained on the average 1.4 (○——○) and 3.6 (□——□) residues 3-NO_2-tyrosine per molecule.

The effect of concentration of BrAcN(Me)Phe on the rates of inactivation of the nitro-carboxypeptidase A derivatives is presented in fig. 1. Nitration of tyrosine-248 appears to weaken slightly reversible binding of BrAcN(Me)Phe to enzyme as suggested by increased values of K_{inact} for the nitro-carboxypepidases (table 1), and to decrease the apparent first order rate constant ($ln2/T$) of the reaction of BrAcN(Me)-Phe with glutamic acid 270 in the enzyme-inhibitor complex (table 1). These effects are slightly more pronounced with the enzyme derivative containing an average of 3.6 residues of 3-NO_2-tyrosine per molecule.

3.2. *Acetyl-carboxypeptidase A*

Acetylation of carboxypeptidase A with *N*-acetylimidazole generates an enzyme derivative of decreased peptidase activity toward Z–Gly–Phe (20% of original) and substantially increased (300% of original) esterase activity using 10 mM Bz–Gly–OPhe as substrate, in reasonable agreement with previous reports [7,9]. Under the conditions employed in these experiments, an average of four tyrosine residues (presumably including tyrosine 248) are acetylated with little modification of other types of residues.

Table 1
Kinetic parameters of the reaction of native and modified carboxypeptidase A with BrAcN(Me)Phe*

Enzyme	T (Min)	K_{inact} (mM)
Native	3.6 ± 0.4	4.8
Acetylated	1.7 ± 0.1	10.2
Acetylated, treated with NH_2OH	3.4 ± 0.5	4.9
Nitrated (3.6 residues of 3-NO_2-Tyr/molecule)	11.7 ± 1.7	13.0
Nitrated (1.4 residues of 3-NO_2-Tyr/molecule)	9.3 ± 0.8	9.3

* T = half-time of inactivation, extrapolated to saturating levels of BrAcN(Me)Phe.
K_{inact} = concentration of BrAcN(Me)Phe producting half-maximal rate of inactivation.
Values for T and K_{inact} were determined by least-squares analysis of plots of $t_{1/2}$ against BrAcN(Me)Phe^{-1}. T and K_{inact} for acetylcarboxypeptidase were estimated at concentrations of BrAcN(Me)Phe (2.5–6.6 mM) at which excess inhibitor protection was not observed. For experimental details see text.

The effect of concentration of BrAcN(Me)Phe on the rate of inactivation of acetyl-carboxypeptidase A is presented in fig. 2. It is apparent that acetylation, like nitration, results in weaker reversible binding of BrAcN(Me)Phe to the enzyme (table 1). However, unlike the case of nitro-carboxypeptidase A, glutamic acid 270 in the acetyl derivative of the enzyme appears to be more reactive toward BrAcN(Me)Phe than in the unmodified enzyme (i.e. T is decreased).

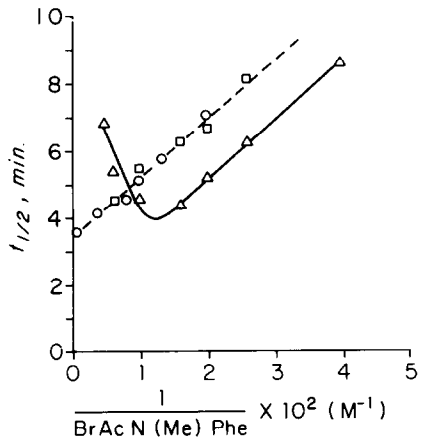

Fig. 2. Half-time of inactivation of (1) carboxypeptidase A (○—○), (2) acetyl-carboxypeptidase A (△—△), and (3) the acetylderivative of carboxypeptidase A after hydroxylamine treatment (○——○) as a function of the reciprocal of BrAcN(Me)Phe concentration.

Additionally, at concentrations of BrAcN(Me)Phe greater than 10 mM, $t_{1/2}$ of acetyl-carboxypeptidase A increases as the inhibitor concentration is increased. This effect may be due to excess inhibitor protection and presumably is analogous to excess substrate inhibition. Thus, acetylation of carboxypeptidase A may generate additional binding sites for BrAcN(Me)Phe allowing formation of complexes of enzyme with two or more molecules of reagent which diminish the reactivity of glutamic acid 270 toward the inhibitor. In contrast, acetylation of carboxypeptidase A apparently *relieves* the inherent excess substrate inhibition toward N-blocked dipeptides [9].

4. Discussion

The proposal that tyrosine 248 serves as a proton donor in the carboxypeptidase A catalyzed hydrolysis of peptide substrates is based in part upon the loss of peptidase activity upon acetylation or nitration of the enzyme. Conceivably, tyrosine 248 could also function by orienting the substrate for nucleophilic attack by the γ-carboxylate of glutamic acid 270. If the latter were the case, the nitro- and acetyl-derivatives of carboxypeptidase A would have diminished peptidase activity due to the inability of the modified tyrosine 248 to properly orient the substrate.

Like peptide substrates, BrAcN(Me)Phe binds at the active site of carboxypeptidase A. The inactivation of the enzyme by this reagent occurs by alkylation of the γ-carboxylate of glutamic acid 270. The favorable orientation of BrAcN(Me)Phe and glutamic acid 270 accounts for most of the enhancement in the rate of reaction relative to that of model systems [4].

If the unmodified form of tyrosine 248 is required for the correct orientation of peptide substrates for nucleophilic attack by glutamic acid 270, it could be expected that a similar requirement would hold for the correct orientation of BrAcN(Me)Phe. Since modification of tyrosine 248 by acetylation or by nitration has relatively little effect on the apparent first order rate constant for the reaction with BrAcN(Me)Phe

(see table 1), it seems likely that tyrosine 248 plays no role in the orientation of the affinity label but functions primarily in a catalytic rather than a steric role in the carboxypeptidase A catalyzed hydrolysis of peptide substrates.

Acknowledgement

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