CHEMICAL EVIDENCE FOR A FUNCTIONAL CARBOXYL GROUP IN CARBOXYPEPTIDASE A*

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Modification of carboxypeptidase A with 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-p-toluene sulfonate at pH 6.0 simultaneously abolishes both esterase and peptidase activities. Inactivation is due to a specific reaction with a single residue in the active center of the enzyme most probably the carboxyl group of glutamic acid 270, previously implicated in the catalytic mechanism by X-ray crystallography.

Chemical modifications of functional tyrosyl, arginyl and histidyl residues of carboxypeptidase A invariably result in a marked decrease or abolition of peptidase activity, while esterase activity usually either remains unchanged or, in fact, increases (1). We have recently found that modification with the carboxyl group reagent, 1-cyclohexyl-3-(2-morpholino-ethyl)-carbodiimide metho-p-toluene sulfonate (CMC), simultaneously alters both activities, decreasing esterase and peptidase activities in parallel (1). The present report indicates that this inactivation is due to the specific modification of a single residue in the enzyme.

EXPERIMENTAL: Modification of carboxypeptidase (Worthington) was generally carried out at 0° using a protein concentration of 0.13 mM and 0.02 M CMC (Aldrich) in 0.05 M 2(N-morpholino)ethanesulfonate (MES)-1M NaCl buffer at pH 6.0. The reaction was stopped by a 100-fold dilution with 0.02 M Veronal-1M NaCl, pH 7.5, buffer or by the addition of one tenth volume of 1.1 M

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β-phenylpropionate followed by successive dialyses against 0.1 M β-phenylpropionate-0.05 M Tris-1M NaCl, pH 7.5 and then the same buffer without β-phenylpropionate. Esterase and peptidase activities were determined using hippuryl-DL-phenyllactate and carbobenzoxyglycyl-L-phenylalanine as substrates respectively, as previously described (2). Incorporation of ^{14}C-CMC (New England Nuclear) was determined on aliquots of samples separated from excess reagent by dialysis or gel filtration on Bio-Gel P4 (Bio-Rad) using a Packard Model 3014 Tri-Carb scintillation counter. Samples were dissolved for counting in 10-15 ml Scintisol-complete (Isolab, Inc.).

RESULTS AND DISCUSSION: Treatment of carboxypeptidase with CMC virtually abolishes both esterase and peptidase activities in about 1 hour, at pH 6, and partially at higher pH values (Fig. 1A). A plot of inactivation vs pH results in a sigmoid curve (Fig. 1B) with an inflection at about pH 7. Due to the intrinsic instability of carboxypeptidase, modification could not be carried out at pH values below 6.

The reaction of carbodiimides with protein usually forms O-acyl urea derivatives of carboxyl groups which undergo acyl transfer to suitable

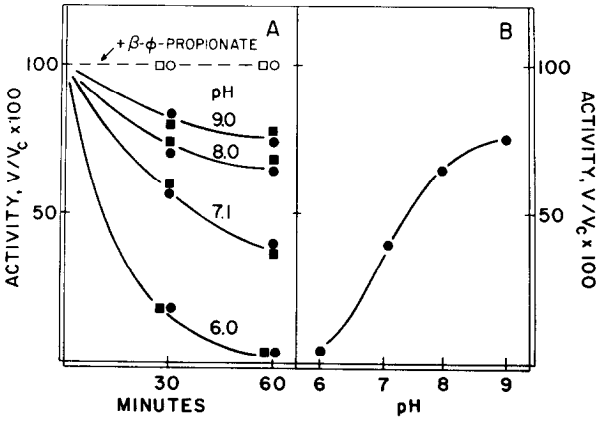

Fig. 1. A. Changes in esterase (●) and peptidase (■) activities on reaction of carboxypeptidase with CMC at the pH values indicated. Open symbols: reaction at pH 6.0 plus 0.1 M β-phenylpropionate.
B. Esterase activity remaining after reaction of carboxypeptidase with CMC for 60 minutes as a function of pH.

nucleophiles (3). Alternatively the O-acyl urea can rearrange to the more stable N-acyl urea. Modification of carboxypeptidase in the presence of 1 M ethyl glycinate did not result either in different rates of inactivation or of activities of the product, compared to those observed in its absence. This suggests that the inactivation may be due to the formation of an N-acyl urea derivative of the enzyme, an interpretation supported by the fact that dialysis does not reverse the inactivation.

The addition to the reaction mixture of 0.1 M β-phenylpropionate, a potent inhibitor of carboxypeptidase prevents inactivation (Fig. 1A), but the same concentration of acetate, a poor inhibitor, does not. The carboxyl group of either acetate or β-phenylpropionate might potentially react with the carbodiimide; however, the failure of acetate to protect against inactivation denotes that most probably the effect of β-phenylpropionate is due to the formation of an adsorption complex with the enzyme.

Incorporation of the reagent into carboxypeptidase was measured directly by means of ^{14}C-CMC. Loss of esterase activity and incorporation of ^{14}C-CMC follows a similar time course; β-phenylpropionate prevents loss of esterase activity completely and ^{14}C-CMC incorporation partially (Fig. 2). In both instances the time course of ^{14}C-CMC incorporation is biphasic. In the absence of β-phenylpropionate, loss of activity is concurrent with the rapid initial incorporation of ^{14}C-CMC. Extrapolation of the two curves representing ^{14}C-CMC incorporation in the presence and absence of β-phenylpropionate to zero time gives a difference of 1.06 residues, correlating with the inactivation of the enzyme. Such selective labeling is reminiscent of that with pseudosubstrates and indicates modification via a specific process. This was confirmed by kinetics.

The time course of inactivation depends upon the initial concentration of CMC (Fig. 3). However, with increasing concentration of reagent the rate of inactivation becomes maximal, indicative of saturation kinetics, implying the formation of a CMC-enzyme adsorption complex prior to inactivation.

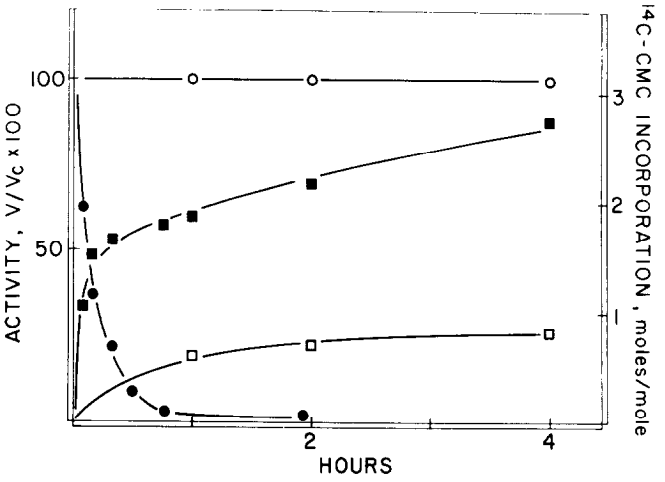

Fig. 2. Changes in esterase activity (●, ○) and incorporation of radioactivity (■, □) on reaction of carboxypeptidase with ^{14}C-CMC at pH 6.0 in the absence (closed symbols) and presence (open symbols) of 0.1 M β-phenylpropionate.

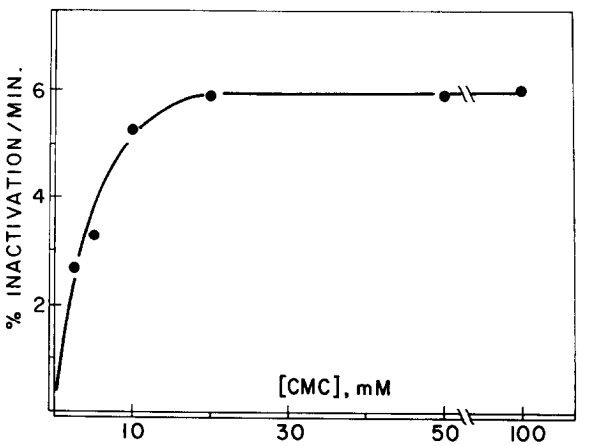

Fig. 3. Rate of inactivation of carboxypeptidase by CMC as a function of reagent concentration. Rates were calculated from the slope of semilogarithmic plots of inactivation vs. time.

Thus, in this instance, CMC exhibits the characteristics of an active site directed, irreversible inhibitor.

A number of different functional groups of the enzyme might conceivably combine with CMC. Primary amines in the unprotonated form will react to give

substituted guanidines. But, at the pH employed it is unlikely that the observed inactivation would be due to lysine modification. Moreover, in all previous studies reagents which react with lysine have failed to alter the activities of carboxypeptidase. Histidyl residues can also give substituted guanidines with CMC, but the pH dependence of inactivation observed here (Fig. 1B) is opposite to that expected for histidine modification. Neither lysine or histidine of CMC-inactivated carboxypeptidase is lost as judged by amino acid analysis, direct evidence that the reagent does not affect these residues. Tyrosyl modification has been ruled out by sequential modifications. Acetylation with acetic anhydride abolishes peptidase activity virtually completely but increases esterase activity five-fold. On treatment with CMC, esterase activity falls to less than 5%. Hence, tyrosine modification does not prevent the inactivation by CMC. Further, subsequent incubation with 1 M hydroxylamine deacylates the tyrosyl residues but does not restore either activity.

Thus, a carboxyl group is the most likely site for the CMC reaction. Indirect evidence for a carboxyl group has been obtained by treating the CMC-labeled enzyme with 2 M hydroxylamine in 8 M urea. Approximately 50% of the radioactivity is released from the protein after 48 hours while the control remains unchanged, typical of N-acyl ureas which are more labile than usual peptide bonds (4). This treatment would readily hydrolyze tyrosyl derivatives but not lysyl or histidyl derivatives. Such conclusions are consistent with analogous recent findings using N-ethyl-5-phenylisoxazolium-3-sulfonate (5). X-ray crystallographic studies have implicated glutamic acid residue 270 as a component of the active center of carboxypeptidase (6), and preliminary peptide isolation experiments are consistant with the location of the label at or near this site.

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