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Mechanistic studies on carboxypeptidase A from goat pancreas Part I: Role of tyrosine residue at the active site

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Abstract. Chemical modification of carboxypeptidase Ag_1 from goat pancreas with N-acetylimidazole or iodine led to loss of enzymic activity. This loss in activity could be prevented when chemical modification was carried out in the presence of β-phenylpropionic acid or substrate NCbz-glycyl-L-phenylalanine, thus suggesting a tyrosine residue at the active site. Chemical modification of tyrosine was confirmed by spectral and kinetic studies. While tyrosine modification destroyed peptidase activity, esterase activity of the enzyme remained unchanged thus indicating non-involvement of tyrosine residue in ester hydrolysis.

Keywords. Tyrosine; active site; goat carboxypeptidase; iodine; N-acetylimidazole.

Introduction

Carboxypeptidase A from bovine pancreas was crystallised in 1937 (Anson, 1937) and its X-ray map was made available in 1968 (Lipscomb *et al*., 1968). Enzyme was shown to contain one atom of zinc per mol, with histidine at 69 and 196 and γ-carboxylate ion of Glu 72 acting as ligand to zinc (Lipscomb *et al*., 1969; Bradshaw *et al*., 1969). Chemical modification studies confirmed these findings (Simpson *et al*., 1963; Riordan and Vallee, 1963; Riordan *et al*., 1967; Sokolovsky and Vallee, 1967; Riordan and Hayashida, 1970; Petra, 1971).

According to the current view of the bovine carboxypeptidase A catalysis, carboxylate ion can act as a general base catalyst and thus accelerate hydrolysis or it can act as a nucleophile generating an anhydride intermediate that would be labile to hydrolysis (Breslow and Wernick, 1977). Acyl anhydride has been demonstrated only in the case of ester substrate at –25° to –40°C (Makinen *et al*., 1976). However, all attempts to detect anhydride with amide substrate or to trap it with alternate nucleophiles had failed (Kaiser and Kaiser, 1972). Modification of active site tyrosine group enhances the esterase activity while peptidase activity is lowered. Substitution of Zn^{2+} by Hg^{2+} also abolishes peptidase activity but increases esterase activity thus suggesting that the mechanism of catalysis in the two cases may not exactly be the same. Carboxypeptidase A from goat pancreas has been purified and resolved into two isozymes $CPAg_1$ and $CPAg_2$ (Dua and Ralhan, 1981a). The goat enzyme is free from some of the kinetic anomalies (Dua and Dixit, 1973) observed in the case of bovine enzyme (Vallee *et al*., 1968; Davies *et al*., 1968; Auld and Vallee, 1971) and hence may provide a simpler system to work with for the elucidation of the catalytic

mechanism. The goat enzyme differs from the bovine enzyme in N-terminus (Dua and Ralhan, 1981a) and product stabilisation (Dua and Ralhan, 1981b). Species differences have also been observed in the case of yeast carboxypeptidase which has no metal ion and has serine and histidine at the active site. Present studies deal with the chemical modification of goat pancreatic carboxypeptidase Ag_1 which show a role for tyrosine residue at the active site.

Materials and methods

N-acetylimidazole, NCbz-glycyl-L-phenylalanine, hippuryl-L-phenyllactic acid, β-phenylpropionic acid, glycyl-L-phenylalanine were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Iodine was obtained from British Drug House, Bombay.

Goat carboxypeptidase Ag_1 was purified as reported earlier (Dua and Ralhan, 1981a). Carboxypeptidase A activity was determined colorimetrically using NCbz-glycyl-L-phenylalanine as a substrate (Folk and Gladner, 1958) while esterase activity was assayed spectrophotometrically using hippuryl-L-phenyllactic acid by the method of McClure *et al*. (1964). Protein concentration was determined colorimetrically by the method of Lowry *et al*. (1951) and spectrophotometrically by the method of Warburg and Christian (1941). Spectrophotometric studies were made using a Pye Unicam SP-500 spectrophotometer.

Modification of carboxypeptidase Ag_1

Acetylation of goat carboxypeptidase Ag_1 with N-acetylimidazole was performed by the method of Simpson *et al*. (1963). N-acetylimidazole (highly hygroscopic) was dissolved in dry benzene. Suitable aliquots of this solution were evaporated in tubes fitted with standard joint under vacuum and weighed. The reagent was prepared fresh each time. Acetylation was carried out in 50 mM borate buffer, pH 7·5 at 30°C in the presence of 300-fold molar excess of the reagent over the enzyme concentration for 45 min unless stated otherwise. Modified protein was desalted on Sephadex G-25 column (0·9 × 40 cm).

Iodination of goat carboxypeptidase Ag_1 was carried out by the method of Vallee *et al*. (1963) with 50-fold molar excess of iodine over the enzyme concentration at 4°C in 20 mM sodium veronal-200 mM NaCl buffer, pH 7·5 for 60 min unless stated otherewise. Iodination was stopped by the addition of sodium thiosulphate solution. Modified protein was desalted by dialysis against 5 mM Tris-hydrochloride-200 mM NaCl buffer, pH 7·5 with several changes of the buffer. Iodine solution was prepared fresh each time by dissolving weighed quantity of iodine in 50 mM potassium iodide solution.

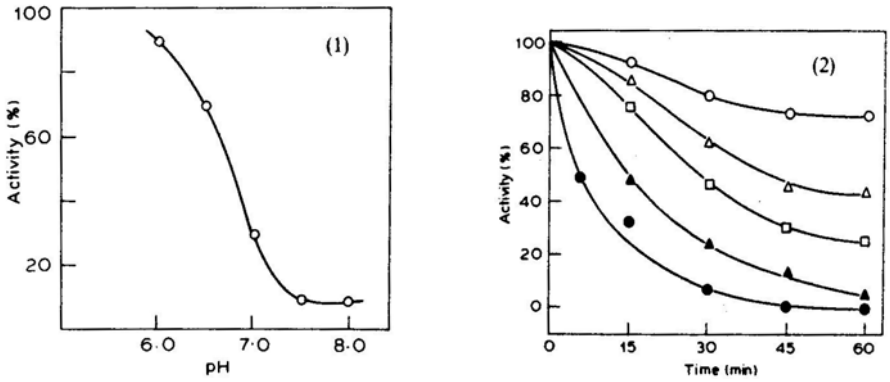
Results and discussion

Treatment of a protein with iodine solution can lead to (i) iodination of tyrosine and/or histidine residues (ii) oxidation of cysteinyl sulphydryl or tryptophan. However,

if the iodination is performed at pH 8·0 or below and in the presence of low concentration of the reagent, iodination may be confined to active site tyrosine. Selective iodination of tyrosyl side chains with loss of respective enzymatic activity has been shown in the case of *Escherichia coli*, asparaginase (Liu and Speer, 1977), bovine carboxypeptidase A (Riordan *et al*., 1967) and D-3-phosphoglyceraldehyde dehydrogenase (Libor and Elodi, 1970).

Preliminary experiments indicated that iodination of tyrosyl residue of goat carboxypeptidase Ag_1 can be optimally carried out at pH 7·5 and 4°C (figure 1). However, this modification which led to loss of enzymic activity was reagent concentration and time dependent. At 20-fold molar excess of the reagent over protein, the enzyme lost 56% of its peptidase activity in 60 min while 100-fold molar excess led to complete loss of enzymic activity within 40 min (figure 2). Iodine is a potent non-specific inhibitor of enzymic activity. Hence that this loss of activity was due to modification and not due to inhibition, was established when exhaustive dialysis of the modified enzymes against 5 mM Tris-200 mM sodium chloride buffer, pH 7·5 for 24 h with four changes of the buffer failed to restore the enzymic activity.

The inactivation due to iodination was prevented when iodination was carried out in the presence of a substrate or its specific inhibitor. Only about 20% activity was lost



Figures 1 and 2. 1. Effect of pH on iodination of goat carboxypeptidase Ag_1. One ml aliquots of the enzyme (protein: 0·2 mg/ml) were incubated for 60 min with 50-fold molar excess of iodine at 4°C at different pHs. Iodination was terminated by addition of 0·2 ml of 50 mM sodium thiosulphate and the reaction mixture dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7·5 for 24 h and assayed for peptidase activity. Controls were run at respective pHs under similar conditions without iodine and per cent activity was calculated by taking control as 100. **2.** Effect of concentration of iodine on the modification of goat carboxypeptidase Ag_1 as a function of time. Four ml aliquots of the enzyme solution (protein: 0·4 mg/ml) were incubated in the absence and presence of 10 (O); 20 (Δ); 30 (□); 50 (▲), and 100 (●) fold molar excess of iodine over the enzyme concentration in 20 mM veronal–200 mM sodium chloride buffer, pH 7·5 at 4°C. Aliquots 0·5 ml were withdrawn at suitable time intervals and dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7·5 for 24 h after terminating iodination by adding 0·2 ml of 0·05 M sodium thiosulphate. Controls were run under similar conditions without the addition of iodine. The per cent activity of the modified enzyme was plotted against time. The control value was taken as 100.

when iodination was done in the presence of β-phenylpropionic acid or NCbz-glycyl-L-phenylalanine as against 93% in their absence (table 1), thus indicating that tyrosine being modified is at the active site and is protected by the presence of substrate or its inhibitor. Tyrosine modification was confirmed by comparing the spectra of the native and modified enzyme which showed an increase in absorption between 290–300 nm (figure 3). Such a shift in absorption from 278 to 290–300 nm has been attributed to tyrosine modification from model experiments (Hughes and Straessle, 1950).

N-acetylimidazole can acetylate ε-amino group of lysine, or OH group of tyrosine. However, it has been shown that acetylation with N-acetylimidazole under controlled

Table 1. Effect of competitive inhibitor or substrate on the modification of goat carboxypeptidase A.

Treatment	Activity[a] (%)
Control	100
Modified with iodine in the absence of inhibitor/substrate	7.2
Modified with iodine in the presence of β-phenylpropionic acid	78·9
Modified with iodine in the presence of NCbz-gly-L-phe	77·5
Modified with N-acetylimidazole in the absence of inhibitor/substrate	15
Modified with N-acetylimidazloe in the presence of β-phenylpropionic acid	96·3
Modified with N-acetylimidazole in the presence of NCbz-glycyl-L-phenylalanine	91·1

[a] Two ml aliquots of the enzyme solution (protein: 0·15 mg/ml) were incubated in the absence and presence of 20 mM β-phenylpropionic acid or NCbz-gly-L-phe in 20 mM veronal – 200 mM sodium chloride buffer, pH 7·5 for 30 min at 30°C and were modified with 50-fold molar excess of iodine at 4°C for 60 min or with 300-fold molar excess of N-acetylimidazole for 45 min at 30°C in 50 mM borate buffer pH 7.5, desalted on Sephadex G-25, dialysed separately against 500 ml of 5 mM Tris-200 mM sodium chloride buffer, pH 7·5 for 18 h with four changes. Peptidase activity was determined in each case. The per cent activity left in the modified enzyme was calculated by taking the activity of unmodified enzyme as 100.

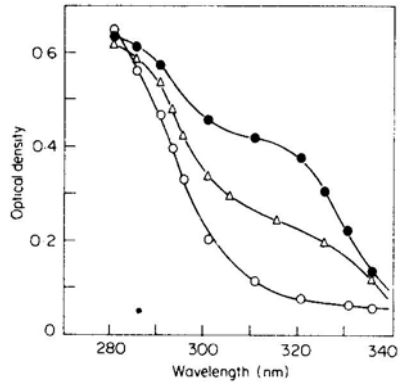


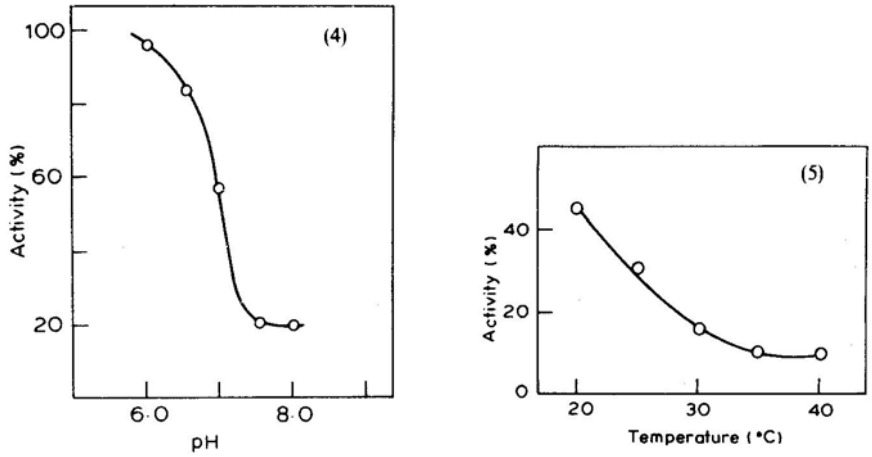
Figure 3. Ultra-violet absorption spectra of native and iodinated goat carboxypeptidase Ag_1. Three ml of enzyme solutions (protein: 0·8 mg/ml) were modified under optimal conditions and dialysed against 5 mM Tris-200 mM sodium chloride buffer, pH 7·5. Ultra-violet spectra of modified and native enzyme were recorded at protein concentrations of 0·6 mg/ml. (O), Native enzyme; (Δ), iodinated enzyme for 30 min; (●), iodinated enzyme for 60min.

conditions leads to selective acetylation of tyrosine residue with loss of respective enzymic activity of rabbit muscle aldolase (Schmid *et al*., 1966; Pugh and Horecker, 1967), bovine carboxypeptidase A (Simpson *et al*., 1963) and rapid loss of allosteric properties of rabbit muscle phosphofructose kinase (Chapman *et al*., 1969).

Preliminary investigation indicated that goat carboxypeptidase Ag_1 was also acetylated on treatment with N-acetylimidazole with concomitant loss of enzymic activity. Highest modification was obtained if the acetylation was carried out between pH 7·5 and 8·0 in 50 mM borate buffer at 30°C (figures 4, 5). However, pH 7·5 was chosen because of the better stability of N-acetylimidazole at this pH. Even at this temperature and pH, the extent of modification depended upon the concentration of the reagent used, *e.g*. incubation with 300-fold molar excess of N-acetylimidazole for 45 min led to 83% loss in activity as against 95% loss in activity in the same period when concentration of the reagent was raised to 450-fold molar excess (figure 6).

The loss in enzymic activity on treatment of carboxypeptidase Ag_1 with N-acetylimidazole was due to modification of the active site amino acid and not due to inhibition by the reagent because dialysis of the treated enzyme against 1 litre of 20 mM Tris-500 mM sodium chloride buffer, pH 7·5 for 24 h with several changes failed to restore the enzymic activity.

That treatment with N-acetylimidazole acetylates specifically tyrosine and not ε-amino group of lysine was confirmed when ultra-violet absorption spectra of the native and acetylated enzyme showed a change in absorption only at 278 nm. Actually,



Figures 4 and 5. 4. Effect of pH on the modification of goat carboxypeptidase Ag_1 by N-acetylimidazole. Two ml aliquots of the enzyme solution (protein: 0·3mg/ml) were incubated with 300-fold molar excess of N-acetylimidazole at 30°C for 45 min at different pHs, desalted on Sephadex G-25 column (0·9 × 40 cm), and their peptidase activity determined. Controls were run at the respective pHs under similar conditions without N-acetylimidazole. **5.** Effect of temperature on the modification of goat carboxypeptidase Ag_1 by N-acetylimidazole. Two ml of enzyme aliquots (protein: 0·3 mg/ml) were incubated with 300-fold molar excess of N-acetylimidazole at different temperatures for 45 min at pH 7·5, desalted on Sephadex G-25 column (0·9 × 40 cm) and assayed for peptidase activity. Control was incubated under similar conditions without N-acetylimidazole. The per cent activity left in each sample was calculated by taking control as 100.

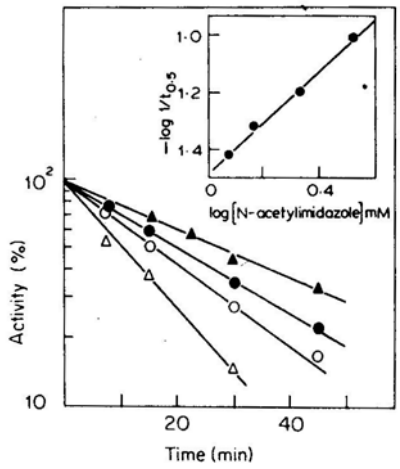


Figure 6. Effect of concentration of N-acetylimidazole on modification of goat carboxypeptidase Ag_1 as a function of time. Eight ml aliquots of the enzyme solution (protein: 0·25 mg/ml) were incubated with 150 (▲); 200 (●); 300 (O), and 450 (Δ) fold molar excess of N-acetylimidazole in 50 mM borate buffer, pH 7·5 at 30°C. Aliquots were withdrawn at different time intervals, desalted on Sephadex G-25 column (0·9 × 40 cm) and assayed for peptidase activity. Control was incubated under similar conditions without N-acetylimidazole. The per cent activity left in each sample was calculated by taking control as 100. *Inset:* Determination of the order of inactivation by N-acetylimidazole.

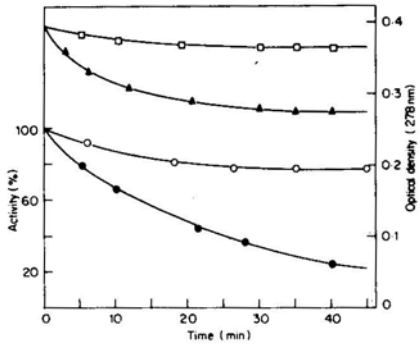


Figure 7. Correlation of loss of peptidase activity and change in optical density at 278 nm on acetylation of carboxypeptidase Ag_1. Three ml of the enzyme solution (protein: 0·4 mg/ml) in 50 mM borate buffer, pH 7·5 was mixed with 0·05 ml of same buffer containing 75 or 300-fold molar excess of N-acetylimidazole over enzyme and incubated under optimal conditions. The absorbance of the enzyme solution containing different amounts of N-acetylimidazole were measured at 278 nm against the blank solution containing 3·0 ml of buffer and 0·05 ml of buffer containing 75 or 300-fold molar excess of N-acetylimidazole.

Aliquots (0·3 ml) were removed from reaction mixture at different time intervals, diluted with buffer to 1·0 ml and assayed for peptidase activity immediately. **A.** Modification in the presence of 75-fold molar excess of N-acetylimidazole. Peptidase activity (O); Change in optical density at 278 nm (□). **B.** Modification in the presence of 300-fold molar excess of N-acetylimidazole. Peptidase activity (O). Change in optical density at 278 nm (▲).

the decrease in optical density at 278 nm and loss in peptidase activity ran very much parallel (figure 7). Thus the enzyme acetylation could be easily followed spectrophotometrically at 278 nm. Treatment of acetylated enzyme with hydroxylamine led to deacetylation of the enzyme with restoration of the peptidase activity from 22 to 97·2% of the original activity (table 2) and also restoration of native enzyme spectrum, thus supporting the view that tyrosine residue is being modified.

Table 2. Deacetylation of acetylated goat carboxypeptidase Ag_1 with hydroxylamine.

Sample	Activity[a] (%)
Native enzyme	100
Acetylated enzyme	22·3
Hydroxylamine treated acetylated enzyme	97·2

[a] One ml of 2 M hydroxylamine solution (pH 7·5) was added (to a final concentration of 330 mM) to 5·0 ml of acetylated protein (0·5 mg/ml) in 50 mM borate – 200 mM sodium chloride buffer, pH 7·5. The reaction mixture was incubated for 15 min at 30°C and excess of hydroxylamine was removed by passing through Sephadex G-25 column (0·9 × 40 cm) and assayed for peptidase activity.

The fact that the tyrosine residue being modified is at the active site was established when the presence of *β*-phenylpropionic acid or NCbz-glycyl-L-phenylalanine in the reaction medium, protected the enzyme from modification. Loss in enzyme activity in their absence was 85% but in their presence it was only 3·7% and 8·9% respectively (table 1). Thus binding of specific inhibitor or substrate at the active site protects the enzyme from acetylation which again suggests that tyrosine being acetylated is at the active site.

Kinetics of modification with N-acetylimidazole

Goat carboxypeptidase Ag_1 was incubated with various concentrations of N-acetylimidazole in 50 mM borate buffer, pH 7·5 at 30°C. Aliquots were withdrawn at regular intervals, desalted on Sephadex G-25 column, and assayed for residual peptidase activity. The inactivation process follows pseudo-first order kinetics at N-acetylimidazole concentrations used (figure 6) since the modifying reagent concentration greatly exceeded that of the enzyme.

The number of molecules of N-acetylimidazole reacting per mol of the enzyme were calculated by plotting $-\log (t_{0\cdot5})^{-1}$ *vs.* the log of N-acetylimidazole concentration (Marcus *et al*., 1976; Hollenberg *et al*., 1971). A slope of 0.9 was obtained (figure 6 inset) indicating that one molecule of N-acetylimidazole reacted with one mol of the enzyme leading to its inactivation. When observed pseudo-first order rate constants (k_{obs}) were

plotted against concentration of N-acetylimidazole (figure 8) a linear plot was obtained suggesting a one step reaction of the following type (Strickland *et al*., 1975):

$$A + B \rightleftharpoons C.$$

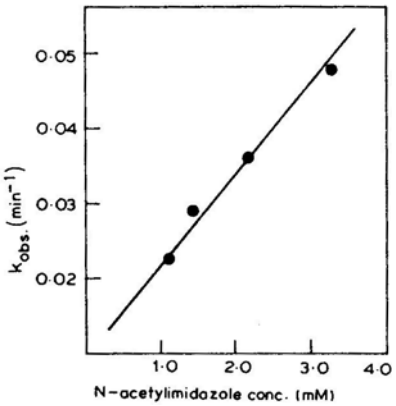


Figure 8. Direct plot of observed pseudo-first order rate constant *vs.* concentration of N-acetylimidazole. Observed pseudo-first order rate constants (k_{obs}) were calculated for 1·09mM, 1·45 mM, 2·18 mM and 3·27 mM concentration of N-acetylimidazole from the semilogarithmic plot of inactivation of goat carboxypeptidase Ag_1 (figure 6). The k_{obs} *vs*. concentration of N-acetylimidazole was plotted and the number of steps involved in the inactivation process determined.

Effect of tyrosine modification on esterase activity

The goat carboxypeptidase Ag_1 has both esterase and peptidase activities. So the question arises whether active site tyrosine is needed for the hydrolysis of both type of substrates or only one type. Hence the enzyme was acetylated under optimal conditions for different time intervals, samples withdrawn, desalted on Sephadex G-25 and assayed for esterase activity. No significant change in esterase activity was observed (table 3). So it can be concluded that tyrosine is not involved in ester hydrolysis. This aspect of enzymic properties was also confirmed by modifying tyrosine residue with iodine. While peptidase activity was almost completely lost on iodination, no significant change in esterase activity was observed.

Such an involvement of a tyrosine residue in bovine carboxypeptidase A catalysed hydrolysis of acyldipeptides has been demonstrated by iodination (Riordan *et al*., 1967), acetylation (Simpson *et al*., 1963), nitration (Sokolovsky and Vallee, 1967), diazotization with diazo-*p*-arsanilic acid (Klesov and Vallee, 1977) or diazotized 5-amino 1-H tetrazole (Cucni and Riordan, 1978). However, in these cases modification of the tyrosine residue led to a significant increase in the esterase activity. No such increase in the esterase activity on modification of a tyrosine residue of goat enzyme has been observed. Hence it is likely that the active site of the enzyme from the two sources differ in detail.

Table 3. Effect of tyrosine modification on esterase activity of goat carboxypeptidase Ag_1.

Time of incubation (min)	Activity[a] (%)
15	92·2
30	97·3
45	95·7
60	93·33
90	92·52
120	90·7

[a] Eight ml aliquot of the enzyme solution (protein: 0·6 mg/ml) was incubated with 300-fold molar excess of N-acetylimidazole under optimal conditions. Aliquots were drawn after 15, 30, 45, 60, 90, 120 min and subjected to gel filtration in Sephadex G-25 column (0·9 × 60 cm). Control was subjected to similar conditions without N-acetylimidazole. Activity of the control was taken as 100.

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