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Functional Arginyl Residues in Carboxypeptidase A. Modification with Butanedione†

James F. Riordan

ABSTRACT: Treatment of carboxypeptidase A with butanedione in borate buffer markedly decreases peptidase activity correlating with the reversible modification of a single arginyl residue. This correlation supports the hypothesis that the specificity of carboxypeptidase is due to interaction of the terminal carboxyl group of peptide substrates with an arginyl residue of the enzyme. However, this modification simultaneously increases apparent esterase activity threefold indicating that the mode of binding of esters differs from that of peptides. If modification is carried out in the absence of borate, peptidase activity still decreases but esterase activity is unchanged. Binding of esters, like that of inhibitors, may involve a metal ion–carboxyl group interaction whereas peptide binding requires an arginyl residue as the positively charged substrate recognition site.

Carboxypeptidases catalyze the hydrolysis of carboxyl-terminal peptide and ester bonds of peptides or their depsipeptide analogs. The presence of a free terminal α-carboxyl group of the substrate is a strict specificity requirement of these enzymes. A positively charged residue in the active center of bovine carboxypeptidase A has long been thought to account for this specificity (Waldschmidt-Leitz, 1931; Smith, 1949; Vallee *et al.*, 1963). Since the zinc atom does not appear to be essential for peptide binding (Coleman and Vallee, 1964), we have considered a positively charged residue such as lysine or arginine to be the most likely alternative. Acylation experiments have excluded lysyl residues from binding functions (Riordan and Vallee, 1963). Hence, it seemed probable that arginine might be important for the specificity of this enzyme, and therefore attempts were made to identify a functional arginyl residue by means of chemical modifications (Vallee and Riordan, 1968; Riordan, 1970).

Reagents suitable for the modification of arginyl residues in proteins under relatively mild conditions have been described only recently (Yankeelov *et al.*, 1968). One of the first of these, butanedione, had been used successfully in the identification of antibody-combining sites and seemed particularly promising (Grossberg and Pressman, 1968).

Indeed, under the conditions to be described this reagent and a number of other α-dicarbonyl compounds block arginyl residues of carboxypeptidase A, and one of these appears to be involved in the productive binding of peptide but not of ester substrates (Vallee and Riordan, 1968). Since then similar effects on activity have been found for carboxypeptidase B suggesting a common basis for their analogous substrate specificities (Werber and Sokolovsky, 1972).

Materials and Methods

Bovine carboxypeptidase A, prepared according to the procedure of Anson (1937), and porcine carboxypeptidase B were obtained from the Worthington Biochemical Corp. Butanedione was a product of Eastman and was distilled immediately prior to use. Crystalline trimeric butanedione was kindly provided by Dr. John A. Yankeelov, Jr. All other chemicals were of the highest grade available. All buffer solutions were extracted with 0.1% dithizone in CCl_4 and other precautions, previously described, were taken to prevent contamination by adventitious metal ions (Thiers, 1957).

In preliminary experiments, a 15% solution of butanedione was incubated overnight at room temperature according to the method of Yankeelov *et al.* (1966) as modified by Grossberg and Pressman (1968) prior to addition to carboxypeptidase. Borate, Tris, Veronal, bicarbonate, and *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonate (Hepes),[1] all at 0.05 M, pH 8.6, were examined for their suitability as buffers. Both borate and Tris were found to interact with butanedione as evidenced by an immediate fall in pH on addition of the reagent to the buffer solution. The remaining three buffers did not appear to react with butanedione nor to have any other specific effects. Subsequently, it was found that preincubation of butanedione was unnecessary for modifying carboxypep-

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received May 8, 1973.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health, of the Department of Health, Education, and Welfare.

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate; Mes, 2-(*N*-morpholino)ethanesulfonate.

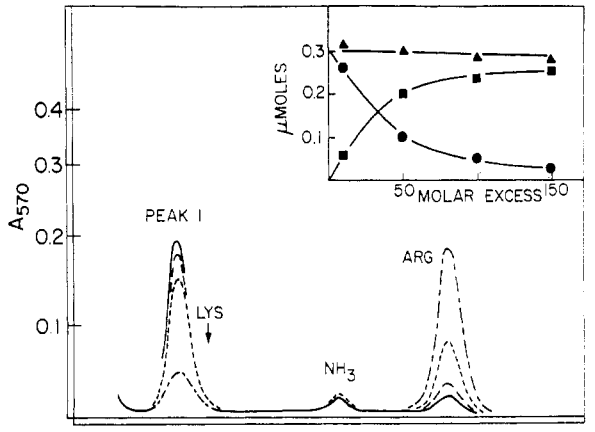


FIGURE 1: Chromatography of arginine–butanedione reaction mixtures. Aliquots containing the equivalent of 0.3 μmol of arginine were applied to the short column of the amino acid analyzer after reaction for 15 min with a 10- (— - —), 50- (- - - -), 100- (— —), and 150- (——) fold molar excess of butanedione. The arrow indicates the normal elution position of lysine. Insert: recovery of arginine (●), peak 1 (■), and arginine plus peak 1 (▲) as a function of the molar excess of butanedione in the reaction mixture.

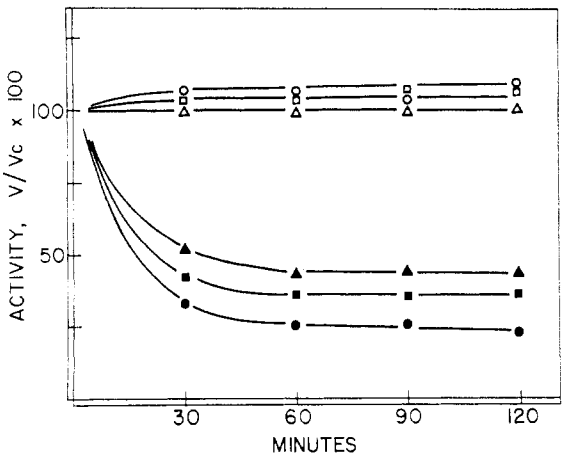


FIGURE 2: Changes in esterase (open symbols) and peptidase (closed symbols) activities on modification of carboxypeptidase (0.15 nM) with 22.5 (▲), 37.5 (■), and 75 (●) mM butanedione in 1 M NaCl, pH 7.5, 20°. Activities are expressed as the ratio of that of the modified enzyme, v, and the unmodified control, v_c, times 100.

tidase and hence standard conditions were adopted which employed freshly prepared solutions of reagent. All reactions were carried out at room temperature (20°) by adding an aliquot of a 10% butanedione solution in an appropriate buffer to a solution of carboxypeptidase (5 mg/ml) in the same buffer. The stock solution of butanedione was adjusted to pH 7.5 prior to addition to the enzyme, and the pH of the reaction mixture was maintained at that value during the course of modification. This adjustment of pH was critical particularly for the reactions carried out in borate buffer. In some instances modification was performed in 1 M NaCl without buffer, and the pH was kept constant by means of an autotitrator.

Peptidase activity was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Cyclo Chemical Corp.) and activity is expressed as a turnover number, k, in units of min^{-1}. Assays were performed either at 0° or 25° by a modification of the ninhydrin method (Snoke and Neurath, 1949) in 0.05 M Tris–1.0 M NaCl (pH 7.5) (Coleman and Vallee, 1961), using a Technicon AutoAnalyzer (Auld and Vallee, 1970). None of the reagents employed interferred with the assay procedure.

Esterase activity was determined by titration (Snoke *et al.*, 1948) with 0.1 M NaOH of the protons released on hydrolysis, using a pH-Stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Assays were performed at 25° using hippuryl L-β-phenyllactate (Cyclo Chemical Corp.) in 0.2 M NaCl–0.005 M Tris (pH 7.5). Activities were calculated as zero-order rate constants with units of equivalents of H^+ released per minute per mole of enzyme.

The reaction of butanedione with arginyl residues of proteins can be followed by amino acid analysis. Previous studies had shown that butanedione oligomers react with arginine, but under the conditions employed complete modification required more than 48 hr (Yankeelov, 1970). It was essential to determine if arginine modification would take place within the much shorter time periods employed under the present conditions. Hence, 1 ml of the amino acid calibration mixture (Spinco Type I) was diluted into 4 ml of 0.05 M borate buffer and reacted for 15 min with 1 mmol of butanedione (approximately 110-fold molar excess with respect to each amino acid) at pH 7.5. An aliquot of the reaction mixture was added to pH 2.2 citrate diluting buffer and applied to the amino acid analyzer. Arginine was 96% modified and all other amino acids were recovered with an average yield of 80%.

A similar procedure was employed to study the modification of arginine, lysine, and an equimolar mixture of the two. Increasing molar excesses of butanedione were added to 5 ml of an aqueous solution of arginine (6×10^{-4} M) and the pH was kept constant at 7.5 for 15 min. The pH was lowered to 2.2 with 1 N HCl and the volume adjusted to 10.0 ml with pH 2.2 citrate diluting buffer. A 1-ml aliquot was applied to the short column of the amino acid analyzer. A new ninhydrin-positive peak could be detected, emerging just ahead of the normal elution volume for lysine (Figure 1). The area under this peak increased in direct proportion to the disappearance of arginine. Using the same integration constant as for arginine, the sum of the micromoles corresponding to peak 1 and arginine was constant (Figure 1, insert). Under the same conditions lysine was not modified, nor did its presence interfere with the modification of arginine. Incubation of the arginine–butanedione reaction mixture for 1 hr in either 1 N HCl or 1 N triethanolamine at 20° prior to dilution with citrate did not regenerate arginine but destroyed the unknown peak 1. These results are similar to those obtained by Yang and Schwert (1972).

Amino acid analysis was also used to determine modification of arginyl residues of carboxypeptidase. Aliquots (0.5 ml) of the reaction mixture were removed at various times and added to 0.2 ml of 6 N HCl to both halt the modification reaction and prevent the regeneration of free arginine (*vide infra*). The precipitated protein was washed with water and suspended in 1 ml of 6 N HCl. Hydrolysis was carried out in sealed, evacuated ampoules at 110° for 18–20 hr and analysis was performed according to Spackman *et al.* (1958) using a Spinco Model 120C amino acid analyzer.

Protein concentrations were measured by absorbance at 278 nm using the Zeiss PMQ II spectrophotometer. A molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} was used for native carboxypeptidase (Simpson *et al.*, 1963).

Carboxypeptidase was acetylated with a 60-fold molar excess of *N*-acetylimidazole in 0.05 M borate–1 M NaCl (pH 7.5) at room temperature (20°) but otherwise as previously described (Simpson *et al.*, 1963). Succinylcarboxypeptidase was

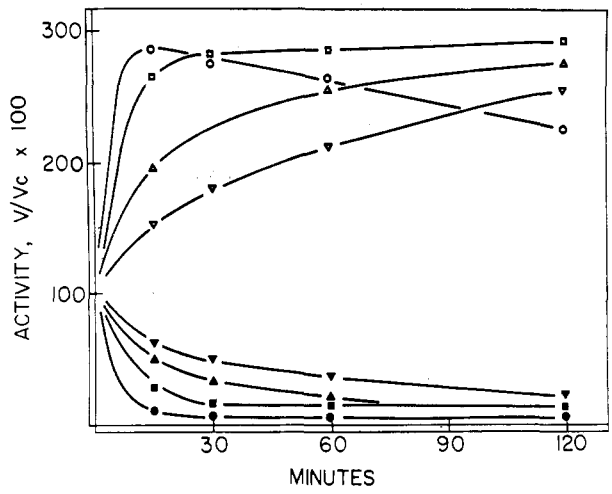


FIGURE 3: Changes in esterase (open symbols) and peptidase (closed symbols) activities on modification of carboxypeptidase (0.15 mM) with 2.25 (▼), 4.5 (▲), 9.0 (■), and 22.5 (●) mM butanedione in 0.05 M borate–1 M NaCl, pH 7.5, 20°. Activities are expressed as in Figure 2.

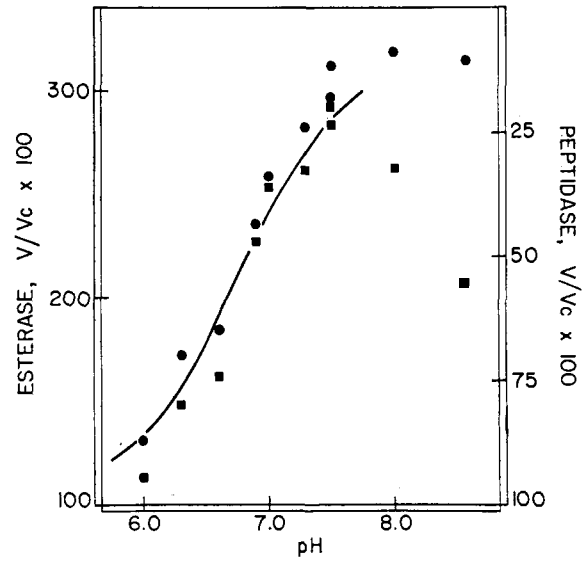


FIGURE 4: Changes in esterase (■) and peptidase (●) activities on modification of carboxypeptidase (0.15 mM) with butanedione (22.5 mM) in 0.05 M borate–0.05 M Mes–1 M NaCl, 15 min, 20° as a function of pH. Activities are expressed as in Figure 2.

prepared according to Riordan and Vallee (1964) and nitrocarboxypeptidase according to Riordan *et al.* (1967). Reaction with 1-cyclohexyl-3(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical Co.) was carried out by adding 0.2 ml of 0.2 M reagent to 2 ml of enzyme (5 mg/ml) in 0.05 M borate–acetate buffer–1 M NaCl (pH 6.0) for 60 min at 0° (Riordan and Hayashida, 1970).

Results

Time Course of Inactivation. The time course for the reaction of different concentrations of freshly distilled butanedione with carboxypeptidase in the absence of any buffer is shown in Figure 2. Peptidase activity is lost progressively, but with a 500-fold molar excess of reagent it is not abolished completely, even after 2-hr reaction. Significantly, esterase activity is hardly affected by butanedione at all concentrations employed. Similar results are obtained when the modification reaction is carried out in Veronal, Hepes, or bicarbonate buffers.

The time course of activities during modification in the presence of borate buffer is shown in Figure 3. With a 150-fold molar excess of reagent peptidase activity decreases within 15 min to about 20% of the control and then more slowly toward zero. During this time period esterase activity first increases up to three times that of the control and declines thereafter. At lower molar excesses of butanedione the reaction is slower. Thus, in the presence of a 60-fold molar excess, the maximum increase in esterase activity occurs after an hour.

Effect of pH. The pH dependence of the modification reaction was examined between pH 6.0 and 9.0 in 0.05 M borate–0.05 M Mes–1 M NaCl buffer using a 150-fold molar excess of reagent for 15 min, 20°. Under these conditions a maximum increase in esterase activity is observed at pH 7.5 (Figure 4). As the pH at which modification is performed is lowered from 7.5 to 6.0 the alterations in both esterase and peptidase activities are diminished simultaneously. The increase in esterase activity is also less when the reaction is carried out above 7.5. However, the decreases in peptidase activity are the same at pH 8.6 and 7.5.

Effect of Borate. The influence of borate buffer on the carboxypeptidase–butanedione reaction prompted an examination of the effect of borate ion concentration. Modifications were carried out with a 150-fold molar excess of reagent at pH 7.5 and aliquots were removed and assayed at 15 min. A maximal decrease in peptidase activity to about 15% of that of the native enzyme occurs in the presence of 0.05 M borate and coincides with the maximal increase in apparent esterase activity which reaches almost 300% of native. With higher borate concentrations the effect on both activities becomes progressively less and in 0.5 M borate there is virtually no change at all relative to the native enzyme. It seems unlikely that borate binds to the active site of carboxypeptidase thereby preventing modification, since 0.5 M borate does not inhibit peptidase activity. Rather, it would appear that borate interacts with butanedione to reduce the effective concentration of reagent. Such a reaction is suggested by the change in the pH from 8.6 to 3.8 on mixing 1.5 ml of butanedione with 8.5 ml of 0.05 M borate. Further, the absorption maximum of aqueous butanedione at 408 nm (ϵ 1.15) is abolished by addition of borate to 0.5 M, pH 7.5, while the intensity of the band at 284 nm (ϵ 9.75) is reduced by about 75%.

Reversibility of the Effects of Borate and Butanedione. The effects of borate on the activities of carboxypeptidase occur subsequent to and are superimposed upon the effects of butanedione alone. Treatment of carboxypeptidase with butanedione in Veronal buffer for 1 hr decreases peptidase activity to 33% while esterase is unchanged (Figure 5A). Subsequent addition of borate to the reaction mixture brings about an instantaneous increase of esterase activity and a further decrease of peptidase activity. On the other hand, gel filtration of enzyme, modified in the presence of borate, through a column of Bio-Gel P-4 equilibrated with 0.02 M Veronal–1 M NaCl buffer (pH 7.5) to remove borate, rapidly changes activities to those which would have been obtained had the enzyme been modified in the absence of borate (Figure 5B). However, gel filtration through a column equilibrated with borate buffer does not affect activities.

Subsequent to the removal of excess reagent and borate by gel filtration the activities of both samples of butanedione-modified carboxypeptidase return to the values characteristic of the native enzyme in a matter of hours (Figure 5A,B). If

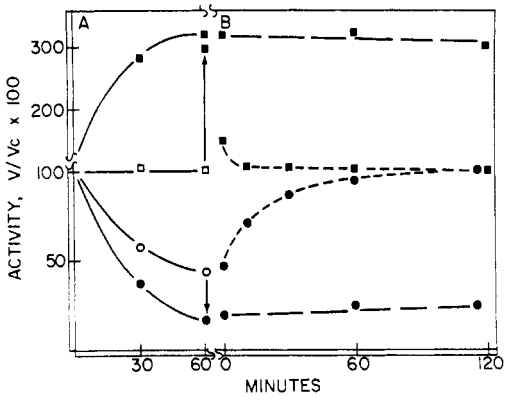


FIGURE 5: (A) Changes in esterase (□,■) and peptidase (○,●) activities on modification of carboxypeptidase A (0.15 mM) with butanedione in 0.05 M borate–1 M NaCl, pH 7.5 (9 mM reagent, closed symbols), or in 0.02 M Veronal–1 M NaCl, pH 7.5 (75 mM reagent, open symbols), 20°. The changes in activity immediately on addition of borate after 1 hr to the sample reacted in Veronal buffer are indicated by the arrows. (B) Changes in activities of the samples reacted in borate buffer subsequent to gel filtration through Bio-Gel P-4 equilibrated either with 0.05 M borate–1 M NaCl, pH 7.5 (— —), or with 0.02 M Veronal–1 M NaCl, pH 7.5 (- - - - -). Activities are expressed as in Figure 2.

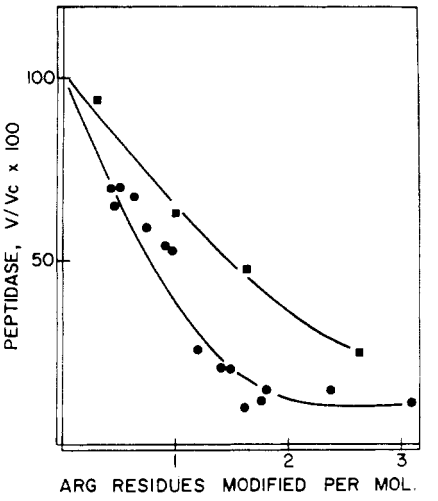


FIGURE 6: Correlation of changes in peptidase activity with modification of arginine residues on reaction of carboxypeptidase (0.15 mM) with butanedione in 0.02 M Veronal–1 M NaCl, pH 7.5 (■), or in 0.05 M borate–1 M NaCl, pH 7.5 (●). Variations either in molar excess of reagent or time of reaction were employed to achieve the different degrees of modification. Activities are expressed as in Figure 2.

borate remains present full native activity is not restored even after 10 days (Figure 5B).

The reversibility of the activity changes is not affected by addition of a 30-fold molar excess of sodium borohydride to the reaction mixture, though it can be prevented by addition of periodate (Table I). If the modified enzyme is mixed with periodate immediately after gel filtration, peptidase activity remains essentially constant for more than one hour while that of a sample not treated with periodate returns to the level of the control. Subsequent dialysis of the periodate treated sample does not restore native activity.

The reversibility of the modification is also affected by prolonging the reaction time to 20 hr. After dialysis against Veronal buffer, such an enzyme has 46% of the native peptidase (and 102% of the esterase) activity (Table I). A similar sample, set to dialyze after only 1 hr of reaction, exhibits native peptidase and esterase activities. Addition of borate to the sample treated with butanedione for 20 hr does not change activity further.

Modification of Arginyl Residues. The changes in activity which occur on modification of carboxypeptidase in borate buffer with increasing molar excesses of reagent correlate with the loss of two of the ten arginyl residues of the enzyme (Figure 6). All other amino acid residues are unaffected. Modification in the absence of borate either in Veronal buffer or on the pH-Stat without any buffer requires much higher concentrations of reagent to achieve decreases in peptidase ac-

TABLE I: Effect of Borohydride, Periodate and Reaction Time on the Reversibility of the Butanedione Modification of Carboxypeptidase.

Treatment	Act., $v/v_c \times 100$	
	Peptidase	Esterase
1. None, control	100	100
2. Butanedione	33	105
3. P-4	45	103
4. P-4, + 1 hr	92	103
5. P-4, + periodate	42	98
6. P-4, + borohydride	94	97
7. Butanedione, 20 hr	46	102
8. Butanedione, 20 hr, + borate	45	96

Modification with a 500-fold molar excess of butanedione in 0.02 M Veronal–1 M NaCl (pH 7.5). After 1 hr, an aliquot was removed for assay (2). Part of the reaction mixture was then gel filtered on a Bio-Gel P-4 column equilibrated with 0.02 M Hepes–1 M NaCl (pH 7.5). A portion of the protein eluate was assayed immediately (3) and again after 1 hr (4). Another portion (5) was mixed with sodium periodate (final concentration 0.01 M) and also assayed after 1 hr. Another portion was treated with 5 mM $NaBH_4$ for 19 hr and then dialyzed overnight against 0.02 M Veronal–1 M NaCl (pH 7.5) and assayed (6). After 20 hr the remaining reaction mixture was dialyzed against 0.02 M Veronal–1 M NaCl (pH 7.5) and then assayed before (7) and after (8) addition of borate. Activities are expressed as in Figure 2.

TABLE II: Changes in Activities and Arginine Content on Reaction of Carboxypeptidase A with Butanedione: Reversal by Dialysis.

	Act., $v/v_c \times 100$		Δ Arginine (Residues/ Molecule)
	Peptidase	Esterase	
Carboxypeptidase	100	100	
↓ butanedione + borate			
Butanedione–carboxypeptidase	17	269	2.2
↓ dialysis[a]			
Butanedione–carboxypeptidase	89	98	1.0

[a] Against 0.02 M Veronal–1 M NaCl (pH 7.5), 4°, 18 hr. Modification was carried out in 0.05 M borate with a 60-fold molar excess of butanedione as described under Methods. Activities are expressed as in Figure 2.

TABLE III: Changes in Peptidase and Esterase Activities on Modification of Carboxypeptidase A with Butanedione: Effect of Added Substrates and Inhibitors.

Addition	Act., $v/v_c \times 100$ Peptidase	Esterase
None[a]	19	307
Gly-L-Tyr, 0.01 M[a]	39	285
β-Phenyl propionate, 0.1 M[a]	51	276
Acetyl-L-Phe, 0.1 M[a]	38	300
L-Phe, 0.1 M[a]	36	302
None[b]	39	98
Gly-L-Tyr, 0.01 M[b]	36	100
β-Phenyl propionate, 0.1 M[b]	60	96

[a] Modification with a 60-fold molar excess of butanedione in 0.05 M borate–1 M NaCl, pH 7.5, 20°, for 1 hr. [b] Modification with a 500-fold molar excess of butanedione in 0.05 M Veronal–1 M NaCl, pH 7.5, 20°, for 1 hr. Activities are expressed as in Figure 2.

TABLE IV: Changes in Peptidase and Esterase Activities on Sequential Chemical Modifications of Carboxypeptidase A.[a]

Derivative	Act., $v/v_c \times 100$ Peptidase	Esterase
Acetylcarboxypeptidase	6	470
Acetylcarboxypeptidase + butanedione	6	360
Succinylcarboxypeptidase	89	106
Succinylcarboxypeptidase + butanedione	8	288
Butanedione–carboxypeptidase	14	300
Butanedione + dialysis	94	98
Butanedione + CMC[b]	0	0
Butanedione + CMC + dialysis	0	0
Butanedione + acetylimidazole	5	240
Butanedione + acetylimidazole + dialysis	7	420

[a] In each case modification with butanedione was carried out in 0.05 M borate with a 60-fold molar excess of reagent as described under Methods. The conditions for the other modifications are also given under Methods. Dialysis was for 18 hr against 0.02 M Veronal–1 M NaCl, pH 7.5, at 4°. Activities are expressed as in Figure 2. [b] CMC, cyclohexylmorpholinoethylcarbodiimide.

tivity comparable to those obtained in borate. Under these conditions, more arginyl residues are modified per unit of peptidase activity lost.

Only one of the modified arginines seems actually to be involved in activity. Amino acid analysis of samples taken either before and after dialysis or during recovery of activity after gel filtration indicate that the restoration of peptidase activity occurs concomitantly with the reappearance of a single arginyl residue (Table II).

Protection against Modification. The presence of inhibitors of carboxypeptidase during modification with butanedione does not completely prevent changes in activity (Table III). Only moderate protection is afforded by β-phenyl propionate and even less by the poorly hydrolyzed peptide substrates, acetyl-L-phenylalanine or glycyl-L-tyrosine.

Other functional residues of the enzyme were chemically modified to examine the possible effects of such procedures on the reaction of carboxypeptidase with butanedione (Table IV). Acetylation of tyrosyl residues with *N*-acetylimidazole increases esterase and decreases peptidase activities (Table IV, line 1) (Simpson *et al.*, 1963). Addition of butanedione in borate buffer to the acetyl-enzyme does not change peptidase activity further, but the esterase activity of acetylcarboxypeptidase decreases to about the value observed for native enzyme, modified directly with butanedione (line 2). Succinylation (Riordan and Vallee, 1964) blocks most of the ε-amino groups of the native enzyme without markedly altering activity (line 3), and subsequent treatment with butanedione (line 4) gives activity changes virtually identical with those obtained with the native enzyme.

The effects of prior modification of the essential carboxyl group of carboxypeptidase on the butanedione reaction cannot be assessed by means of activity data alone, since the resulting derivative exhibits negligible esterase and peptidase activities. However, it is possible to examine the effects of prior arginine modification on the carboxyl group reaction. Carboxypeptidase was modified with butanedione under the usual conditions (line 5) and the reaction mixture was then adjusted to pH 6.0. Dialysis against Veronal buffer overnight restores both activities virtually completely (line 6). However, if cyclohexylmorpholinoethylcarbodiimide is added prior to dialysis (Riordan and Hayashida, 1970) both activities fall to near zero after 1 hr, and subsequent dialysis *vs.* Veronal buffer does not restore them (lines 7 and 8). Hence, blocking the arginyl residue does not appear to interfere with subsequent carboxyl group modification.

In an analogous series of experiments, the enzyme was first modified with butanedione in borate and then acetylated with *N*-acetylimidazole (line 9). On removing the arginine blocking group by dialysis, the activities become characteristic of acetylcarboxypeptidase, *i.e.*, esterase activity is increased 4–5-fold and peptidase activity is less than 10% relative to the native enzyme (line 10) indicating that prior arginine modification doesnot prevent tyrosine acetylation.

Esterase and Peptidase Kinetics. Modification with butanedione in the presence of borate increases the K_m for hippuryl L-phenyllactate hydrolysis approximately 10-fold and correspondingly shifts substrate inhibition to higher concentrations (Figure 7). The k_{cat} is about the same as for the native enzyme and the 2- to 3-fold increase in esterase activity observed on modification under routine assay conditions, would seem to be due to a shift in the inhibition by substrate and the increased K_m which combine to generate a new activity optimum at about 3 mM hippuryl L-phenyllactate. Modification in the absence of borate does not seem to affect esterase kinetics between 2×10^{-4} and 5×10^{-2} M hippuryl L-phenyllactate.

Kinetic complexities are also apparent in the substrate concentration dependence profile for the hydrolysis of the peptide substrate, carbobenzoxyglycyl-L-phenylalanine (Figure 8). Maximal activities for the native enzyme, that modified in Veronal, and that modified in borate, all occur at about 0.02 M carbobenzoxyglycyl-L-phenylalanine with substrate inhibition evident at higher concentrations. The multiphasic nature of the respective Lineweaver–Burk plots precludes definitive kinetic analyses. However, extrapolation of data

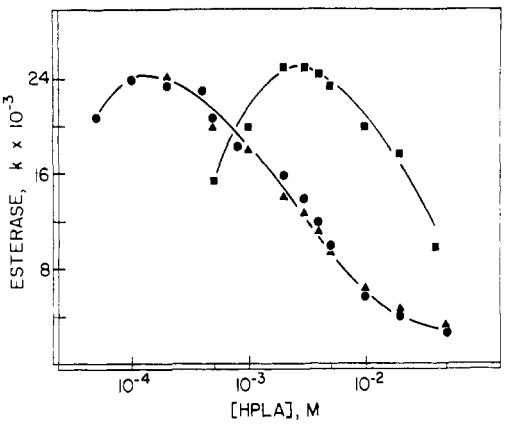


FIGURE 7: Substrate concentration dependence of the hydrolysis of hippuryl L-phenyllactate (HPLA) by carboxypeptidase (●) and carboxypeptidase modified with a 500-fold molar excess of butanedione in 0.02 M Veronal–1 M NaCl, pH 7.5 (▲) or a 60-fold molar excess in 0.05 M borate–1 M NaCl, pH 7.5 (■).

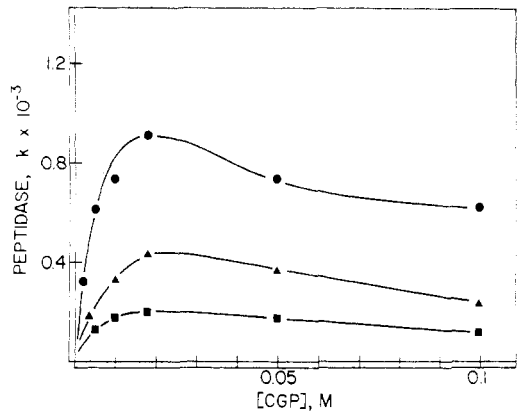


FIGURE 8: Substrate concentration dependence of the hydrolysis of carbobenzoxyglycyl-L-phenylalanine (CGP) by carboxypeptidase (●) and carboxypeptidase modified with a 500-fold molar excess of butanedione in 0.02 M Veronal–1 M NaCl, pH 7.5 (▲), or a 60-fold molar excess in 0.05 M borate–1 M NaCl, pH 7.5 (■). Activities were measured at 0°.

collected between 0.01 and 0.001 M carbobenzoxyglycyl-L-phenylalanine give K_m values of $5 \pm 2 \times 10^{-3}$ M for each of the three enzymes and k_{cat} values of 1400, 700, and 350 min^{-1}, respectively. Arginine modification did not seem to alter the substrate specificity of carboxypeptidase. Neither the native nor the modified enzyme catalyzed the hydrolysis of hippuryl-L-phenyllactyl-L-alanylmethyl ester, an ester substrate for the zinc endopeptidase thermolysin (Holmquist, 1970). Loss of its presumable terminal carboxyl group specificity site did not convert carboxypeptidase from an exo- to an endopeptidase.

Preliminary experiments using dansylglycyl-L-tryptophan and dansylglycylglycyl-L-phenylalanine according to the stopped-flow procedures of Latt *et al.* (1970) indicate that the loss of peptidase activity which occurs on modification with butanedione closely parallels a loss of peptide binding.

Modification with Other Dicarbonyl Compounds. Several other reagents were examined for their potential usefulness in the modification of arginyl residues of carboxypeptidase (Table V). Only α-dicarbonyl compounds give results similar to those with butanedione and with the accompanying borate effect. The β-diketone, 2,4-pentanedione, modifies neither the activity of carboxypeptidase nor its arginyl residues. Some α-dicarbonyl compounds such as phenanthraquinone, 1,2-cyclohexanedione, 1,4-dibromo-2,3-butanedione, and glyoxal are also nonreactive toward the enzyme. Esterase and peptidase activities are both altered slightly by methylglyoxal and to a greater extent by phenylglyoxal.

TABLE V: Changes in Peptidase and Esterase Activities of Carboxypeptidase A on Reaction with Dicarbonyl Compounds.[a]

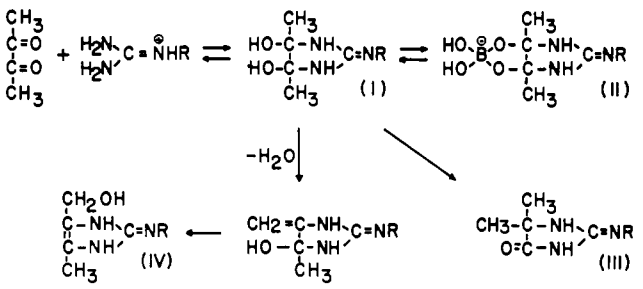
Reagent	Reaction Time (min)	Molar Excess Reagent	Act., $v/v_0 \times 100$ Peptidase	Esterase
2,3-Butanedione	15	150	14	290
2,3-Pentanedione	15	150	32	266
1-Phenyl-1,2-propanedione	60	60	28	285
2,4-Pentanedione	15	150	92	90
Phenanthraquinone	30	60	100	100
1,2-Cyclohexanedione	30	150	99	108
1,4-Dibromo-2,3-butanedione	30	150	95	95
Glyoxal	30	150	109	106
Methylglyoxal	30	150	89	84
Phenylglyoxal	60	150	37	64
Butanedione trimer	60	150	16	323

[a] All modifications were carried out using 0.15 mM carboxypeptidase in 0.05 M borate–1 M NaCl, pH 7.5, 20°. Activities are expressed as in Figure 2.

Butanedione undergoes self-condensation in the presence of alkalinized powdered glass to form a trimer which is reactive toward arginyl residues in proteins (Yankeelov, 1970). Treatment of carboxypeptidase with a 150-fold molar excess of this reagent for 1 hr in borate decreases peptidase activity to 16% and increases esterase activity to 323% of those of the native enzyme. Such changes are quite similar to those observed with monomeric butanedione (Table V). After dialysis of this derivative against Veronal buffer for 24 hr, peptidase and esterase activities return to about 55 and 130% of the control, respectively. Under similar conditions the activities of butanedione-modified carboxypeptidase return completely to those of the native enzyme. When the modification with trimer is carried out in Veronal buffer, the resultant product exhibits 59% peptidase and 105% esterase activity. Hence, the trimer both behaves like and unlike the monomeric reagent.

Modification of Carboxypeptidase B. Porcine carboxypeptidase B exhibits the same α-carboxyl group specificity as does carboxypeptidase A. Therefore, it was of interest to compare the effects of butanedione on the activities of these two enzymes. Under the same modification conditions, in Veronal buffer, peptidase activities are decreased by 70 and 50% for the A and B enzymes, respectively, while both their esterase activities are virtually unchanged. In borate buffer, the decrease in peptidase activities are even greater, 84 and 75%, respectively, while esterase activity increases to 300% of the control for the A enzyme but decreases by 44% for the B enzyme. Thus, for both enzymes, only peptidase activity appears to be altered when the modification is carried out in Veronal, while both activities are altered, albeit differently, when borate is present.

SCHEME I



Discussion

A large number of different amino acid side chains have been recognized to participate in the interaction of enzymes with their substrates. Until recently, reagents suitable for the chemical modification of arginyl residues were not available. Hence, no enzyme was known to have active-center arginyl residues. However, during the past several years, a number of reagents have been proposed for the selective chemical modification of arginyl residues of proteins under mild conditions. In particular, the suitability and relative advantages of butanedione, phenylglyoxal, and glyoxal have each been considered.

The present work with butanedione demonstrates that arginyl residues are involved in substrate binding and specificity of carboxypeptidase. As part of a study to determine the existence of positively charged recognition sites on enzymes acting on anionic substrates, it has recently been shown that two arginyl residues are essential to the activity of *Escherichia coli* alkaline phosphatase (Daemen, 1973). Functional arginyl residues may well be a more general phenomenon than recognized so far.

Butanedione was first shown to react with arginyl residues of proteins more than 60 years ago (Harden and Norris, 1911), but this observation received surprisingly little attention. Recently, Yankeelov and coworkers reinvestigated this reagent and showed that under their conditions the reactive species is an oligomer, *i.e.*, a dimer or a trimer (Yankeelov *et al.*, 1966, 1968; Yankeelov, 1970). The present evidence indicates that the monomeric reagent can effectively modify both free arginine and arginyl residues in carboxypeptidase. In both instances the reaction is even more rapid than that observed with oligomeric butanedione.

Based on amino acid analysis butanedione modifies only arginyl residues of carboxypeptidase. This is consistent with the fact that ϵ-amino groups are not altered when either lysine or a mixture of arginine and lysine is treated with this reagent. Spectra of native and butanedione-modified carboxypeptidase in the region from 260 to 300 nm are identical and would tend to rule out a reaction with phenylalanine, tyrosine, or tryptophan.

The influence of borate buffer on the time course of the reaction of carboxypeptidase with butanedione also signifies modification of a functional arginyl residue. The reaction of butanedione with arginine would seem to be analogous to that with benzamidine (Cornforth and Huang, 1948; Diels and Schleich, 1916) proceeding *via* the formation of a 4,5-dimethyl-4,5-dihydroxy-2-imidazoline (Scheme I, I). A similar reaction scheme has been proposed for the condensation of glyoxal or benzil with urea (Pauly and Sauter, 1930). The formation of a borate complex (II) with such an intermediate could then account for the specific effects which were observed with this buffer. Based on such a tentative scheme butanedione could first react reversibly with the guanido group of arginine to form the *cis*-diol, dihydroxyimidazoline derivative; this would then complex with borate by means of a rapid process (Roy *et al.*, 1957) which is also readily reversible (Figure 6). Thus, in the presence of borate the reaction would proceed faster due to product stabilization. The intermediate itself would have to be moderately stable, since it apparently still reacts with borate even after remaining at room temperature with excess reagent for 1 hr (Table III). If, at that time, excess reagent is removed, the intermediate dissociates slowly to regenerate free arginine (Figure 5A,B). However, if the reaction is allowed to continue for 20 hr, then the intermediate likely rearranges to a nondissociable product which no longer reacts with borate (Table I). This product is probably formed by a pinacol-type rearrangement by analogy to earlier studies (Diels and Schleich, 1916) and would have the structure shown for compound III in the scheme. A closely related product was obtained by reacting arginine with cyclohexanedione under strong alkaline conditions (Toi *et al.*, 1967).

Evidence for the glycol nature of I is given by the effect of periodate on the butanedione-modified enzyme (Table I). Periodate has no effect on the activities of either carboxypeptidase itself or the modified enzyme. However, it prevents the restoration of peptidase activity to the enzyme modified in Veronal buffer, which would otherwise occur on dialysis. Presumably, periodate acts by cleaving the glycol.

Acidification of the reaction mixture can also prevent regeneration of arginine, and, under the conditions employed for protein hydrolysis, the dihydroxyimidazoline derivative is probably dehydrated to form a methylene intermediate which in turn, undergoes an anionotropic shift to give the hydroxymethylimidazoline (compound IV) (Cornforth and Huang, 1948). This product does not break down to regenerate arginine and, hence, it is possible to measure the degree of arginine modification in proteins by routine amino acid analysis. It should be emphasized that the structures indicated in Scheme I, although reasonable, have not been rigorously established in the present instance.

The effect of varying the borate ion concentration on the enzyme modification reaction can best be accounted for by two separate processes. Esterase activity increases and peptidase activity decreases in direct proportion to the borate concentration up to a concentration of 0.05 M. This likely reflects the formation of the postulated borate–dihydroxyimidazoline complex. Above this concentration of borate, however, the changes in both esterase and peptidase activities are much less. In all probability, borate begins to reduce the effective concentration of butanedione, forming a complex with it as well as with the product. Virtually all of the reagent is complexed in 0.5 M borate and therefore very little modification occurs. Spectral evidence is consistent with this idea. The anomalous reaction kinetics observed by Yang and Schwert (1972) may derive, in part, from a similar process.

The pH dependence of the modification reaction indicates the importance of an ionization which occurs between pH 6 and 8 (Figure 4). It is unlikely that this reflects the pK of an arginyl residue of the enzyme since free arginine (p$K > 12$) is modified readily even at pH 7.5. However, it could be related to the ionization of another group of the enzyme which might dominate the local environment and affect the reactivity of active-center arginyl residues. A similar pH dependence for the reactivities of both the functional carboxyl group and the tyrosyl residue may well be related. Alternatively, this pH dependence could be due to an effect on borate buffer. In particular this might account for the changes which are ob-

served above pH 7.5 where polyborate formation is known to occur.

Modification of arginyl residues also correlates with the changes in carboxypeptidase activities. Prior chemical substitution of tyrosyl or lysyl residues does not prevent the subsequent effects of butanedione (Table IV). Nor does treatment with this agent interfere with modification of the active-center carboxyl group. Thus, each of the residues known so far to be functional components of the active center of carboxypeptidase exhibit unusual reactivities which can be observed independently of that of the others.

Changes in the activities of carboxypeptidase on reaction of tyrosyl, carboxyl, or histidyl residues have been prevented in the past by carrying out the modification reaction in the presence of 0.1 M β-phenyl propionate. In the present instance this and other inhibitors afford only partial protection against modification (Table III) supporting the conclusion that the reaction affects a residue different from those just cited.

Modification in the presence of borate reduces peptidase activity to between 10 and 20% of that of the native enzyme concomitant with the loss of approximately two arginyl residues. When the modification is carried out in veronal buffer loss of peptidase activity correlates more nearly with the loss of three arginyl residues (Figure 6). However, not all of the arginines modified seem to be involved in activity. On dialysis or gel filtration to remove both excess reagent and borate, activity is recovered but only a single arginyl residue reappears (Table II). Parameters are not known which might promote the dissociation of this particular modified residue, allow it to remain as a readily reversible species and/or prevent it from rearranging to the more stable derivative. In any case, only a single arginyl residue exhibits this unique behavior which leads to a one to one correlation between structure and function. It is of interest that only 2 or 3 of the 10 arginyl residues of the enzyme, all of which are thought to have some contact with solution, are modified whereas under the same conditions free arginine reacts almost completely. No explanation for the lack of reactivity of the other residues is available.

In the mechanism of action of carboxypeptidase one arginyl residue, Arg-145, has been thought to bind the terminal carboxyl group of the substrate, accounting for the specificity of the enzyme (Lipscomb *et al.*, 1968). Other arginyl residues, Arg-127 and Arg-71, have also been located within the peptide substrate binding groove, but no functional role has been assigned to them. Modification of any one of these residues might potentially alter the activity of the enzyme. Sequence determination would be the most direct approach to localize the specific residue(s) affected, but the ready reversibility of the present modification reaction precludes that procedure in this instance. For this reason alternative reagents are currently under investigation in an attempt to obviate this problem.

Studies of the effect of butanedione modification of carboxypeptidase on the kinetics of peptide hydrolysis are consistent with a role of an arginyl residue in substrate binding. When modified either in borate or in Veronal the enzyme exhibits a K_m value for carbobenzoxyglycyl-L-phenylalanine, virtually identical with that of the native enzyme. The observed changes in activities are reflected only as an apparent decrease in k_{cat}. This would be expected if activity were to be abolished by enzyme modification but if only a fraction of the enzyme molecules were actually modified. Preliminary experiments, designed to examine substrate binding directly by means of the stopped-flow fluorescence procedure of Latt *et al.* (1970), support the view that loss of peptidase activity is due to interference with substrate binding. The proposition that an arginyl residue is essential for specificity gains support from the fact that arginine modification affects the peptidase activities of both carboxypeptidase A and B in much the same manner (Werber and Sokolovsky, 1972).

However, while the peptidase activity of carboxypeptidase depends on at least one arginyl residue, its esterase activity does not. Within the range of substrate concentration examined the esterase activity of carboxypeptidase, modified in the absence of borate, is the same as that of the native enzyme. Significantly, butanedione modification not only does not inhibit esterase activity of carboxypeptidase but it actually increases it to about 300% of the control in the presence of borate. This is clear evidence that the ester substrate cannot bind to the same carboxyl group recognition residue which binds the peptide. The marked increase of esterase activity on modification in the presence of borate could be due to the introduction of a negative charge into the active-center region. Charge repulsion could then decrease both productive and nonproductive substrate binding. Whatever the mechanism for this increased esterase activity the modes of peptide and ester binding to carboxypeptidase must be different. If Arg-145 recognizes the terminal carboxyl group of the peptide then there has to be another site to recognize the ester.

This conclusion is entirely consistent with a variety of other experiments which have indicated to us different mechanisms for the dual specificity of carboxypeptidase A (Vallee and Riordan, 1968). Chemical modification of tyrosyl residues has long been known to affect the hydrolysis of esters and peptides differentially (Vallee and Riordan, 1968) and both kinetic and binding studies suggest different modes of binding (Vallee *et al.*, 1968).

Metal binding to the apoenzyme and ensuing restoration of the holoenzyme and of its activity is prevented by peptides but not by esters or by inhibitors (Felber *et al.*, 1962; Coleman and Vallee, 1962). Other studies have indicated, moreover, that certain of these inhibitors likely bind through their carboxyl group to the active-site metal atom (Coleman and Vallee, 1964; Schulman *et al.*, 1966). Kinetic studies of metal-substituted carboxypeptidase have demonstrated recently that the metal functions primarily in the catalytic step of peptide hydrolysis but primarily in the binding step of ester hydrolysis (Auld and Holmquist, 1973). This would be expected if ester binding, like that of inhibitors, involved a carboxyl-metal interaction rather than an arginine residue. The present data are consistent with the positively charged recognition site for peptides being an arginine and that for esters being the metal.

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Synthesis and Biological Evaluation of Poly-γ-glutamyl Derivatives of Methotrexate†

M. G. Nair and Charles M. Baugh*

ABSTRACT: Chemical methods for the synthesis of a family of poly-γ-glutamyl derivatives of methotrexate (*N*-[*p*-[((2,4-diamino-6-pteridyl)methyl)methylamino]benzoyl]glutamic acid) which are also metabolites of the drug, are reported. The lability of the 4-amino group to both acid and base initially presented a major obstacle to these syntheses. Attempts were made to prepare the N^2,N^4-ditrifluoroacetyl-N^{10}-methylpteroic acid derivative by reaction of the appropriate pteroic acid analog with trifluoroacetic anhydride under anhydrous conditions. The desired product was obtained in only 10% yield; the remaining was degraded. These problems were circumvented by the discovery of a suitable solvent system for 2,4-diamino-N^{10}-methylpteroic acid (equal volumes of tetrahydrofuran and Me_2SO). Subsequent studies established that protection for the 2- and 4-amino groups was unnecessary, and a simple direct route to these derivatives was at hand. A comparison of the effectiveness of these derivatives with methotrexate as inhibitors of growth of the folate-requiring bacterium *Streptococcus faecium* ATCC 8043 has shown that these derivatives become increasingly less toxic to *S. faecium* as the γ-glutamyl chain is lengthened. A study of the reactivity of hog kidney conjugase (pteroylglutamyl-γ-glutamyl hydrolase) toward the methotrexate derivative with six additional γ-glutamyl residues has been carried out. Column chromatography of an incomplete enzymic (conjugase) hydrolysis mixture suggests varying affinities of the enzyme for different chain lengths with a pronounced preference for the derivative with a total of seven glutamyl residues. These studies also permit the conclusion that the γ-glutamyl residues are hydrolyzed one at a time.

In the course of attempts to identify certain newly discovered and previously unreported metabolites of methotrexate from the rat (Baugh *et al.*, 1973), it became necessary to obtain authentic samples of a variety of poly-γ-glutamyl derivatives of this drug. The approach to their synthesis utilized the solid-phase peptide synthetic procedures employed earlier to

† From the Nutrition Program, University of Alabama in Birmingham, School of Medicine, University Station, Birmingham, Alabama 35294. *Received April 18, 1973.* Principal support for these investigations was received from the American Cancer Society Grant No. 1C-3M. This paper is also a contribution (No. 1162) from the U. S. Army Research Program on Malaria. Present address for both authors: Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Ala. 36688.