The Activation of Bovine Procarboxypeptidase A. II. Mechanism of Activation of the Succinylated Enzyme Precursor*

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ABSTRACT: Succinyl fraction I, the zymogen precursor of succinylated carboxypeptidase, is rapidly activated by catalytic quantities of a variety of proteolytic enzymes. The activation by trypsin occurs in two stages as judged by measurements of activity and sedimentation in the ultracentrifuge. Complete tryptic activation leads to the formation of an enzyme which has an amino acid composition nearly identical with that of carboxypeptidase A_α. The release of low molecular weight products totalling some 60 amino acid residues accompanies this activation. Acetylation of the zymogen, followed by tryptic activation, results in the same changes in enzymatic function as occur subsequent to the acetylation of succinylcarboxypeptidase (Riordan, J. F., and Vallee, B. L. (1964), *Biochemistry 3*, 1768). These changes are prevented by β-phenylpropionate, a competitive inhibitor of carboxypeptidase which also acts to retard the tryptic activation of succinyl fraction I. The effects of certain metals on the activation of succinyl fraction I have been investigated.

The disaggregation of procarboxypeptidase A by succinylation and the isolation of succinyl fraction I have been described (Freisheim *et al.*, 1967). The latter is the precursor of succinylcarboxypeptidase A and can be converted to the active form by trypsin. The present paper is concerned with the mechanism of the activation reaction including the specificity of the activating enzyme, the characterization of the products of activation, and the possible preexistence in the zymogen of certain components of the active site. This problem has been approached through investigation of the effects of chemical modification of the zymogen and their prevention by a competitive inhibitor.

Materials and Methods

Succinyl fraction I, the immediate precursor of succinylcarboxypeptidase, was isolated and purified as described in the accompanying paper (Freisheim *et al.*, 1967). Preparations of the succinylated zymogen were dialyzed for 48 hr against 0.0125 M Tris-chloride–0.05 M KCl (pH 8.0) to remove any labile *O*-succinyl groups (Riordan and Vallee, 1964).

Substrates. Hippuryl-dl-β-phenyllactic acid (HPLA)[1] was prepared in this laboratory or purchased from the Cyclo Chemical Corp. Chromatographically pure *carbobenzoxyglycyl-L-phenylalanine* (CGP) was obtained from Mann Research Laboratories, Inc.

Reagents and Enzymes. Salt-free, twice-crystallized, lyophilized *trypsin*, thrice-crystallized *α-chymotrypsin*, *papain*, a crystalline suspension in 0.03 M cysteine, and *soybean trypsin inhibitor* (STI) were obtained from the Worthington Biochemical Corp. *Subtilisin* (Nagarse) was obtained from the Biddle-Sawyer Co. *Succinyltrypsin*[2] was prepared according to the method of Fraenkel-Conrat (1949). *Fraction II*, the potential endopeptidase in procarboxypeptidase A, was prepared according to the method of Brown *et al.* (1963) and was activated by trypsin (trypsin:zymogen weight ratio of 1:2000) for 5.5 hr at 0° just prior to use. *1,10-Phenanthroline* (OP) was the crystalline dihydrochloride (G. F. Smith Co.) and was used without further purification. *Buffers* used in the metal exchange experiments were prepared from reagent grade chemicals, adjusted to pH 7.0, then extracted with diphenylthiocarbazone (Eastman) in carbon tetrachloride to remove contaminating metal ions. Excess diphenylthiocarbazone was removed by repeated extraction with carbon tetrachloride. *N-Acetylimidazole* (mp 103–105°) was prepared according to the method of Boyer (1952) and was recrystallized from minimal isopropenyl acetate (Matheson Coleman and Bell). Diisopropylphosphorofluoridate (DFP) was obtained from the Merck Chemical Co. and was diluted

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[1] Abbreviations used: HPLA, hippuryl-*dl*-β-phenyllactic acid; CGP, carbobenzoxyglycyl-L-phenylalanine; STI, soybean trypsin inhibitor: TPCK, tosyl-2-amidophenylethyl chloromethyl ketone; DIP, diisopropylphosphoryl; OP, 1,10-phenanthroline.

[2] Succinyl fraction I possessed 8–9% carboxypeptidase activity (*vs.* HPLA) prior to tryptic activation. This finding may represent an intrinsic property of the zymogen since Yamasaki *et al.* (1963) have similarly observed an initial activity of 5–6% for procarboxypeptidase prior to tryptic activation.

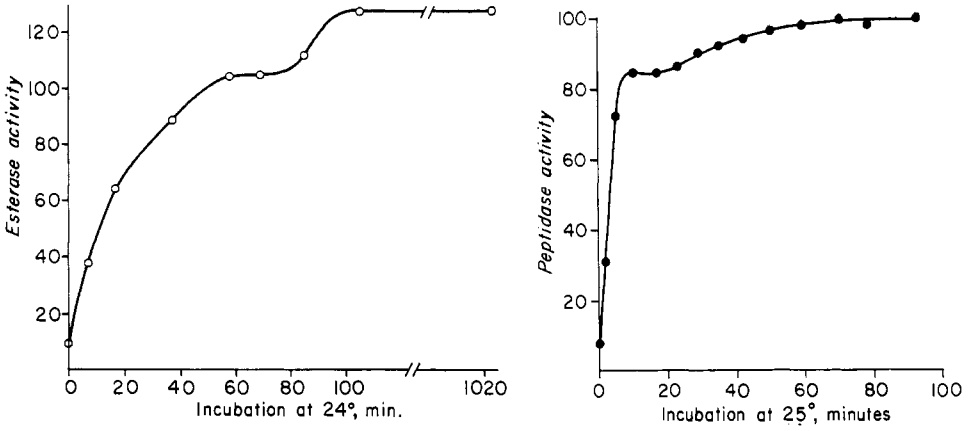

FIGURE 1: Activation of succinyl fraction I by bovine trypsin. HPLA esterase (O) and CGP peptidase (●) activities are given in terms of a per cent of that of native carboxypeptidase A_α. (A) Esterase activity. Succinyl fraction I: trypsin (161:1, w/w). (B) Peptidase activity. Succinyl fraction I: trypsin (112:1, w/w).

to 1 M with anhydrous isopropyl alcohol before use. 1-Fluoro-2,4-dinitrobenzene (FDNB) was purchased from Matheson Coleman and Bell and was used without further purification. Sephadex G-75 (Pharmacia) was prepared and columns were packed as advised by the manufacturer.

Precautions were taken to ensure that no chymotryptic activity was associated with the trypsin preparations employed in the activation studies. Trypsin was treated with tosyl-2-amidophenylethyl chloromethyl ketone (TPCK) (Schoellman and Shaw, 1963) according to Kostka and Carpenter (1964). To ensure against any slight contamination of succinyl fraction II in the succinyl fraction I preparations used in these studies, the procarboxypeptidase A-S6 preparations were activated to the endopeptidase stage according to Yamasaki *et al.* (1963) and completely inhibited with DFP. Under these conditions, no measurable amounts of carboxypeptidase were formed. The succinyl fraction I was then purified subsequent to succinylation of the DIP-procarboxypeptidase.

Protein concentrations were measured spectrophotometrically at 280 mμ assuming an absorbancy index of 18.3 for a 1% solution of succinyl fraction I (Freisheim *et al.*, 1967).

Amino acid analyses were performed as described in the accompanying paper (Freisheim *et al.*, 1967).

Assay Procedures. Esterase activities for trypsin, chymotrypsin, and carboxypeptidase A were determined as outlined in the accompanying paper (Freisheim *et al.*, 1967). Peptidase activity was determined by the rate of release of phenylalanine from CGP (0.01 M in 0.01 M sodium Veronal–0.05 M sodium chloride, pH 7.5) by development of the ninhydrin color according to Putnam and Neurath (1946). Alternatively, CGP peptidase activity was evaluated spectrophotometrically at 223 mμ according to the procedure of McClure *et al.* (1964).

Amino-terminal analyses were carried out using the FDNB method of Sanger (1945) as modified by Fraenkel-Conrat *et al.* (1955).

Sedimentation analyses were performed in a Beckman Model E ultracentrifuge equipped with absorption optics as described by Lamers *et al.* (1963).

Results

Activation by Bovine Trypsin. Succinyl fraction I (0.1–0.4 mg/ml) in 0.04–0.05 M potassium phosphate (pH 6.5) was rapidly activated by bovine trypsin. When the activation was followed by the appearance of either esterase activity (Figure 1A) or peptidase activity (Figure 1B), an apparent two-stage process was observed. The absolute rates of appearance of esterase and peptidase activity are not strictly comparable in these two sets of data. However, as indicated in Figure 1A,B, a discrete plateau in the activation occurred at approximately 80–85% of maximal activity followed

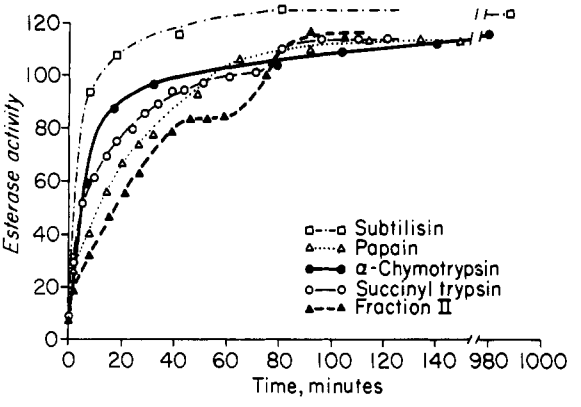

FIGURE 2: Activation of succinyl fraction I by subtilisin, papain, α-chymotrypsin, succinyltrypsin, and fraction II. Esterase activity is given in terms of per cent of that of carboxypeptidase A_α. For experimental details, see the text.

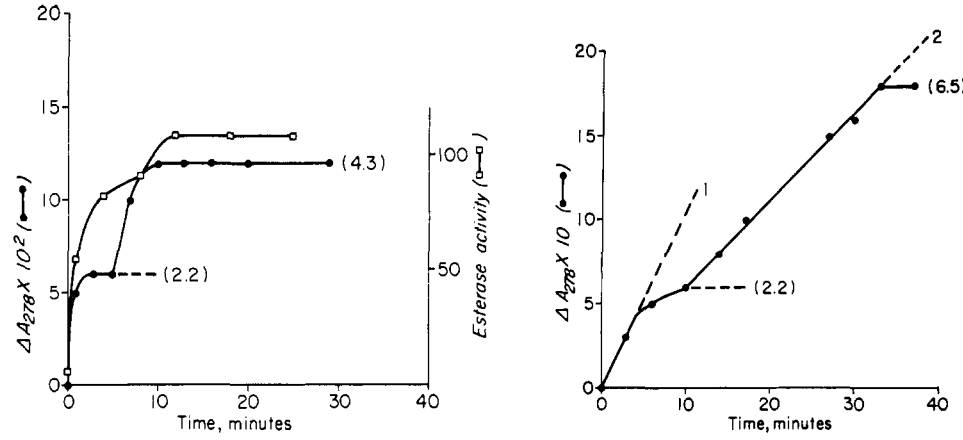

FIGURE 3: Deacylation of *O*-succinyltyrosine residues of succinyl fraction I. Numbers in parentheses indicate residues deacylated. For further details see the text. (A) Deacylation during activation by trypsin. (B) Deacylation with 0.15 M hydroxylamine. Slope of curve 1 = 1.04 ΔA_{278} min^{-1}. Slope of curve 2 = 0.52 ΔA_{278} min^{-1}.

by a further increase to full activity. The fact that the carboxypeptidase activity remained constant after 17-hr incubation (Figure 1A) suggests that the enzyme formed resisted further degradation either by trypsin or by autolysis.

Results of sedimentation studies during different stages of activation are shown in Table I. No change in sedimentation coefficient was observed when succinyl fraction I was activated to *ca.* 80% of maximal activity by incubation at a trypsin:succinyl fraction I weight ratio of 1:100 for 30 min at 25° (activation stopped by the addition of STI). Activation under conditions which gave rise to full activity (trypsin:succinyl fraction I, 1:100, 2 hr at 25°) resulted in a concomitant decrease in sedimentation coefficient from 4.0 to 3.1 S, a value identical with that reported for carboxypeptidase A (Rupley and Neurath, 1960).

Activation by Other Proteolytic Enzymes. It was of interest to determine if the conversion of succinyl fraction I to succinylcarboxypeptidase was specifically mediated by trypsin or whether other proteolytic enzymes would also catalyze this transformation. Subtilisin, papain, succinyltrypsin, α-chymotrypsin, and fraction II, the endopeptidase derived from procarboxypeptidase A-S6 (Brown *et al.*, 1963), were compared with regard to their effect on the activation of succinyl fraction I. These studies were performed using 0.03–0.05 M potassium phosphate buffers (pH 6.5) and 0.1–0.3% solutions of succinyl fraction I. The final concentrations in terms of weight ratios of succinyl fraction I:enzyme were 14:1 for succinyltrypsin, 16:1 for α-chymotrypsin, 10:1 for papain, 20:1 for subtilisin, and 24:1 for fraction II. The results of these studies are shown in Figure 2. The enzymes resulting from these activations possessed approximately the same specific activity (HPLA as substrate) except in the case of subtilisin which gave rise to a species of slightly higher maximal activity. The actions of both succinyltrypsin and fraction II on succinyl fraction I resulted in apparent two-stage activations as was observed during tryptic activation (Figure 1).

TABLE 1: Effect of Activation of Succinyl Fraction I on Sedimentation Coefficient.[a]

% Act.[b]	$s_{20,w}$ (S)
9	4.0
79	4.0
100	3.1

[a] Succinyl fraction I concentrations were 0.3 mg/ml in these experiments. [b] Activities were measured prior to sedimentation analysis. Further details are given in the text.

Deacylation of O-Succinyltyrosines during Activation. Results from the preceding paper (Freisheim *et al.*, 1967) indicated that approximately six to seven phenoxy groups of tyrosyl residues remained succinylated in succinyl fraction I. Calculation of the number of *O*-succinyl groups released following treatment with hydroxylamine was based on an increase in molar absorbancy at 278 mμ of 1160 for the transition *N,O*-diacetyl- to *N*-acetyltyrosine (Simpson *et al.*, 1963). Using this spectrophotometric criterion, activation of succinyl fraction I was found to be accompanied by a spontaneous release of succinyl groups from phenolic hydroxyls of tyrosines as described below.

Succinyl fraction I (1.0 mg/ml) in 0.03 M potassium phosphate (pH 6.5) was incubated at 23° with TPCK-treated trypsin (33:1, w/w). The results shown in Figure 3A indicate that the two-stage tryptic activation of succinyl fraction I is paralleled by a two-stage deacylation of *O*-succinyltyrosines. At 80% of maximal activity about two such residues have become deacylated; after complete activation approximately four residues have been deacylated.

The deacylation of succinyl fraction I in 0.15 N

hydroxylamine–0.038 M Tris (pH 7.5) is indicated in Figure 3B. The total number of tyrosines deacylated was calculated to be 6.5 (*vide supra*). The data shown in Figure 4B indicate a discontinuity in rate of deacylation, the initial rate being twice that of the final rate. The inflection point in the curve coincides with the release of two succinyl groups. This rapid deacylation of two *O*-succinyltyrosines by hydroxylamine correlates with the rapid deacylation of two such residues during tryptic activation. Thus the apparent two-stage tryptic activation of succinyl fraction I may simply reflect a parallel biphasic deacylation of *O*-succinyltyrosines rather than a discrete two-stage hydrolysis of peptide bonds.

Products of Tryptic Activation. The tryptic activation of succinyl fraction I is associated with the appearance of new amino-terminal residues. These were identified as follows. A solution containing 0.41 μmole (3.3 mg/ml) of succinyl fraction I in 0.05 M Tris-HCl–10^{-4} M $ZnCl_2$ (pH 8) was incubated at 25° with TPCK-treated trypsin (270:1, w/w). Zinc ions were included to minimize any exchange of native zinc with extraneous metals. The solution was assayed for enzymatic activity using HPLA as substrate. When *82% of maximal activity* was reached the activation was stopped by the addition of 0.2 mg of STI and adjusting the solution to 10^{-3} M DFP. The protein fraction was subjected to filtration on a 3 × 106 cm Sephadex G-75 column (equilibrated with 0.01 M Tris-HCl–10^{-5} M $ZnCl_2$). The pooled protein fraction exhibited 84% of maximal activity when assayed *vs.* HLPA. Amino-terminal analysis of the protein by treatment with FDNB revealed the presence of several ether-soluble DNP-amino acids (Table II). Of these, DNP-alanine and DNP-serine were the major new N-terminal residues found. Only ε-DNP-lysine was found in the aqeuous phase. These data indicate that several peptide bonds in the N-terminal region of succinyl fraction I are susceptible to tryptic attack.

TABLE II: N-Terminal Analysis of Succinyl Fraction I after 80% Activation.

DNP-Amino Acid	Moles of DNP-Amino Acid/Mole of Protein (uncor)
Alanine	0.34
Serine	0.26
Leucine/Isoleucine	0.13
Aspartic acid	0.16
Glycine	0.08

The complete tryptic conversion of succinyl fraction I to succinylcarboxypeptidase was carried out as follows. A solution containing 1.04 μmoles of succinyl fraction I (8.3 mg/ml) in 0.05 M Tris-HCl–10^{-4} M $ZnCl_2$ (pH 8.0) was incubated with TPCK-treated trypsin (231:1, w/w) at 25°. The activation was stopped after 2.5 hr by the addition of DFP to 10^{-3} M and 0.2 mg of STI. The activation mixture was then applied to a 3 × 125 cm Sephadex G-75 column which had been previously equilibrated with 0.01 M Tris-HCl–10^{-5} M $ZnCl_2$ (pH 8). The results are shown in Figure 4. Those protein fractions which exhibited constant specific activity were pooled and the material was further characterized.

TABLE III: N-Terminal Analysis of Succinyl Fraction I Following Complete Activation.

DNP-Amino Acid	Moles of DNP-Amino Acid/Mole of Protein (uncor)
Alanine	0.38
Serine	0.31
Aspartic acid	0.12

TABLE IV: Amino Acid Composition of the Protein Fraction Obtained after Full Activation of Succinyl Fraction I.

	Amino Acid Residues/ 35,000 g of Protein[a]	Nearest Integral No. of Amino Acid Residues	Amino Acid Residues of CP A_α/ 34,600 Mol Wt
Lysine	15.2	15	15
Histidine	7.9	8	8
Arginine	10.8	11	11
Aspartic acid	27.9	28	28
Threonine[b]	25.2	25	25
Serine[b]	31.0	31	31
Glutamic acid	27.7	28	25
Proline	10.9	11	10
Glycine	23.2	23	23
Alanine	19.7	20	20
Valine	15.9	16	16
Isoleucine	19.7	20	20
Leucine	23.0	23	23
Tyrosine	18.7	19	19
Phenylalanine	16.2	16	16
Half-cystine	1.98	2	2
Methionine	3.02	3	3

[a] Assuming that the protein had approximately the same molecular weight of CP A_α. [b] Linear extrapolation of values obtained after 24 and 72 hr of hydrolysis to zero time.

TABLE V: Amino Acid Composition of the Peptide Fraction Following Complete Activation of Succinyl Fraction I.

	Extrapolated (μmoles)[a]	Residues (0.440 μmole)[b]	Nearest Integral No. of Residues	Residues Predicted from Zymogen − Enzyme Differences[d]
Lysine	1.144	2.6	3	2
Histidine	0.970	2.2	2	1
Arginine	1.635	3.7	4	3
Aspartic acid	2.120	4.8	5	5
Threonine	0.780	1.8	2	
Serine	1.221	2.8	3	
Glutamic acid	4.994	11.4	11	12
Alanine	1.630	3.8	4	4
Valine	1.670	6.1	6	6
Methionine[c]	0.443	1.0	1	1
Tyrosine	0.436	1.0	1	1
Isoleucine	1.466	3.3	3	2
Leucine	2.442	5.6	6	7
Glycine	1.672	3.8	4	4
Phenylalanine	1.239	2.8	3	2
Proline	1.630	3.7	4	4
			62	54
			(6764)[e]	(5862)[e]

[a] The data are expressed as micromoles of residue per total material recovered. [b] Assuming that 0.440 μmole = 1 residue (methionine sulfone, 0.443 μmole; 0.436 μmole of tyrosine). The assumption made is that the conversion of succinyl fraction I (four methionines per mole) to succinylcarboxypeptidase A (three methionines per mole) involves the release of one methionyl residue. [c] Determined by performic acid oxidation. [d] Differences in the amino acid compositions of succinyl fraction I and succinylcarboxypeptidase. [e] Molecular weights calculated from the number of residues of amino acid times residue molecular weight.

N-Terminal analysis of fully activated succinyl fraction I revealed the presence of the DNP derivatives of alanine, serine, and minor amounts of aspartic acid (Table III). The uncorrected recoveries indicate that alanine and serine were the major new amino-terminal groups formed following complete tryptic activation.

The amino acid composition of fully activated succinyl fraction I was calculated as summarized in Table IV. The minimum molecular weight of 34.631 ± 368 is in good agreement with the value of 34,600 reported for carboxypeptidase A_α by Bargetzi *et al.* (1963).

A striking resemblance is apparent when the amino acid compositions of fully activated succinyl fraction I and carboxypeptidase A_α are compared (Table IV). Except for three residues of glutamic acid (or glutamine) and one proline, the amino acid compositions are identical.

The peptide fraction liberated following complete tryptic activation (Figure 4) was also subjected to amino acid analysis. The number of residues was calculated from the extrapolated micromoles for each amino acid assuming that 0.440 μmole = 1.0 g residue of amino acids (methionine sulfone, 0.443 μmole; tyrosine, 0.436 μmole). The corollary of this assumption is that the conversion of succinyl fraction I (four methionines per mole of protein) to succinyl carboxypeptidase (three methionines per mole of protein) involves the release of one methionyl residue. The data are shown in Table V. The peptide fraction contains virtually all amino acids except "half-cystine" and tryptophan and is particularly rich in dibasic amino acids. A comparison of the differences in amino acid composition of succinyl fraction I and succinylcarboxypeptidase with that of the peptide fraction is also shown in Table V. This difference amounts to 54 amino acid residues, as compared to 62 residues calculated from amino acid analysis of the peptide fraction.

Metal Replacement Studies of Succinylcarboxypeptidase. The substitution of zinc by various metals of the first transition series in carboxypeptidase results in significant changes in relative activities of the enzyme characteristic of the specific metal (Coleman and Vallee, 1961). More recently, Piras and Vallee (1966, 1967) have reported analogous metal replacement studies on procarboxypeptidase A-S6 and have concluded that the esterase and peptidase activities observed are independent of the order in which metal substitution and tryptic activation are carried out, except that a

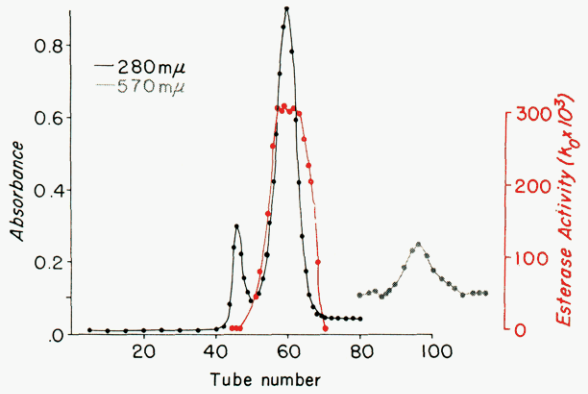

FIGURE 4: Sephadex G-75 gel filtration of the products of complete tryptic activation of succinyl fraction I. The protein fraction was located by means of absorbance measurements at 280 mμ (black line) and specific esterase activities were determined using HPLA as substrate (red line). To determine the position of the peptide fraction, aliquots of effluent fractions (tubes 80–120) were subjected to alkaline hydrolysis and ninhydrin color values, absorbance at 570 mμ, were determined gray line). For further details, see the text.

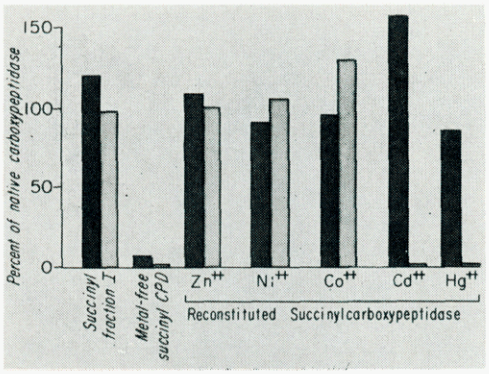

FIGURE 5: Reconstitution of metal-free succinylcarboxypeptidase with certain metal ions. Following tryptic activation of metal-free succinyl fraction I, 2-ml aliquots (0.9 mg/ml) were dialyzed against 350 ml of 0.1 M metal-free Tris-HCl (pH 7.0) containing 1×10^{-4} M metal ion. Peptidase activity (white bars) and esterase activity (shaded bars) were determined using CGP and HPLA, respectively, as substrates. For further details, see the text.

metal atom must apparently occupy the metal-binding site of the zymogen for enzymatic activity to be observed. Since succinyl fraction I, in contrast to procarboxypeptidase A, is free of potential endopeptidase activity, similar analyses were carried out, using succinyl fraction I as the parent zymogen. A solution of 0.64 μmole of succinyl fraction I (0.92 mg/ml) in 0.1 M Tris-chloride buffer (pH 7.0) containing 2×10^{-3} M OP, was dialyzed against two changes of 500 ml each of the same buffer at 4° for 21 hr. To 0.32 μmole of the dialyzed zymogen solution was added TPCK-trypsin (100:1, w/w). The activation was allowed to proceed for 0.5 hr at 24° and then stopped by the addition of 0.2 mg of STI. Aliquots (2 ml) (0.9 mg/ml) of the activation mixture were dialyzed against 350 ml of 0.1 M metal-free Tris-chloride buffer (pH 7.0) containing, respectively, the following divalent metals (all chlorides) in concentrations of 10^{-4} M: Zn, Ni, Co, Cd, and Hg. A separate 2-ml aliquot was dialyzed against 350 ml of metal-free 0.1 M Tris-chloride buffer (pH 7.0) containing no metal ion. The activity of native carboxypeptidase was the basis of comparison. The results are summarized in Figure 5. Esterase and peptidase activities are in general agreement with the results obtained by Coleman and Vallee (1961) on the corresponding metallocarboxypeptidases and procarboxypeptidases (Piras and Vallee, 1966, 1967; Coleman *et al.*, 1966). Although metal analyses were not performed on these metal-reconstituted succinylcarboxypeptidases, the similarity in enzymatic changes in esterase and peptidase activities *known* to be associated with the various metal replacements in the native enzyme indicate that such replacements have, in fact, occurred in the succinlyated enzyme. The activation of succinyl fraction I in the absence of bound zinc results in a product which, upon addition of certain metal ions, including zinc, possesses the characteristic biological specificity associated with each of these metallocarboxypeptidases.

Acetylated of Succinyl Fraction I with N-Acetylimidazole. Acetylation of either carboxypeptidase or succinylcarboxypeptidase with *N*-acetylimidazole results in significant changes in the enzymatic properties of the enzyme (Simpson *et al.*, 1963; Riordan and Vallee, 1964). The peptidase activity is almost completely destroyed while the esterase activity (HPLA as substrate) as measured with 0.01 M *dl*-HLPA is increased by about 500–600% of that of the native enzyme. Simpson and co-workers (1963) have correlated these changes in enzymatic activity with the acetylation of two particular tyrosyl residues at or near the active center of carboxypeptidase. These changes in enzymatic function could be prevented by the inclusion of 0.05 M β-phenylpropionate during the acetylation, or reversed by incubation with hydroxylamine.

In order to determine whether these tyrosyl residues of the active site are also available and reactive in the zymogen, succinyl fraction I was acetylated with *N*-acetylimidazole in the presence and absence of β-phenylpropionate and then subjected to the activation procedure. Solutions of succinyl fraction I (2 mg/ml) in 0.05 M sodium Veronal–2.0 M sodium chloride were treated with varying amounts of *N*-acetylimidazole and subsequently dialyzed for 24 hr against 2 l. of the same buffer. The acetylated succinyl fraction I solutions were then incubated at 25° with TPCK-treated trypsin. Peptidase activity was measured at times of activation

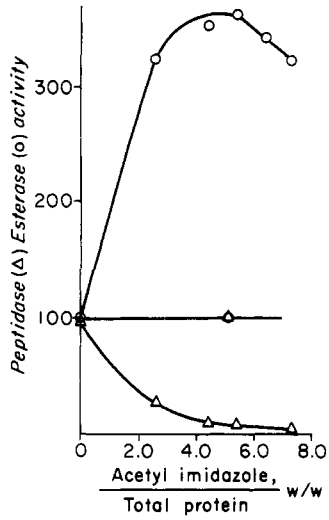

FIGURE 6: Effects of acetylation of succinyl fraction I with acetylimidazole on the esterase and peptidase activities obtained after activation with TPCK-treated trypsin (10:1, w/w) at 25°. Enzymatic activities are given in terms of per cent of that of activated succinyl fraction I. The horizontal line originating at 100% activity shows the protective effects of 0.1 M β-phenylpropionate on the effects of acetylation.

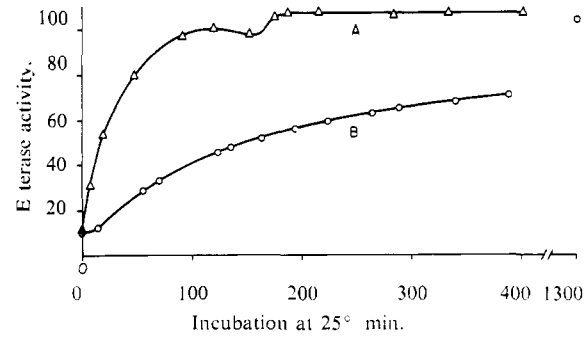

FIGURE 7: Activation of succinyl fraction I by trypsin in the presence (curve B) and absence (curve A) of 0.20 N β-phenylpropionate. For experimental details, see the text. Esterase activity is expressed in terms of per cent of that of native carboxypeptidase A_α.

corresponding to maximal esterase activity for each concentration of the acetylating agent (Figure 6). At a weight ratio of *N*-acetylimidazole to succinyl fraction I of 5:1, the peptidase activity was almost completely lost while the esterase activity increased by approximately 360% over that of the control succinyl fraction I. These changes in enzymatic function, induced by acetylation, were prevented by prior addition of 0.10 M β-phenylpropionate to the zymogen at 0° (Figure 6). As in the case of acetylcarboxypeptidase, reversal of these enzymatic changes could be effected by incubation at 24° with 1.0 M hydroxylamine for 10 min.

Since the activation of β-phenylpropionate prior to activation prevents the effects of acetylation, it appeared of interest to examine the action of this competitive inhibitor on the rate of activation of succinyl fraction I as a probe for a possible relationship of the binding site to the site of activation. The activation at 25° of solutions containing 0.94 mg/ml of succinyl fraction I and TPCK-trypsin (100:1, w/w) was followed by measurements of esterase activity. The buffers employed were 0.40 M ammonium acetate (pH 7.5) (curve A, Figure 7) and 0.20 M ammonium acetate–0.2 M β-phenylpropionate (pH 7.5) (curve B, Figure 7). Samples for assay were sufficiently diluted that the final concentrations of β-phenylpropionate were not inhibitory as determined in control samples. As indicated in Figure 7, after 190-min incubation maximal activity was reached in the sample containing no inhibitor (curve A). The sample containing 0.20 M β-phenylpropionate had only reached 52% of maximal activity after 190-min incubation (curve B), but was fully active after 1300-min incubation. Controls showed that tryptic activity toward BAEE was not inhibited by β-phenylpropionate. These data indicate that β-phenylpropionate interacts with succinyl fraction I in such a way that the rate of conversion of the zymogen to active enzyme is greatly diminished.

Discussion

Succinyl fraction I is rapidly and quantitatively converted by trypsin to a product having the known properties of succinylcarboxypeptidase A. This conversion proceeds two orders of magnitude faster than that of the procarboxypeptidase A-S6 aggregate. It is complete within 1.5 hr at 25° at a zymogen:trypsin ratio of 160:1 (w/w) whereas full activation of procarboxypeptidase A-S6 requires 12 hr at 36° at a zymogen:trypsin ratio of 19:1 (w/w) (Yamasaki *et al.*, 1963). In further contrast to the zymogen aggregate, activation of succinyl fraction I does not require the participation of the endopeptidase in fraction II. However, several proteolytic enzymes, besides trypsin, can catalyze the activation of succinyl fraction I, such as chymotrypsin, papain, subtilisin, and activated fraction II. These results demonstrate that the conversion of succinyl fraction I to an active enzyme has no unique relationship to the specificity of the activating enzyme and suggest that a multiplicity of chemically distinct forms of the enzyme may arise depending upon which peptide bond or bonds are cleaved by each of these proteolytic enzymes.

The tryptic activation of succinyl fraction I apparently occurs in two stages. The sedimentation coefficient remains unchanged in the first stage (up to 80–85% of maximal activity) and is the same as that of succinyl fraction I (4.0 S). Upon full activation, in the second stage, the sedimentation coefficient decreases to a value of 3.1 S, which is identical with that reported for native carboxypeptidase A (Rupley and Neurath, 1960). This behavior is analogous to that seen in the tryptic activation of bovine procarboxypeptidase B

(Wintersberger *et al.*, 1962; Cox *et al.*, 1962) which likewise occurs in two stages as defined by activity and sedimentation coefficients.

The two stages in the activation process may also be related to the deacylation of *O*-succinyltyrosine residues, as shown in Figure 3A. In the first stage, two residues become deacylated, and in the second stage an additional two. The presence of *O*-succinyltyrosines in succinyl fraction I was unexpected since Riordan and Vallee (1964) had previously shown that in succinylcarboxypeptidase, these residues undergo a time-dependent deacylation, and the present preparations had been extensively dialyzed. If, in the succinylated enzyme, hydrolysis of *O*-succinyltyrosines occurs *via* a mechanism involving intramolecular nucleophilic catalysis by the carboxylate group of the succinic ester (Riordan and Vallee, 1964; Bruice and Pandit, 1960) it must be concluded that in the succinylated zymogen the steric relationships among the participating groups are unfavorable for such a reaction. The release of succinyl groups during activation suggests that a conformational change occurs during this first stage of the activation process, which induces both enzyme activity and deacylation of two *O*-succinyltyrosine residues.

The second, slower step of activation no longer represents an obligatory aspect of the activation proper. The attendant molecular changes include the deacylation of two additional succinyltyrosines, the release of peptides containing approximately 60 amino acid residues, and a concomitant decrease in sedimentation coefficient from 4 to 3 S.

Succinyl fraction I contains two lysines and three arginines in addition to those of carboxypeptidase A_α. Thus, there are at least three possible sites potentially susceptible to tryptic cleavage. The multiplicity of N-terminal residues of the activated species and the fact that several proteolytic enzymes can effect a transformation of succinyl fraction I to an active enzyme suggest that, indeed, several chemically distinct, active forms of the enzyme result from tryptic activation of succinyl fraction I and have amino acid compositions similar to carboxypeptidase A_α. The N-terminal residues are preponderantly those found for the α and β forms of carboxypeptidase A and have probably arisen from tryptic cleavage of bonds indicated by the arrows in the N-terminal sequence (Sampath Kumar *et al.*, 1964)

$$\overset{\uparrow(\alpha)}{\text{Ala-Arg}}\overset{\uparrow(\beta)}{\text{Ser-Thr--}}$$

Several inherent difficulties became apparent in attempts to identify the peptide fraction released following the complete tryptic activation of succinyl fraction I. The finding of several N-terminal amino acid residues after activation would indicate that a mixture of peptides was released. In addition, the succinylated enzyme formed could liberate carboxyl-terminal free amino acids from either succinyl fraction I or from succinylcarboxypeptidase (Bargetzi *et al.*, 1964). Since the mixture of low molecular weight material liberated during activation contained eight amino acids more than predicted from differences in the amino acid compositions of the zymogen and the normal enzyme, exopeptidase action is a distinct possibility.

Evidence for the preexistence of substrate and inhibitor binding sites in zymogens of proteolytic enzymes has been accumulating in recent years. The first indication of this rather unexpected characteristic of zymogens came from the work of Vaslow and Doherty (1953) on chymotrypsinogen and has been recently extended by Deranleau and Neurath (1966) as well as by several other investigators (Weiner and Koshland, 1965; Glazer, 1965). The present experiments on the acetylation of succinyl fraction I and its prevention by β-phenylpropionate are supporting the view that the binding site for β-phenylpropionate also exists in this zymogen. Thus acetylation led to a product which, upon activation with trypsin, had activity characteristics of acetylcarboxypeptidase. Hence the tyrosine residues of the active site (Simpson *et al.*, 1963) appear to be available for acetylation in the succinylated zymogen. More significantly, the effects of acetylation were prevented by β-phenylpropionate, as in the native enzyme, and were reversed by hydroxylamine. These data are entirely consistent with the behavior of native carboxypeptidase during acetylation and indicate that the effects of acetylation and activation of the succinylated zymogen are independent of the sequence in which these operations are performed. β-Phenylpropionate also caused a marked retardation in the rate of tryptic activation of succinyl fraction I. If the inhibitor is assumed to bind only at the active site, this result indicates that the inhibitor binding site and the site of activation may be in close proximity in the succinylated zymogen.

Recent experiments of Piras and Vallee (1966, 1967) strongly support the concept that the substrate (or inhibitor) binding site of carboxypeptidase preexists in the zymogen. These investigators have shown that ^{65}Zn freely exchanges with the single zinc atom in procarboxypeptidase A-S6 but competitive inhibitors or specific substrates markedly retard this exchange.

Incubation of aposuccinyl fraction I with trypsin resulted in a species which, upon addition of zinc or other metals, possessed the full enzymatic activity associated with each of the respective metallosuccinylcarboxypeptidases. Proteolysis of the correct peptide bond(s) undoubtedly takes place; presumably addition of the metal, absolutely required for catalytic function, permits the formation of the chelate at the active site (Coleman and Vallee, 1961). In contrast to these results, apoprocarboxypeptidase A-S6 treated with trypsin exhibited no carboxypeptidase activity even after addition of zinc (Piras and Vallee, 1966, 1967). Since there is a free exchange of Zn^{2+} with the bound zinc atom in native procarboxypeptidase A-S6 (*vide supra*), it is possible that the Zn binding site has become altered, perhaps as a consequence of proteolysis by the endopeptidase formed in stoichiometric amounts during activation of the procarboxypeptidase aggregate.

Alternate explanations for these differences in behavior of the apozymogens during tryptic attack are suggested by the physicochemical differences between succinyl fraction I and procarboxypeptidase A-S6. Not only does the former lack the potential endopeptidase but also it has a major redistribution of charges and a much lower molecular weight. The observations that activation proceeds much more quickly in the disaggregated system and is a two-step process suggest differences in the mechanistic detail of the activation reaction. A precise molecular explanation of the differences in metal restoration must await further examination of the nature and role of metal ligands in the activation mechanism of the disaggregated zymogen.

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