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Induction of Polymer Formation in Solutions of Bovine Pancreas Carboxypeptidase A by Aromatic Compounds*

J. L. Bethune

ABSTRACT: In solutions containing β-phenylpropionic acid, carboxypeptidase A_γ and A_δ form polymers stabilized by hydrophobic bonds. The extent of polymer formation varies directly with the temperature and the concentrations of protein, β-phenylpropionic acid, and NaCl in the solvent. The active center is one of the sites of polymerization, since removal of the zinc atom greatly depresses polymer formation. Acetylation with acetylimidazole also depresses formation; hence tyrosyl residues appear to be components of the other site(s). This investigation reveals binding site(s) accessory to those utilized by synthetic di- and tripeptides [Coleman, J. E., and Vallee, B. L. (1964), *Biochemistry 3*, 1874] which are called upon when large molecules are attached to the enzyme, thus explaining the stabilities of polypeptide–enzyme–substrate complexes, large compared to those of dipeptide–enzyme complexes [Coombs, T. L., and Wacker, W. E. C. (1965), *Federation Proc. 24*, 410].

The physical chemistry of polymerization reactions of proteolytic enzymes and their potential pertinence to the chemical basis of catalytic specificity has been discussed recently (Bethune, 1965). Carboxypeptidase A, in particular, has been shown to undergo polymerization to either one of two products, depending upon the NaCl concentration in the solvent. In 1–2 M NaCl, the integrity of certain tyrosyl residues of the protein is required for polymer formation. The polymer formed in 2.5 M NaCl is stabilized by hydrophobic bonds.

The present investigation reports a third mode of polymerization of both carboxypeptidase A_γ and A_δ induced by certain aromatic compounds. The catalytically active zinc atom and the "free" tyrosyl residues of carboxypeptidase participate in the formation of the polymer which is stabilized by hydrophobic bonds as shown by its temperature dependence. A preliminary account has been rendered (Bethune, 1964).

Materials and Methods

Bovine pancreatic carboxypeptidase A_δ was prepared by the method of Allan *et al.* (1964). *Bovine pancreatic carboxypeptidase A_γ*, prepared by the method of Anson (1937), was obtained commercially (Worthington Biochemical Corp., Freehold, N. J.). *Protein concentrations* were measured by absorbance at 278 mμ. A molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} was used (Simpson *et al.*, 1963). *Apocarboxypeptidase* was prepared as described (Coleman and Vallee, 1961). All chemicals were of reagent grade and used without further purification.

Electrophoresis was carried out in a Spinco Model H diffusion and electrophoresis apparatus at 18°. *Sedimentation* was carried out at 59,780 rpm in a Spinco Model E ultracentrifuge, equipped with a phase plate and an RTIC unit. Sedimentation coefficients were determined by projection and tracing of the patterns, while areas were determined from these by planimetry. Suitable base lines were run in all cases. All sedimentation coefficients were corrected to those which would obtain in a solvent with the viscosity and density of water at 20° (Svedberg and Pedersen, 1940). *pH measurements* were made with a Radiometer pH meter. All measurements are referred to National Bureau of Standards pH 7 buffer.

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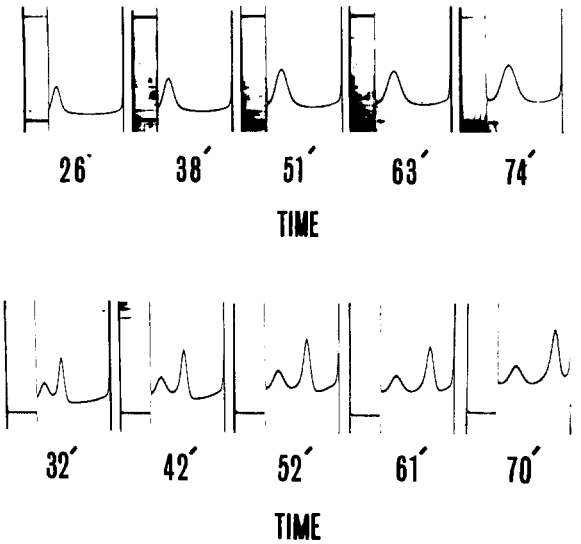

FIGURE 1: Sedimentation of carboxypeptidase A_γ. Upper, 2 M NaCl, 0.02 M sodium Barbital, pH 7.5, protein concentration, 17 mg/ml. Phase-plate angles, from left to right, 80°, 75°, 70°, 65°. Lower, in 1 M NaCl, 0.02 M sodium Barbital, 0.1 M β-phenylpropionic acid, pH 7.5. Protein concentration, 19 mg/ml. Phase-plate angles, from left to right, 75°, 70°, 65°, 65°, 60°; at 59,780 rpm. Here and in all similar illustrations sedimentation proceeds from left to right.

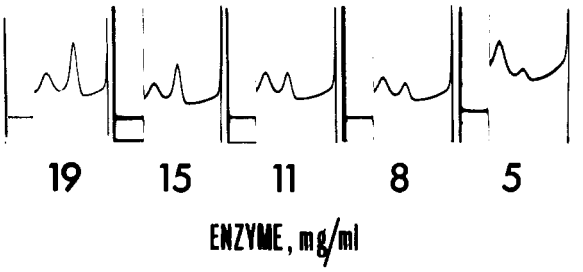

FIGURE 2: Effect of varying carboxypeptidase A_γ concentration. In 1 M NaCl, 0.02 M sodium barbital, 0.1 M β-phenylpropionic acid, pH 7.5, at 59,780 rpm. Times (in minutes) and phase-plate angles, from left to right, 52, 65°; 40, 70°; 39, 65°; 37, 65°; 40, 50°.

Results

Carboxypeptidase A in 2 M NaCl, 0.02 M sodium Barbital, pH 7.5, sediments as a single, symmetrical boundary (Figure 1, top). On addition of 0.1 M β-phenylpropionic acid two boundaries appear (Figure 1, bottom).

The relative areas under the two boundaries are functions of the protein concentration (Figure 2 and Table I). The areas are expressed as mg/ml. As the total protein concentration increases, the areas under *both* boundaries increase. The sedimentation coefficients of each boundary are shown in Table I, columns 3 and 5. The slow boundary sediments at the same rate as carboxypeptidase in the absence of β-phenylpropionic acid, *i.e.*, 3.3 S, while the sedimentation coefficient of the second boundary is much greater, *i.e.*, 10 S. With increasing protein concentration both coefficients decrease. As the total protein concentration varies, the changes in concentration represented by the area under the slow boundary and the changes in the sedimentation coefficient of the rapid boundary are not consistent with a rapidly reversible polymerization reaction (Gilbert, 1955).

On the basis of present theory, such a result must be regarded either as a polymerization, the half-time of which is large compared to that of separation in the centrifuge, *i.e.*, about 15 min, or as a pseudopolymerization of the type $aA + bB = C$, where the concentration of B, in this case β-phenylpropionic acid, is virtually constant across the cell, and a and b are stoichiometric coefficients.

Figure 3 shows a plot of the actual areas *vs.* protein concentration. The areas are expressed in arbitrary units at a fixed plate angle. Areas obtained for the protein in the absence of β-phenylpropionic acid are included. All points lie on one line. For those samples which did not contain β-phenylpropionic acid, the slope of the regression line is 21.7 ± 0.4 area units/mg per ml, that for the samples with β-phenylpropionic acid is 21.9 ± 0.5, while that for the whole set is 21.5 ± 0.3. Thus, there is no statistically significant difference in slope in the presence and absence of β-phenylpropionic acid and, hence, micelle formation by the β-phenylpropionic acid cannot be held responsible for the appearance of the rapid boundary.

Polymer formation is also a function of β-phenylpropionic acid concentration (Figure 4). The rapid boundary is not detected at concentrations lower than 1×10^{-3} M, a concentration which is well above that at which the enzymatic activity is inhibited completely (Coleman and Vallee, 1964). At concentrations of 5×10^{-2} M β-phenylpropionic acid and above, the ratio of the two areas remains constant. The dependence upon β-phenylpropionic acid concentration is completely reversible, the same area distribution being obtained if a given concentration of this agent is approached from either side.

TABLE I: Sedimentation Data for Carboxypeptidase in 1 M NaCl, 0.01 M β-Phenylpropionic Acid, 0.02 M Sodium Barbital, pH 7.5.

Protein Concn (mg/ml)	Slow Boundary		Rapid Boundary	
	Area (mg/ml)	$s_{20,w}$ (S)	Area (mg/ml)	$s_{20,w}$ (S)
18.7	6.5	3.2	12.2	9.1
14.6	5.4	3.3	9.2	9.2
10.6	5.2	3.3	5.4	9.6
7.8	4.1	3.3	3.7	9.7
5.4	4.0	3.3	1.4	9.9

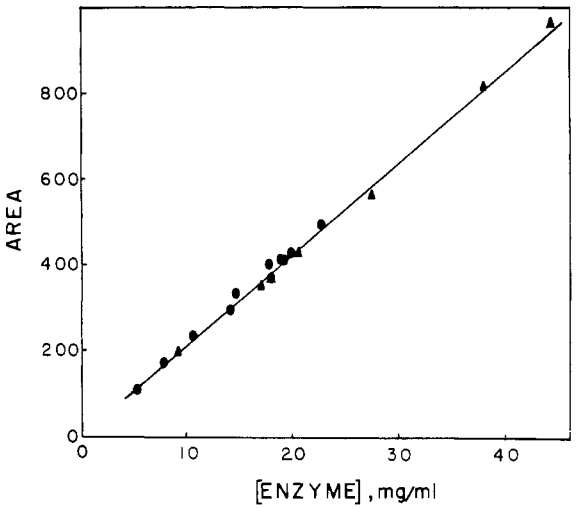

FIGURE 3: Total area *vs.* carboxypeptidase A_γ concentration. In the presence of 0.1 M β-phenylpropionic acid (●); in its absence (▲). The areas are in arbitrary units.

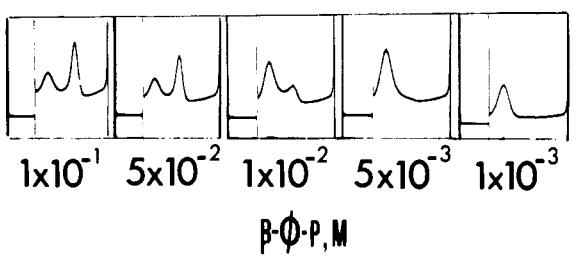

FIGURE 4: Effect of varying the β-phenylpropionic acid (β-ϕ-P) concentration. Enzyme concentration 18 mg/ml, 1 M NaCl, 0.02 M sodium Barbital, pH 7.5; at 59,780 rpm. Times (in minutes) and phase-plate angles, from left to right, 52, 65°; 42, 70°; 42, 70°; 42, 70°; 45, 80°.

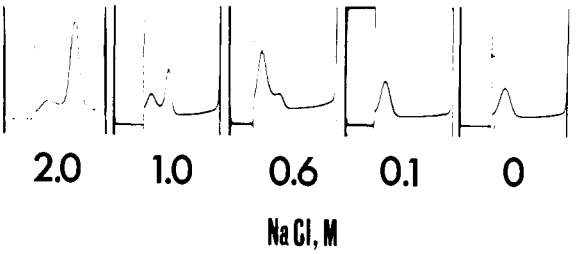

FIGURE 5: Effect of varying the NaCl concentration. 0.1 M β-phenylpropionic acid, 0.02 M sodium Barbital, pH 7.5. Enzyme concentration 14–17 mg/ml; at 59,780 rpm. Time (in minutes) and phase-plate angles, from left to right, 63, 60°; 32, 75°; 30, 65°; 33, 75°; 37, 75°.

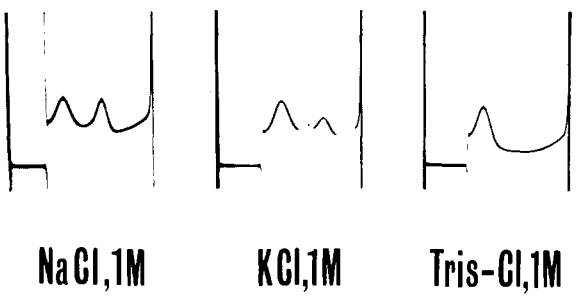

FIGURE 6: Effect of varying the cation species. 0.1 M β-phenylpropionic acid, 0.02 M sodium Barbital. Enzyme concentration 11 mg/ml; at 59,780 rpm. Time (in minutes) and phase-plate angles, for NaCl, 50, 60°; for KCl, 50, 65°; for Tris-Cl, 46, 75°.

Since carboxypeptidase A is generally soluble to a significant extent only in solutions of high ionic strength the above experiments were performed in 1 M NaCl–0.02 M sodium Barbital buffer, pH 7.5, to which the appropriate quantity of β-phenylpropionic acid has been added. It was found, however, that the enzyme is soluble in solutions of sodium Barbital and β-phenylpropionic acid in the absence of NaCl, therefore permitting the study of the effects of variation of NaCl concentration. In the absence of NaCl the second rapid boundary is not seen. It first appears when the NaCl concentration reaches 0.5 M, the relative area under this boundary increasing with salt concentration up to 2 M (Figure 5). Remarkably, this phenomenon is not only an effect of ionic strength, since it also depends upon the particular cation present (Figure 6). Li^+ and Na^+ are both equally effective in inducing polymer formation, and both are more effective than K^+, which in turn is more effective than NH_4^+. In the presence of Tris-H^+ the second boundary is not detected. A change of the anion from, *e.g.*, NaCl to $NaNO_3$ to sodium acetate does not however affect the relative areas under the boundaries.

The presence or absence of the Barbital buffer does not alter the extent of polymer formation. Sedimentation of the protein in a 1 M NaCl–0.1 M β-phenylpropionic acid solution, adjusted to pH 7.5 with NaOH, or in 1 M NH_4Cl–0.1 M phenylpropionic acid solution, adjusted to pH 7.5 with NH_4OH, results in the same area distribution under the boundaries as that seen when either sodium or ammonium Barbital is additionally present.

Over the pH range 6.5–9.5 the polymerization is independent of pH. Sedimentation patterns at a protein concentration of 18 mg/ml in 1 M NaCl, 0.1 M β-phenylpropionic acid, 0.02 M sodium Barbital or Tris, adjusted to the required pH, are superimposable at any pH value between 6.5 and 9.5.

The zinc atom must be present for the polymerization to occur. Its removal almost completely abolishes the polymerization, as shown by sedimentation of the native and apoenzymes in the presence of β-phenylpropionic acid (Figure 7). The pattern for the apoenzyme on the right is greatly magnified over that for the native enzyme to show the small slow boundary, which, in effect, is only readily visible when the bar angle is 60°, much lower than in the control. Native

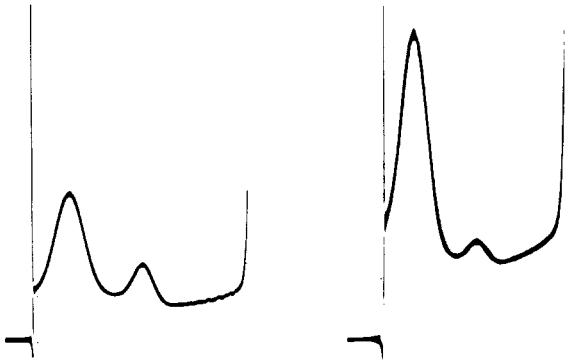

FIGURE 7: Effect of removal of zinc upon the polymerization of carboxypeptidase A_δ. Left, native enzyme; right, apoenzyme. In 1 M NaCl, 0.1 M β-phenylpropionic acid, 0.02 M sodium Barbital, pH 7.5. Enzyme concentration 23 mg/ml; at 59,780 rpm. Native enzyme, after 42 min, phase-plate angle 75°; apoenzyme, after 37 min, an angle of 60°.

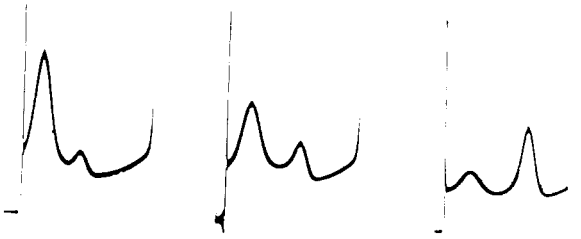

FIGURE 8: Effect of temperature. Left, 4°; center, 15°; right, 30°. In 1 M NaCl, 0.02 M sodium Barbital, 0.1 M β-phenylpropionic acid, pH 7.5. Enzyme concentration 12 mg/ml; at 59,780 rpm. Time (in minutes) and phase-plate angle, from left to right, 62, 60°; 70, 60°; 48, 65°.

carboxypeptidase A binds 1 mole of β-phenylpropionic acid at the active center while apocarboxypeptidase A does not (Coleman and Vallee, 1964). Hence, this must be one of the sites through which polymerization occurs.

The polymerization in β-phenylpropionic acid is temperature dependent (Figure 8). Similar to the polymerization in 2.5 M NaCl (Bethune, 1965), the relative area under the rapid boundary varies *directly* and reversibly with temperature. This behavior would be predicted for a polymer stabilized by hydrophobic bonds, and this constitutes important confirmation of this type of bonding in a protein–protein interaction.

The temperature dependence of the system also permits the determination of possible changes in charge as a result of the polymerization reaction. At a concentration and temperature giving equal areas under the two boundaries in the centrifuge, free boundary electrophoresis of the enzyme shows only a single symmetrical boundary. Moreover, if a solution of the enzyme in 1 M NaCl, 0.1 M β-phenylpropionic acid, under nitrogen, is adjusted to pH 7 at 20° and the temperature is then lowered to 0°, the pH of the resultant solution does not differ within ±0.02 pH unit from that of the solvent after undergoing the same temperature change. Thus, there is no detectable change in charge to mass ratio or in the balance uptake or release of hydrogen ions as a result of the reaction.

As is apparent from Table II, β-phenylpropionic acid is not the only agent giving rise to this process. While some of these agents, like β-phenylpropionic acid, are inhibitors of the enzyme others have no effect on activity. There is no correlation between capacity to serve as substrates or inhibitors and ability to induce polymerization. Thus, β-phenylpropionic acid and 3-indoleacetic acid both inhibit the enzyme and both induce polymerization; but iodoacetic acid and bromacetic acid, while inhibiting the enzyme (Coleman and Vallee, 1964), do not induce polymerization. The products of hydrolysis of the synthetic substrates carbobenzoxyglycyl-L-phenylalanine and hippuryl-DL-phenyllactic acid induce double boundary formation; however, the two substrates glycyl-L-phenylalanine and glycyl-L-leucine do not.

TABLE II: Effect of Various Compounds on Carboxypeptidase A.[a]

Compounds Inducing Polymerization	Compounds not Inducing Polymerization
Benzoic acid	Bromoacetic acid
Carbobenzoxyglycine	Caprylic acid
Hippuric acid	Glycyl-L-leucine
Indole-3-acetic acid	Glycyl-L-phenylalanine
Indole-3-propionic acid	*p*-Hydroxyphenoxyacetic acid
Phenylacetic acid	*p*-Hydroxy-β-phenylpropionic acid
L-Phenylalanine	Iodoacetic acid
L-Phenylalaninamide	β-Iodopropionic acid
D-Phenyllactic acid	*p*-Nitrophenylacetic acid
L-Phenyllactic acid	D-Phenylalanine
Phenoxyacetic acid	
β-Phenylpropionic acid	

[a] All compounds were used in 2 M NaCl, 0.02 M sodium Barbital, pH 7.5, 20–22°, at a concentration of 0.1 M, except for glycyl-L-leucine, 0.19 M, and iodoacetic acid, 0.1 and 0.5 M.

A terminal aromatic ring which is not *para*-substituted is a common feature of the substances which induce polymerization. While these structural requirements are necessary features they are not sufficient of themselves. D-Phenylalanine, an inhibitor of the enzyme exhibiting the requisite characteristics, does not cause double boundary formation, though the hydrolysis product of the synthetic substrate, L-phenylalanine, is effective in inducing it. Further, L-phenylalaninamide is more effective than L-phenylalanine. Both D- and

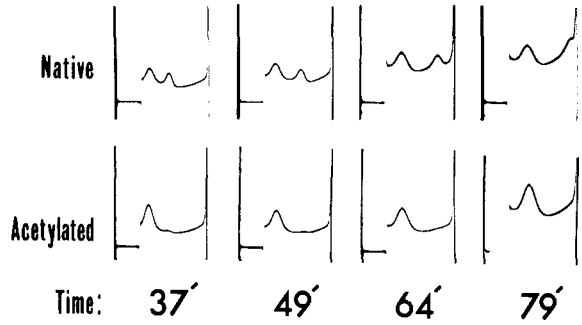

FIGURE 9: Effect of acetylation. Top, native carboxypeptidase A_γ; bottom, acetylated enzyme. 1 M NaCl, 0.02 M sodium Barbital, 0.1 M β-phenylpropionic acid, pH 7.5. Enzyme concentration 8 mg/ml; at 59,780 rpm. Phase-plate angles from left to right, for the native enzyme, 65°, 60°, 50°, 45°; for the acetylated enzyme, 65°, 65°, 60°, 45°.

L-phenyllactic acids cause polymerization, demonstrating that the orientation of the positive charge on the amino group and the negative charge on the carboxylate group in the phenylalanines largely determines the stereospecificity.

The absolute requirement of a nonsubstituted terminal aromatic ring in the induction of polymerization suggests that the agent interacts primarily with aromatic side chains of amino acid residues on the protein. The possible involvement of tyrosyl residues can be readily examined since the acetylating agent acetylimidazole specifically modifies 6–7 tyrosine residues of carboxypeptidase and does not acetylate other residues in this enzyme (Simpson *et al.*, 1963). However, only 4–5 tyrosyl residues *outside* the active center of the enzyme which binds dipeptide substrates are acetylated when this reaction is carried out in the presence of β-phenylpropionic acid. The inhibitor still binds at the active center of carboxypeptidase acetylated in the presence of β-phenylpropionic acid (Coleman and Vallee, 1964). Sedimentation patterns of carboxypeptidase acetylated in the absence of β-phenylpropionic acid are shown in Figure 9, together with a control for the native enzyme at this protein concentration. The polymerization is almost completely abolished. The same is true for the partially acetylated enzyme. Thus, β-phenylpropionic acid must bond to sites on the surface of the carboxypeptidase molecule in addition to those at the active site where synthetic dipeptide substrates and inhibitors bind. It may be concluded that these ancillary sites, containing tyrosine residues as an important constituent, are responsible in part for the β-phenylpropionic acid mediated polymerization of the enzyme.

Discussion

The first two modes of polymerization of carboxypeptidase A have been discussed previously (Bethune, 1965). The third mode of polymerization is that induced by compounds such as β-phenylpropionic acid. While referring to this phenomenon as a polymerization of the enzyme, it is obviously an inadequate description, since β-phenylpropionic acid is known to bind to the enzyme. Thus, the monomer species in solution is a complex between β-phenylpropionic acid and enzyme. This first reaction is of the type:

$$E + xA \rightleftharpoons C$$

where E = carboxypeptidase, A = β-phenylpropionic acid, C = enzyme–β-phenylpropionic acid complex, x = number of moles of β-phenylpropionic acid bound. The polymerization reaction then follows:

$$nC \rightleftharpoons P$$

where P = polymer, n = degree of polymerization. Assuming the simplest case, that no intermediate complexes of polymers exist, formal equilibrium constants may be written[1]

$$K_1 = \frac{(C)}{(E)(A)^x}$$

$$K_2 = \frac{(P)}{(C)^n}$$

Then

$$K_1^n K_2 = \frac{(P)}{(E)^n(A)^{nx}}$$

and if a concentration plateau of A exists in the cell, the ratio of $(P)/(E)^n$ is constant and the polymerization may be described by a single pseudoequilibrium constant. Calculation of the concentration distribution of a molecule of the size of β-phenylpropionic acid at equilibrium in the applied field shows that the maximum concentration change, for an initial concentration of 0.1 M, is from 0.08 to 0.12 M. The data shown in Figure 4 illustrates that, above a concentration of 0.05 M, further increase in the concentration of β-phenylpropionic acid has no effect upon the relative areas under the boundaries. Therefore, the existence of a concentration range of 0.08 to 0.12 M does not affect the results. The same considerations apply to the distribution of NaCl in the cell.

Under these conditions, the reaction leading to the formation of a polymer can be studied as an isolated system. The data in Table I, therefore, demonstrate that

[1] The third pattern of Figure 4, that at 1×10^{-2} M β-phenylpropionic acid, may show the presence of an intermediate, since here the refractive index gradient does not approach the base line between the two boundaries. Above 5×10^{-2} M β-phenylpropionic acid, however, this possible intermediate is present in, at most, negligible amounts, since here the gradient closely approaches the base line.

this polymerization cannot belong to the rapidly equilibrating class since in such systems the area under the slow boundary remains constant with increasing protein concentration after the second boundary has appeared. Moreover, the sedimentation coefficient of the rapid boundary should increase with increasing protein concentration (Gilbert, 1955).

The exact formulation of the process must await further theoretical investigation, since the observed effects may be due to a kinetically controlled reaction (Oberhauser *et al.*, 1965).

Since a terminal aromatic ring is the structural feature of the agent necessary for effective polymerization, it may be hypothesized that the effective agents are bound to hydrophobic (aromatic?) sites of the enzyme. Each enzyme molecule must contain at least two interacting sites since a polymer larger than a dimer is formed. The formation of a finite polymer,[2] however, makes the existence of more than two sites of interaction improbable: more than two sites would allow the formation of many different polymers. Moreover, to yield a finite polymer, the angle between the interaction sites on any one molecule cannot be 180°, since this would also allow very large polymers to form.

The active center itself may be immediately identified as one of the sites. It is known that 1 mole of β-phenylpropionic acid binds at the active site, with a dissociation constant of 1×10^{-4} M. Furthermore, in the zinc-free enzyme binding of β-phenylpropionate at the active site does not occur (Coleman and Vallee, 1964), and on sedimentation of the apoenzyme in the presence of this agent a remarkable diminution in polymer formation is seen (Figure 7).

The existence of an additional site(s) is identified by the acetylation experiments. If acetylation is performed in the absence of β-phenylpropionic acid, six–seven "free" tyrosine residues are acetylated while other reactive groups are not modified (Simpson *et al.*, 1963). Only two of these residues are at the active catalytic center for dipeptide substrates; the other four to five are not. When the active center is acetylated, β-phenylpropionic acid no longer binds to this locus (Vallee, 1964). In this case polymerization is repressed. If the acetylation is performed in the presence of β-phenylpropionic acid, only four–five tyrosine residues, not located at the "active center," are acetylated. In such partially acetylated carboxypeptidases the two active center residues are not acetylated and β-phenylpropionic acid binds at the active center (Vallee, 1964). Sedimentation of this material reveals that polymerization again does not occur, indicating that another site(s) of interaction, which also binds β-phenylpropionic acid, is present on the enzyme. Since those sites are affected by acetylation with acetylimidazole, which modifies only the tyrosines of carboxypeptidase, they must contain tyrosyl residues as does the active center.

Since polymer formation occurs only when the concentration of β-phenylpropionic acid exceeds 1×10^{-3} M and is complete at 5×10^{-2} M, the dissociation constant of the second molecule necessary for polymerization appears to be of the order of 10^{-2} M, much higher than that for binding to the active center, 1×10^{-4} M (Coleman and Vallee, 1964). The firmer binding of β-phenylpropionic acid to the active site undoubtedly reflects the contribution of the zinc atom to binding. A binding constant of the order of 10^{-2} M would also explain the observation that concentrations of β-phenylpropionic acid greater than 0.05 M do not affect the distribution of area under the two boundaries.

Since the dependence of β-phenylpropionic acid binding at any site on temperature is not known, conclusions cannot as yet be drawn regarding the nature of the forces responsible for binding. *Polymer* formation, however, is heavily dependent upon temperature, the ratio of polymer to monomer increasing with increasing temperature. This temperature dependence is that predicted for structures stabilized by hydrophobic bonds (Kauzmann, 1959; Némethy and Scheraga, 1962) and is one of the most dramatic physical manifestations of this type of bonding. Even though the nature of this temperature dependence for hydrophobic bonding is well understood from model systems, the experimental demonstration of its occurrence in protein–protein interaction is still scant (Murayama, 1956; Lauffer *et al.*, 1958; Kirkman and Hendrickson, 1962; Von Eys *et al.*, 1964). Hence, the present data constitute significant confirmation of the existence of these forces in a protein system.

This temperature dependence is the same as that found for the polymerization of carboxypeptidase A in 2.5 M NaCl (Bethune, 1965) and, since the β-phenylpropionic acid induced polymerization also requires a concentration of NaCl greater than 0.5 M before being apparent, these may be manifestations of one basic molecular process, a change in the hydration of the enzyme molecule.

The actual binding of one monomer unit to another is not accompanied by changes in electrophoretic mobility nor are hydrogen ions detectably involved in the actual polymerization itself, since minimal pH changes are observed as the temperature is increased from 4 (very little polymer) to 20° (predominantly polymer). It is, therefore, highly probable that the high concentration of salt is not required to overcome charge repulsion but is necessary to lower the activity of the solvent sufficiently to allow intramolecular bonding. Moreover, no charged residue of the enzyme molecule is masked during the polymerization by movement to a region in which its pK_a differs from its normal pK_a. This contrasts with the situation observed in the polymerization of tobacco mosaic virus protein, where the dependence of the polymerization on temperature is similar to that observed here but in which changes in both electrophoretic mobility and in pH accompany the polymerization (Ansevin *et al.*, 1965).

The specific nature of the polymerization induced by aromatic agents is supported by several lines of evidence. The difference in the effects of D- and L-phenyl-

[2] A study of the variation of the weight-average molecular weight as a function of all the relevant parameters shows that the polymer formed is a hexamer (J. L. Bethune, to be published).

alanines demonstrates that the polymerization induced by the presence of the agents is stereospecific. The acetylation experiments, moreover, show that this process is not a result of nonspecific binding to heterogeneous loci since, in carboxypeptidase, only loci incorporating "free" tyrosyl residues are acetylated by acetylimidazole (Riordan *et al.*, 1965).

These findings are not only significant in regard to the physicochemical interaction of proteins and small molecules under experimental conditions. Carboxypeptidase is a proteolytic enzyme whose biological substrates are polypeptides and proteins. Detailed studies of substrate binding, utilizing small di- and tripeptides, have demonstrated that tyrosyl residues at the active site are involved at least in their binding (Coleman *et al.*, 1964). The present studies clearly demonstrate that the carboxypeptidase molecule will react with another protein molecule (in this case, another molecule of carboxypeptidase) to form a polymer. Here again, tyrosyl residues, additional to those at the active site, appear to be essential for binding. Such a protein–protein interaction suggests that binding of one of the natural protein substrates may well occur by multiple attachment to the enzyme. Thus, larger protein substrates would be bound not only at the active site but also at the ancillary, tyrosine-containing binding sites here identified. The exceedingly firm binding of protein substrates, whose dissociation constants are an order of magnitude lower than those for synthetic dipeptides (Coombs and Wacker, 1965), are explicable on such a basis. Moreover, the alterations in the molecular state of a protein accompanying temperature changes, such as that observed here and in other proteins (Ansevin *et al.*, 1964; Townend *et al.*, 1960), may serve as the explanation for the marked temperature dependence of biological events in some species (J. L. Bethune, in preparation).

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