The Polymerization of Carboxypeptidase A in Solutions Containing Sodium Chloride*

J. L. Bethune

ABSTRACT: Depending upon the NaCl concentration of the solvent, carboxypeptidase A polymerizes predominantly to either one of two products, as revealed by sedimentation. In 1–2 M NaCl only a single symmetrical boundary is seen, which together with the concentration dependence of the sedimentation coefficient implies the formation of a dimer. Apparently, one molecule of the enzyme, acting as an enzyme, interacts with a second molecule of enzyme acting as "substrate," the dimer, therefore, representing an "enzyme–substrate" complex. Acetylation of the "free" tyrosine residues of the enzyme, known to abolish synthetic dipeptide binding (Vallee, B. L. (1964), *6th Intern. Congr. Biochem. New York 32*, 255), also abolishes the polymerization, in accord with this interpretation. However, in 2.5 M NaCl, carboxypeptidase forms a higher polymer, which is in rapid equilibrium with the monomer. Here two boundaries are seen at high enzyme concentrations and here the magnitude of the area under the slow boundary varies inversely with temperature, suggesting that the polymer is stabilized by hydrophobic bonds. The formation of this polymer, containing more than two monomer units, demonstrates that the surface of carboxypeptidase has more than one binding site for interaction with additional protein molecules.

Under appropriate conditions three proteolytic enzymes, mercuripapain (Smith *et al.*, 1954), chymotrypsin (Massey *et al.*, 1955; Nichol and Bethune, 1963), and carboxypeptidase A (Bethune, 1963), manifest bimodal boundary formation in the ultracentrifuge, reflecting a rapidly equilibrating polymerization (Gilbert, 1955, 1959).

Two proteolytic enzymes, trypsin (Cunningham *et al.*, 1953; Nord and Bier, 1953) and native papain (Smith *et al.*, 1954), while not exhibiting bimodality during sedimentation, polymerize nevertheless since their sedimentation coefficients increase with increasing enzyme concentration. Chymotrypsin (Schwert, 1949; Schwert and Kaufmann, 1957; Massey *et al.*, 1955; Steiner, 1954) and carboxypeptidase A (Rupley and Neurath, 1960) behave similarly under conditions where double boundary formation is not seen, leading to the conclusion that, under different experimental conditions, differing modes of polymerization exist. Thus, different conditions may lead to phenomenologically different polymers, but both the mechanisms of formation and the degrees of aggregation are usually undefined.

Detailed physicochemical studies of these polymerizations have been carried out on chymotrypsin, mainly to investigate the theoretical predictions derived from behavior of interacting systems (Rao and Kegeles, 1958; Bethune and Kegeles, 1961; Nichol and Bethune, 1963; Kegeles and Safare, 1965).

This paper, while concerned with the polymerization of carboxypeptidase A, is the first reporting on a continuing study of the characteristics of the various polymerizing states directed at the discernment of the conditions which may lead to the identification of the sites responsible for these interactions in this proteolytic enzyme.

Materials and Methods

Bovine Pancreatic Carboxypeptidase A_γ. The crystalline enzyme (Worthington Biochemical Corp.) was washed three times with distilled water, dissolved in the appropriate solvent, and dialyzed to the final salt concentration desired. *Peptidase activity and esterase activity* were determined as described (Coleman and Vallee, 1960). *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). *Acetylation* with acetylimidazole was carried out using the method of Simpson *et al.* (1963). *Sedimentation* was carried out in a Model E ultracentrifuge equipped with RTIC and with a phase plate as the schlieren diaphragm. The distance from the center of rotation to the cell center in the rotor used was determined by the method of Kegeles and Gutter (1951), as modified by Schachman (1959).

In all cases 4° aluminum single sector centerpieces ranging in thickness from 3 to 12 mm were used. The rotor was accelerated linearly in an identical manner for each run. The timer was started at 40,000 rpm to give a total lapsed time corresponding to an instantaneous

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Peter Bent Brigham Hospital, Boston, Massachusetts. *Received June 24, 1965; revised September 2, 1965.* This work was supported by Grant-in-Aid HE-07297 from the National Institutes of Health of the Department of Health, Education and Welfare.

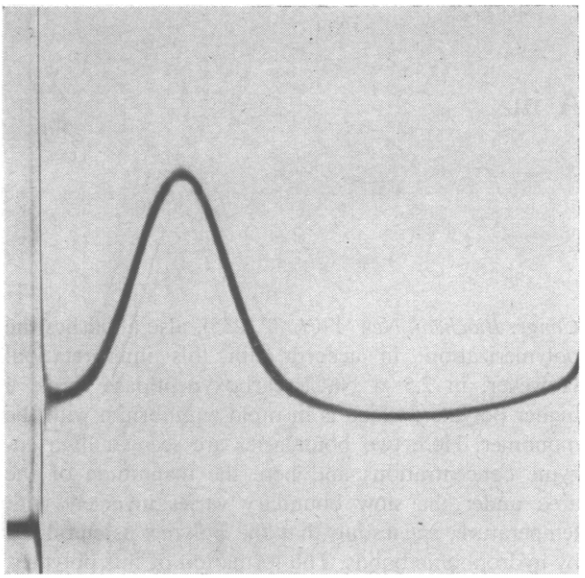

FIGURE 1: Sedimentation of carboxypeptidase. Concentration 18.5 mg/ml in 1 M NaCl, 0.02 M Veronal, pH 7.5; 63 min at 59,780 rpm. In this and all similar patterns sedimentation proceeds from left to right.

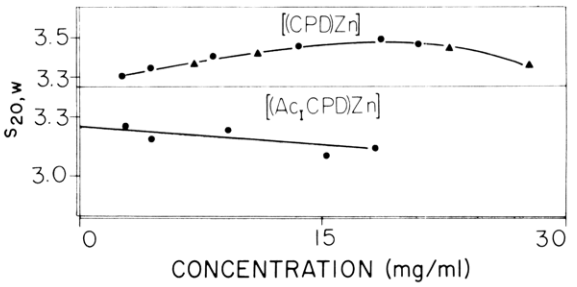

FIGURE 2: Concentration dependence of the sedimentation coefficient for carboxypeptidase A ([(CPD)Zn]), above, and acetylated carboxypeptidase A [(Ac_1CPD)-Zn]), below. In 1 M NaCl, 0.02 M Veronal, pH 7.5 (●), or in 2 M NaCl, 0.02 M Veronal, pH 7.5 (▲).

application of the full field at zero time (Schachman, 1959). Photographs were taken at appropriate times and phase-plate angles on metallographic plates (Eastman-Kodak), developed for 4 min in D-19 developer, were fixed for 10 min in acid fixer and washed for 30 min. The exposure time for different runs was 8–10 sec. Following a sedimentation run or series of runs in which the protein concentration was the only variable, the cell was reassembled, torqued to the same pressure, filled to the same level with the solvent against which the protein had been dialyzed, and the run repeated to obtain suitable base lines. The base-line photographs were taken at the same times and angles as those in the velocity run. Plates were read either in a two-dimension projection comparator (David D. Mann Co.) or were projected and traced on graph paper, followed by planimetry to obtain desired areas. When plates were read in the comparator, the plate was first aligned using the image of the meniscus as the reference line. Readings of the refractive index gradient, Z, were referred to the image of the phase plate in the reference lines from the counter balance. In the calculation of sedimentation coefficients in 1–2 M NaCl the rate of movement of the peak maximum was generally followed, since it was found that utilization of the rate of movement of the square root of the second moment (Goldberg, 1953) gave results which did not differ significantly (± 0.01 S) from those obtained by the first method. In determinations of symmetry the pattern was read at regular increments of 90 μ, as was that of the separate base-line run. Each point was read from two to six times. The standard deviation of a single reading was 0.0005 cm. The base line was then subtracted from the velocity pattern to give the corrected values of the refractive index gradient, Z_r, which were plotted. The values of Z_r were normalized, *i.e.*, the plots show $Z_r/Z_{r,max}$. This procedure of base-line correction was followed, rather than simultaneous registration of solution and base line in a double sector aluminum-filled epoxy centerpiece, since it has been observed that, even with the most careful filling of these cells, while there is first obtained a single narrow meniscus, after running with solvents of the density used here (1.05–1.1 gl/ml) at 59,780 rpm there occurred a gradual broadening of the meniscus, attributable to shifts in the liquid levels on either side resulting from distortion of the centerpiece. To check this effect a cell was deliberately loaded unevenly with 1 M NaCl and a run performed at 59,780 rpm. The positions of the two air–liquid menisci as a function of time are given in Table I. The inner meniscus moved 370 μ centrifugally while on the other hand the outer meniscus moved centripetally by the same amount. This type of movement imparts a velocity to the liquid as a whole which is superimposed on the actual movement of the sedimenting molecules (J. L. Bethune, in preparation).

TABLE I: Radial Position of Meniscus.

Time (sec)	Meniscus I (cm)	Meniscus II (cm)
1140	6.2220	6.4170
1620	6.2359	6.4034
2280	6.2442	6.3927
3000	6.2537	6.3847
3660	6.2592	6.3796

In the case of solutions in 2.5 M NaCl the patterns were projected and traced, after which the base line was projected and traced on the same sheet of graph paper using the counterbalance images, the meniscus, and the phase-plate image as reference points. In determina-

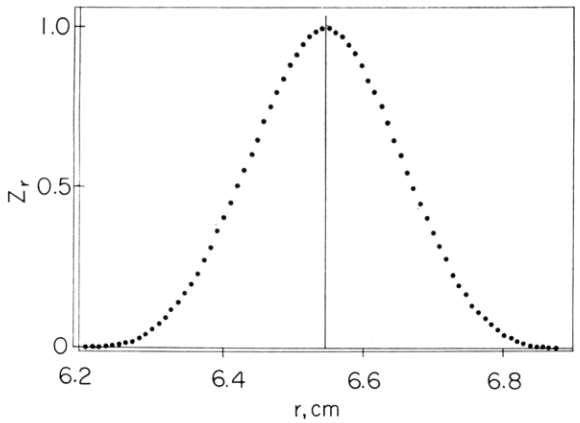

FIGURE 3: Sedimentation pattern for carboxypeptidase A corrected for salt redistribution. Z_r is the normalized corrected refractive index gradient at a distance r from the center of rotation. Enzyme (27.8 mg/ml) in 2 M NaCl, 0.02 M Veronal, pH 7.5; after 108 min at 59,780 rpm. The vertical axis of symmetry is drawn as the bisector of the horizontal distances between conjugate levels of the pattern.

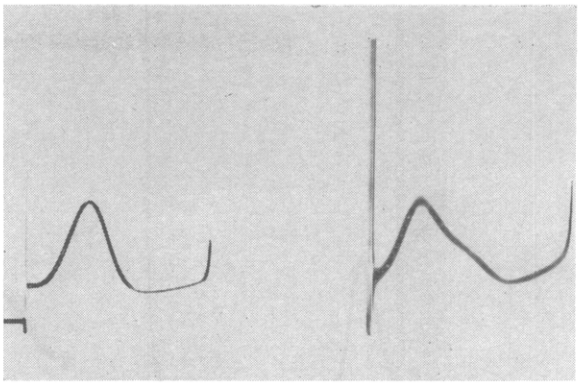

FIGURE 4: Effect of NaCl concentration upon sedimentation of carboxypeptidase. Left, 27.8 mg of enzyme/ml in 2 M NaCl, 0.02 M Veronal, pH 7.5, 22°. Right, 27.4 mg of enzyme/ml in 2.5 M NaCl, 0.02 M Veronal, pH 7.5, 22°; after 94 min at 59,780 rpm.

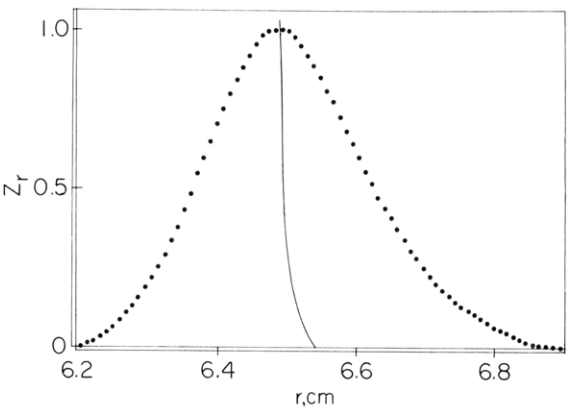

FIGURE 5: Sedimentation pattern for carboxypeptidase A corrected for salt redistribution. Z_r is the normalized corrected refractive index gradient at a distance r from the center of rotation. Concentration 13.7 mg/ml in 2.5 M NaCl, 0.02 M Veronal, pH 7.5; after 104 min at 59,780 rpm. The vertical axis was constructed as in Figure 3.

tions of the concentration represented by the areas under the two maxima, where they occurred, the total area was divided into two at the position of the minimum between the two maxima, since this describes the value that the total protein concentration must achieve before two maxima appear (Gilbert, 1955; Nichol *et al.*, 1964). All sedimentation coefficients are corrected to $s_{20,w}$ (Svedberg and Pedersen, 1940), assuming a partial specific volume of 0.75 ml/g.

Viscosities were determined at 20 ± 0.01° using a 70-sec outflow time Cannon–Fenske pipet. The bath temperature was controlled to ±0.002°. All time determinations were reproducible to $\pm 1.5 \times 10^{-2}$ sec. *Densities* were determined in a 25-ml jacketed pycnometer at 20°. *Chemicals* used in these experiments were of reagent grade and were used without further purification, except for those used in the assay of enzymatic activity, which were purified as described (Coleman and Vallee, 1960).

Results

Carboxypeptidase A is virtually insoluble in water; hence most studies concerning its physicochemical state have to be performed at high salt concentrations. When carboxypeptidase A is sedimented in 1 or 2 M NaCl only one symmetrical boundary is observed (Figure 1), but the concentration dependence of the sedimentation coefficient obtained under these conditions is that characteristic of polymerizing systems (Figure 2). The sedimentation coefficient $s_{20,w}$, determined in 1 M NaCl–0.02 M Veronal, 22°, increases from 3.29 S at 2.7 mg/ml to 3.49 S at 19 mg/ml, due to increasing concentrations of polymer. However, a normal hydrodynamic dependence occurs above a concentration of 19 mg/ml up to 28 mg/ml of enzyme, the solubility limit of the enzyme in 2 M NaCl, here employed. At this concentration the sedimentation coefficient falls to 3.31 S. As is shown in Figure 2, the curve obtained in 2 M NaCl overlaps smoothly with that in 1 M NaCl. The boundary is symmetrical over the whole concentration range examined. One pattern, taken after 108 min of sedimentation at a protein concentration of 28 mg/ml, after correction for salt redistribution (see Methods), is shown in Figure 3. The axis of symmetry (the vertical line) was calculated by bisection of the horizontal distance between conjugate levels on the two sides of the curve. This symmetry suggests that a dimer is formed since the presence of any higher polymers would distort the leading edge of the pattern. More-

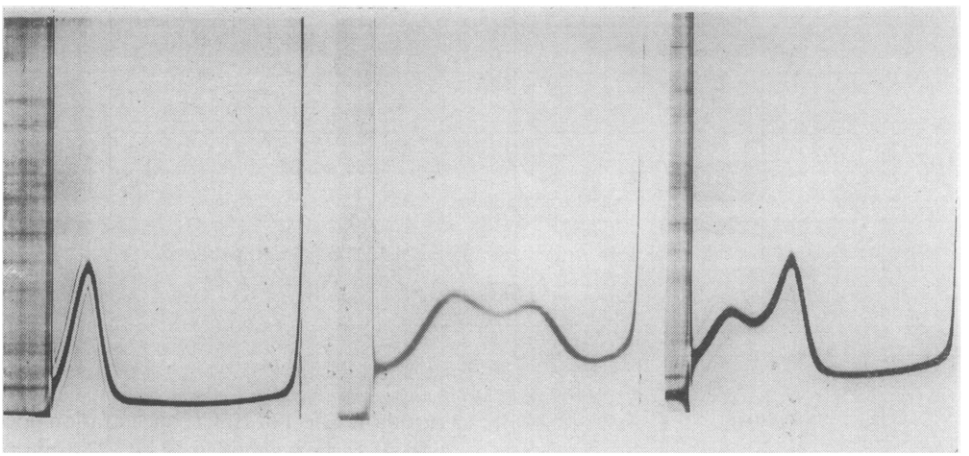

FIGURE 6: Temperature dependence of the polymerization of carboxypeptidase A. Left, at 4°, after 79 min; center, at 22°, after 118 min; right, at 30°, after 51 min. All in 2.5 M NaCl, 0.02 M Veronal, pH 7.5, at 59,780 rpm. Enzyme concentration 44–46 mg/ml.

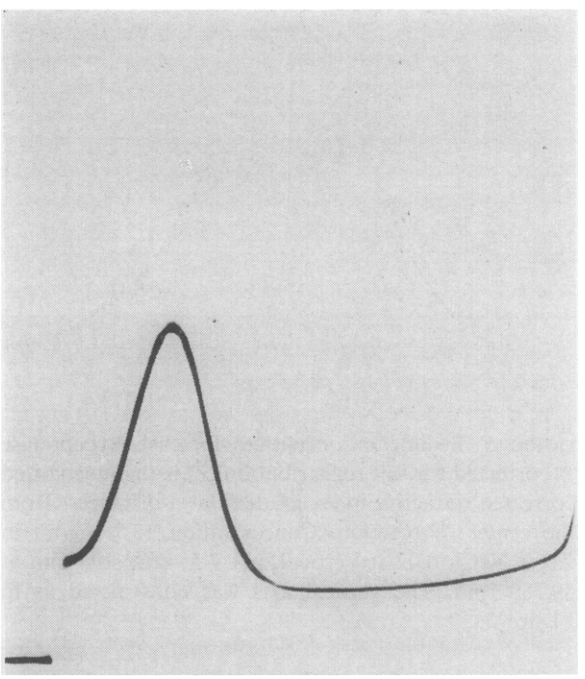

FIGURE 7: Sedimentation of acetylated carboxypeptidase. Concentration 18 mg of enzyme/ml in 1 M NaCl, 0.02 M Veronal, pH 7.5; after 52 min at 59,780 rpm, 23°.

over, it must be in rapid equilibrium with the monomer, *i.e.*, the exchange must be more rapid than the time required for separation in the centrifuge, since otherwise a second boundary due to polymer should be detected.

In order to evaluate the effects both of temperature and of different buffer ions, the sedimentation coefficient was also determined at 4° at a protein concentration 10 mg/ml in 1 M NaCl and either 0.1 M Tris or 0.02 M sodium barbital buffer. In both of these buffers, at 4°, $s_{20,w}$ was 3.31 S, as compared to 3.44 S at 22°. The lowering of the temperature results in a significant difference, but the substitution of Tris for sodium Barbital has no effect.

Studies of the solubility of native carboxypeptidase A_γ have previously employed 2 M NaCl as the upper limit of salt concentration, and 28 mg/ml was the maximum amount of protein which could be dissolved under these conditions. It has been reported briefly that approximately 45 mg/ml of enzyme may be dissolved in 2.5 M NaCl (Bethune, 1963). Two boundaries are seen on sedimentation of the system at such higher enzyme concentrations. This phenomenon, too, is attributable to a rapidly equilibrating polymerization. Upon dilution to lower enzyme concentrations the area under the slow boundary remains constant but that under the rapid boundary decreases, as does its sedimentation coefficient (Table II). However, this polymerization differs from that observed at low salt and enzyme concentrations, since now two boundaries are detected. Thus, this polymer must consist of at least three monomer units of carboxypeptidase (Gilbert, 1955).

TABLE II: Sedimentation of Carboxypeptidase A (2.5 M NaCl, 0.02 M Veronal, pH 7.5).

Protein Concentration (mg/ml)	Slow Boundary		Fast Boundary	
	Concn (mg/ml)	$s_{20,w}$ (S)	Concn (mg/ml)	$s_{20,w}$ (S)
44.3	28	3.4	16	7.1
37.9	28	3.3	10	6.9
27.4	27	3.3		
20.9	21	3.3		
13.3	13	3.3		

These polymerizations and depolymerizations are not accompanied by any denaturing effects upon the enzyme, as can be determined by measurements of enzymatic activity. Dilution of these concentrated solutions (1.0×10^{-3} M) to 1.0×10^{-8} M, the concentration for enzymatic measurements and immediate assay, does not show any lowering of the specific enzymatic activity. The esterase and peptidase activities are the same as those found when the enzyme, which has been maintained at 1.0×10^{-4} or 1.0×10^{-5} M in 1 or 2 M NaCl, is diluted and assayed.

The sedimentation patterns for corresponding protein concentrations in 2 M and 2.5 M NaCl, 22°, are shown in Figure 4. The marked asymmetry of the boundary in 2.5 M NaCl, as compared to that in 2 M NaCl, is that expected for a system undergoing a polymerization (Gilbert, 1955). In addition, in Figure 5 is shown the corrected pattern for the enzyme after 104 min at 59,780 rpm, at a protein concentration of 13 mg/ml in 2.5 M NaCl. The axis of symmetry again, as in Figure 3, is drawn as the locus of the bisectors of the horizontal distances between the two sides of the curve. In comparison to Figure 3, this pattern, at a much lower protein concentration, is clearly asymmetric, indicating the presence of a higher polymer than the dimer. The species present in solution under these two conditions are clearly different.

When solutions containing 45 mg/ml of enzyme are sedimented in 2.5 M NaCl, the relative area under the rapid boundary depends directly upon temperature, as shown by studies at 4, 20, and 30°. At 4°, the second rapid boundary, observed at 20°, is not seen, while at 30° the area under it is increased relative to that seen at 20° (Figure 6). The same pattern is obtained if the temperature at which the solution was maintained is either above or below the temperature at which the run is performed. In all cases, the protein present accounts for the total area under the boundaries. Thus, on lowering the temperature, the polymer dissociates; on raising, more of it forms. Such negative temperature dependence of reactions is typical of hydrophobically stabilized systems.

To investigate possible components of the sites of interaction, chemical modification of the enzyme has been employed. It has been shown (Rupley and Neurath, 1960) that removal of the catalytically essential zinc atom from carboxypeptidase A does not affect the dependence of the sedimentation coefficient on concentration, as determined in 1 M NaCl; therefore, this polymerization is not dependent upon the presence of the metal atom.

Simpson *et al.* (1963) have reported that six out of the total 19 tyrosyl residues of the enzyme can be acetylated with acetylimidazole. In response to this chemical modification peptidase activity is lost. Concomitantly, the extent of polymerization of this modified enzyme in 1 to 2 M NaCl is greatly diminished. Acetylcarboxypeptidase, like the native enzyme, sediments as one symmetrical boundary (Figure 7), but throughout the concentration range studied here the concentration dependence of the sedimentation coefficient of the acetylated enzyme is negative (Figure 2). This is typical of systems in which protein interactions are either negligible or do not occur at all.

Discussion

Carboxypeptidase A dissolved in sodium chloride solutions can exhibit two different modes of polymerization, depending upon the molarity of the salt. Up to a maximum protein concentration of 28 mg/ml, all present evidence indicates that a monomer–dimer reaction occurs in 1 or 2 M NaCl, while at protein concentrations up to 45 mg/ml in 2.5 M NaCl a higher polymer is present in the solution.

On the basis of present data, the first mode of polymerization, that found in 1–2 M NaCl solutions, is best interpreted as a rapidly equilibrating dimerization: as the protein concentration is increased up to 19 mg/ml, the sedimentation coefficient increases, to decrease normally thereafter (Figure 2). In the presence of these concentrations of NaCl, however, only a single symmetrical boundary is seen over the concentration range 3 to 28 mg/ml (Figure 3), implying first that no significant amounts of the higher polymer seen in 2.5 M NaCl are formed since in the former case the boundary should exhibit skewness on the leading edge, in the latter case on the trailing edge (Fujita, 1962) (compare Figures 3 and 5), and second, that the dimer content is quite low (Figure 3) (Gilbert, 1959; Nichol *et al.*, 1964), accounting for the symmetry observed in 1–2 M NaCl. These considerations make the presence of a dimer more probable than that of a higher polymer. In any case, this polymer may well be an intermediate in the formation of the higher polymer, seen in 2.5 M NaCl, the presence of which is immediately apparent in this solvent, since in this case the reaction causes a distortion of the leading edge of the boundary, even at 13 mg/ml (Figure 5).

Only one investigation of the concentration dependence of the sedimentation coefficient of carboxypeptidase A is on record (Rupley and Neurath, 1960). In that instance, carboxypeptidase A_δ was studied. Determinations were carried out over an enzyme concentration range from 2 to 19 mg/ml in 1 M NaCl, 0.1 M Tris, pH 7.0, at 4°. The $s^0_{20,w}$ was 3.06 ± 0.04 S. Smith *et al.* (1949) performed the earliest investigation of what is now known as carboxypeptidase A_γ. The studies were performed in a variety of supporting buffers, pH 7.2, between 22 and 25°. The $s^0_{20,w}$ was found to be 3.07 ± 0.14 S. At the time of the latter study it was not known, however, that the method of temperature measurement employed did not detect the temperature drop (~0.8°) due to adiabatic expansion of the rotor at high speeds (Waugh and Yphantis, 1952; Biancheria and Kegeles, 1954). As a consequence the experimental values, when corrected to give $s_{20,w}$, would give values that would be low. In this case as in many other similar instances a specific retrospective calculation is not possible, but general experience suggests that the correction should be of the order of 2–3%, bringing the

$s^0_{20,w}$ to ~3.2 ± 0.14 S, which is in satisfactory agreement with the present data obtained at 22°.

However, the difference between the results obtained at 22° and those obtained at 4° may not be solely the consequence of a possible inadequacy in the equation used to correct from $s_{t,b}$ to $s_{20,w}$ (Schachman, 1959). If ΔH for the reaction is finite, this may be the result of the temperature dependence of the chemical reaction upon the weight-average sedimentation coefficients (Nichol *et al.*, 1964).

The temperature effect is independent of the buffer. The results in Tris and in sodium Barbital at 4°, at an enzyme concentration of 10 mg/ml, were identical; the $s_{20,w}$ value in both instances is 3.31 S and is in good agreement, moreover, with that of 3.30 S of Rupley and Neurath (1960) (Figure 3). At 22°, the value is 3.44 S for this concentration.

When the NaCl concentration is raised to 2.5 M, a different situation becomes apparent. The existence of two boundaries demonstrates the formation of a higher polymer which, like that in 1–2 M NaCl, is also in a state of rapid re-equilibration with monomer. This is apparent from the fact that the area under the slow boundary is constant and the sedimentation coefficient of the rapid boundary increases with increasing protein concentration. Both theory and the study of model compounds with a strong hydrophobic bond stabilization (Kauzmann, 1959; Klotz, 1960; Némethy and Scheraga, 1962) predict the observed temperature dependence of the equilibrium constant for the second mode of polymerization of carboxypeptidase. Tobacco mosaic virus protein apparently forms a similar hydrophobically bonded polymer (Stevens and Lauffer, 1965).

Such polymer formation cannot be attributed to the establishment of salt linkages, since these should be weakened as the ionic strength is raised (Kauzmann, 1959), nor can it be due to diminution of an electrostatic repulsion. In insulin, where the increase in sedimentation coefficient with increasing ionic strength has been attributed to this effect (Oncley *et al.*, 1952), the temperature dependence of the polymerization is the reverse of that found here: polymerization is enhanced at *lower* temperatures (Doty and Myers, 1953).

The hypothesis that the results observed are a consequence of solvent rearrangements is reinforced by a consideration of hydration studies of DNA (Hearst and Vinograd, 1961). It has been shown that the number of moles of water associated with each base of DNA is dramatically dependent upon the activity of the water over the range 0.9 to 1.0. The water activity of a 2.5 M sodium chloride solution, in which the higher polymer is detectable, is 0.92, while that of a 1 M NaCl solution is 0.97, and in this solvent the higher polymer is not detectable from the sedimentation patterns.[1] If the polymerization observed at high salt concentrations is indeed the result of a hydrophobic interaction, then lowering the activity of the solvent by addition of a neutral salt could strengthen this association (Kauzmann, 1959).

While all the evidence suggests that the polymer that occurs in 2.5 M NaCl is hydrophobically stabilized, no such direct evidence exists in the case of the polymerization detected in 1–2 M NaCl. The small differences observed between the corrected values for experiments performed at 4° (3.31 S) and 22° (3.44 S) cannot unambiguously be attributed to the temperature dependence of a chemical reaction, since application of the usual correction has no strong theoretical basis (Schachman, 1959).

If, however, this polymer is stabilized by hydrophobic bonds involving the participation of tyrosine residues, acetylation of these should modify the sedimentation properties in 1–2 M NaCl.

Acetylation of six tyrosine residues by acetylimidazole abolishes both the peptidase activity of the enzyme (Simpson *et al.*, 1963) and, indeed, gives a product with a more normal dependence of $s_{20,w}$ upon concentration (Figure 2) while no detectable changes in conformation occur (Bethune *et al.*, 1964). It may be concluded that tyrosine residues are indeed involved in this polymerization. It would appear, moreover, that the polymer represents an enzyme–substrate complex in which both components are contributed by the enzyme. A similar suggestion has been made in the case of trypsin (Nord and Bier, 1953; Desnuelle, 1960). Since the acetylated enzyme polymerizes to a lesser extent,[2] the acetylation of these residues has abolished protein and peptide substrate binding, a conclusion supported by studies of metal restoration (Coleman *et al.*, 1964; Vallee, 1964).

Thus, the fundamental basis of this polymerization is identical with that which results in substrate binding as one aspect of the catalytic event. The active centers of enzymes are conceived of as those locations on an enzyme at which substrate binding and catalysis occur. From the relative sizes of enzymes and the substrates the active center is thought to comprise only a small portion of the whole enzyme molecule (Koshland, 1960). The occurrence of a relatively small catalytic site is well substantiated through these studies of, for example, proteolytic enzymes. In these cases, however, the investigations have been carried out using small synthetic di- and tripeptides, and the only binding site investigated is, naturally, that at the catalytic site. Proteolytic enzymes, however, can also attack large peptides or proteins which may well require attachment to the enzyme at more than one locus on the enzyme surface. These accessory binding sites, not located near the catalytic site and used only when large substrates are hydrolyzed, would not be utilized in investigations using only small substrates. Moreover, the nature of the

[1] The solvent activities are calculated from tabulated values of the osmotic coefficients (Robinson and Stokes, 1963).

[2] These sedimentation studies alone do not permit an absolute statement, of course, that polymerization is completely abolished (Gilbert, 1956), nor that only a dimer is formed (Rao and Kegeles, 1958; Bethune and Kegeles, 1961). For unambiguous proof a determination of the weight-average molecular weight as a function of concentration will be necessary.

forces governing the interaction of a small or a large substrate with an enzyme have not been investigated to any great extent. The natural conditions of temperature and concentration under which these enzymes play their role in hydrolysis of proteins usually differ greatly from those usually employed in experimental investigations. As a result binding forces, which may be unimportant under these experimental conditions, may well be extremely important in the natural catalytic event.

To explore these hypotheses, we have chosen to investigate the interactions of carboxypeptidase A_δ and A_γ of bovine pancreas with large substrates, by means of physicochemical procedures, beginning with the modes of self-interaction of the enzyme, *i.e.*, of the types of polymerization occurring under varying conditions. The results presented here show that, when carboxypeptidase is dissolved in 1 to 2 M NaCl, the concentrations of salt used conventionally to solubilize the enzyme, a polymerization ensues through a process which is strictly analogous to that found in the binding of small peptide substrates to the enzyme. Removal of the zinc atom does not abolish the polymerization (Rupley and Neurath, 1960) or substrate binding (Coleman and Vallee, 1960), while acetylation of tyrosine residues abolishes both the polymerization and peptide substrate binding (Coleman *et al.*, 1964).

Since acetylation of carboxypeptidase is not feasible at high protein concentrations, the polymerization occurring in 2.5 M NaCl could not be investigated in similar manner.[3] However, the formation of a polymer the degree of polymerization of which is greater than two geometrically necessitates the existence of more than one site of interaction. Moreover, since the polymer is finite, these sites must be arranged spatially in a manner which does not permit infinite extension of the polymer chain, as occurs, *e.g.*, in precipitation reactions of antibody and antigens.

Thus, one mode of polymerization indicates that more than one binding site exists on the protein molecule, while the other mode of polymerization shows that tyrosine residues are necessary for that polymerization to take place.[4]

Acknowledgments

The author is deeply indebted to Dr. B. L. Vallee for his constant encouragement, advice, and criticism during this work. The excellent technical assistance of Mrs. Janet M. Carlson is gratefully acknowledged.

[3] The chemical modification and its functional consequences are dependent upon protein concentration, becoming progressively less effective at high protein concentrations, thus precluding the preparation of homogeneously modified material at those concentrations at which double boundary formation is detected. Attempts to crystallize the modified enzyme, which would enable the preparation of solutions of high concentration, have not been successful, and the use of precipitation reagents, such as ammonium sulfate, unfortunately bring about a high concentration of a nucleophile, resulting in turn in deacetylation (J. F. Riordan, private communication).

[4] A further mode of polymerization of carboxypeptidase A, induced by the presence in the solvent of certain aromatic compounds, both involves these tyrosine residues at more than one site on the molecule and displays the same temperature dependence of polymer formation as does the polymerization occurring in 2.5 M NaCl (Bethune, 1965).

Bibliography

Bethune, J. L. (1963), *J. Am. Chem. Soc. 85*, 4047.
Bethune, J. L. (1965), *Biochemistry 4*, 2698 (this issue; following paper).
Bethune, J. L., and Kegeles, G. (1961), *J. Phys. Chem. 65*, 1761.
Bethune, J. L., Ulmer, D. D., and Vallee, B. L. (1964), *Biochemistry 3*, 1764.
Biancheria, A., and Kegeles, G. (1954), *J. Am. Chem. Soc. 76*, 3737.
Coleman, J. E., Pulido, P., and Vallee, B. L. (1964), *Federation Proc. 22*, No. 2, 594.
Coleman, J. E. and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.
Cunningham, L. W., Tietze, F., Green, N. M., and Neurath, H. (1953), *Discussions Faraday Soc. 13*, 58.
Desnuelle, P. (1960), *Enzymes 4*, 119.
Doty, P., and Myers, G. E. (1953), *Discussions Faraday Soc. 13*, 51.
Fujita, H. (1962), Mathematical Theory of Sedimentation Analysis, New York, Academic.
Gilbert, G. A. (1955), *Discussions Faraday Soc. 20*, 68.
Gilbert, G. A. (1959), *Proc. Roy. Soc. (London) A250*, 377.
Goldberg, R. J. (1953), *J. Phys. Chem. 57*, 194.
Hearst, J. E., and Vinograd, J. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 825.
Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.
Kegeles, G., and Gutter, F. J. (1951), *J. Am. Chem. Soc. 73*, 3770.
Kegeles, G., and Safare, P. S. (1965), *Federation Proc. 24*, No. 2, 472.
Klotz, L. M. (1960), *Brookhaven Symp. Biol. No. 13*, 25.
Koshland, D. E., Jr. (1960), *Advan. Enzymol. 22*, 45.
Massey, V., Harrington, W. F., and Hartley, B. S. (1955), *Discussions Faraday Soc. 20*, 24.
Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem. 66*, 1773.
Nichol, L. W., and Bethune, J. L. (1963), *Nature 198*, 880.
Nichol, L. W., Bethune, J. L., Kegeles, G., and Hess, E. L. (1964), *Proteins 2*, 305.
Nord, F. F., and Bier, M. (1953), *Biochim. Biophys. Acta 12*, 56.
Oncley, J. L., Ellenbogen, E., Gitlin, D., and Gurd, F. R. N. (1952), *J. Phys. Chem. 56*, 85.
Rao, M. S. N., and Kegeles, G. (1958), *J. Am. Chem. Soc. 80*, 5724.
Robinson, R. A., and Stokes, R. H. (1963), Electrolyte Solutions, 2nd ed., New York, Academic.
Rupley, J. A., and Neurath, H. (1960), *J. Biol. Chem. 235*, 609.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, Academic.
Schwert, G. W. (1949), *J. Biol. Chem. 179*, 655.

Schwert, G. W., and Kaufmann, S. (1957), *J. Biol. Chem. 190*, 807.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Smith, E. L., Brown, D. M., and Hanson, H. T. (1949), *J. Biol. Chem. 180*, 33.
Smith, E. L., Kimmel, J. R., and Brown, D. M. (1954), *J. Biol. Chem. 207*, 533.
Stevens, C. L., and Lauffer, M. A. (1965), *Biochemistry 4*, 31.
Steiner, R. F. (1954), *Arch. Biochem. Biophys. 53*, 457.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London and New York, Oxford University Press, New York, Johnson Reprint Corp.
Vallee, B. L. (1964), Abstracts, *6th Intern. Congr. Biochem. New York 32*, 255.
Waugh, D. F., and Yphantis, D. A. (1952), *Rev. Sci. Instr. 23*, 609.

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.

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Peter Bent Brigham Hospital, Boston, Massachusetts. *Received July 20, 1965; revised September 2, 1965.* This work was supported by grants-in-aid (HE-07297) from the National Institutes of Health of the Department of Health, Education and Welfare.