Studies on ϵ-Peptidase*

Joseph D. Padayatty† and Harold Van Kley

ABSTRACT: Dipeptides of glycine, L-alanine, L-phenylalanine, L-leucine, and L-aspartic acid coupled to the ϵ-amino group of L-lysine were employed as substrates for the study of ϵ-peptidases. The activity was found in extracts of the kidneys of rats and hogs; it could be distinguished from aminopeptidase by a suitable choice of substrates. An ϵ-L-lysine acylase obtained from *Achromobacter pestifier* EA catalyzed the hydrolysis of ϵ-peptides of lysine but not α-peptides. A comparison of enzymatic properties showed that a single protein was responsible for both hydrolytic activities. The energies of activation for the hydrolysis of ϵ-peptides, calculated from Arrhenius plots, were dependent on the amino acid coupled to lysine.

Peptide bonds to the ϵ-amino group of lysine are found in bacitracin A (Hausmann *et al.*, 1955), bovine growth hormone (Li, 1957), and collagen (Mechanic and Levy, 1959). The nature of ϵ-peptide bonds in proteins would be best studied with an enzyme specific for the hydrolysis of such bonds. In addition, such an enzyme might provide a specific method to remove polypeptide chains built onto protein molecules by reaction with *N*-carboxyamino acid anhydrides (Becker and Stahmann, 1953).

An ϵ-peptidase has not been reported, but it is conceivable that known enzymes with broad specificity would hydrolyze ϵ-peptide bonds. In order to look for such enzymatic activity, a variety of commercially available proteolytic enzymes and tissue extracts were tested. L-Directed ϵ-lysine acylases (ϵ-*N*-acyl-L-lysine amidohydrolase, EC 3.6.1.e) have been detected in mammals, birds, mushrooms, and bacteria (Paik, 1963). The enzyme was purified from rat kidney (Paik *et al.*, 1957), hog kidney (Paik and Benoiton, 1963), and *Achromobacter pestifer* EA (Ishikawa *et al.*, 1962a). If the specificity of ϵ-lysine acylase is for amide bonds involving the ϵ-amino group of lysine, then hydrolysis of ϵ-peptide bonds would be probable.

Experimental Section

Peptides of Lysine. ϵ-Peptides of lysine were synthesized by coupling *N*-carbobenzoxy derivatives of amino acids or amino acid benzyl esters with the copper complex of lysine by means of the mixed carboxylic–carbonic acid anhydride procedure. The copper complex was broken with H_2S and the product was subjected to catalytic hydrogenation to yield the dipeptides (Theodoropoulos, 1958). α-Peptides of lysine were synthesized by the same procedure except that ϵ-carbobenzoxylysine benzyl ester hydrochloride was employed instead of the copper complex of lysine (Levin *et al.*, 1956).[1]

ϵ-Benzoyl-L-lysine. This compound was prepared according to the method of Neuberger and Sanger (1943).

Assay of Lysine. Lysine was assayed by the acidic ninhydrin method of Work (1957). During the enzymatic hydrolysis of ϵ-phenylalanyllysine and ϵ-benzoyllysine, the observed absorbancies for the assay of lysine were always higher than the theoretical values. Hence standard curves for the assay of lysine liberated by the enzymatic hydrolysis of ϵ-phenylalanyllysine and ϵ-benzoyllysine were determined. When other ϵ-peptides were used, there was no interference in absorbancies by the peptide or the liberated amino acid.

Enzymatic Hydrolysis of the Peptides of Lysine. The α- and ϵ-peptides of lysine were used as substrates for trypsin (EC 3.4.4.4) (Worthington Biochemicals, Freehold, N. J.), carboxypeptidase A (EC 3.4.2.1) and B (EC 3.4.2.2) (Worthington Biochemicals), leucine aminopeptidase (EC 3.4.1.1) (Sigma Chemical Co., St. Louis, Mo.), and ϵ-peptidase in the extracts of kidneys of hogs and rats and in the cells of *A. pestifer* EA. Solutions of the substrate, enzyme, and the activating metal, with the appropriate buffer solution, were incubated at 37 or 42° for a definite period. If the incubation period was longer than 1 hr, the incubation mixture was covered by a thin layer of toluene to prevent bacterial action. In all cases enzyme and substrate blanks were included. The liberated lysine was assayed colorimetrically.

ϵ-Peptidase from Kidneys of Hogs. Hog kidney acetone powder (100 mg) (General Biochemicals, Chagrin Falls, Ohio) was suspended in 10 ml of 0.1 M Tris–chloride

* From the Edward A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri. *Received August 24, 1965; revised January 3, 1966.* This investigation was supported by Public Health Service Research Grant No. GM-10604 from the Institute of General Medical Sciences.

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[1] Details of the preparation of the chromatographically pure peptides will be published elsewhere.

buffer, pH 8.0, stirred, centrifuged at 5000*g* at 0°, and the supernatant solution was tested for ε-peptidase activity.

ε-Peptidase from Kidneys of Rats. Three adult rats from the St. Louis University colony, of average weight 378 g, were decapitated and the kidneys weighing a total of 9.7 g were suspended in 25 ml of cold 0.067 M phosphate buffer, pH 7.2. The tissue was homogenized twice for 15 sec in a Waring blendor, filtered through cheese cloth, and centrifuged at 0° at 44,000*g* for 30 min. The clear dark supernatant solution was used as the source of ε-peptidase.

ε-Peptidase from Achromobacter pestifer EA. Cells of *A. pestifer* EA[2] were grown according to the method of Ishikawa *et al.* (1962a). An extract of the cells in 0.067 M sodium sulfate solution was used as the enzyme source.

Purification of ε-Peptidase from A. pestifer EA. The ε-L-lysine acylase was obtained from cells of *A. pestifer* EA by the procedure of Ishikawa *et al.* (1962a) with the addition of gel filtration in 0.1 M sodium acetate buffer, pH 6.0, on Sephadex G-200 before column zone electrophoresis.

Action of ε-Peptidase on Bacitracin A, Proteins, and Modified Proteins. The purified bacterial enzyme was incubated with solutions of bacitracin A, casein, bovine serum albumin, collagen, bovine growth hormone,[3] polyglutamyl bovine serum albumin[4] or polyglycyl bovine serum albumin[4] and 0.1 M sodium acetate buffer, pH 5, at 42° for 1–2 hr; enzyme and substrate blanks were included. At the end of the incubation period, the reaction was stopped by plunging the tubes into ice and the free amino groups were assayed according to the ninhydrin method of Troll and Cannan (1953).

Results

Hydrolysis of α- or ε-peptides of lysine was not detected after incubation for 3 hr with trypsin using 0.1 M phosphate buffer, pH 7.8; the result was expected from the known specificity of trypsin in which the bond involving the carboxyl group of lysine is cleaved (Desnuelle, 1960). Carboxypeptidase A removes amino acid residues one at a time from the C-terminal end of a peptide chain but is inert if a basic amino acid such as lysine is C-terminal (Neurath, 1960). The results of an incubation for 3 hr of the peptide substrates and the enzyme in 0.02 M Veronal buffer, pH 7.5, gave no measurable hydrolysis. Carboxypeptidase B is specific for the hydrolysis of basic amino acids at the C-terminal end (Neurath, 1960). This enzyme hydrolyzed α-peptides of lysine, but not ε-peptides. Leucine aminopeptidase hydrolyzes a wide variety of amino acid amides and peptides at varying rates (Smith and Hill, 1960). The enzyme hydrolyzed α-peptides of lysine, ε-phenylalanyllysine, and ε-leucyllysine; the rates of hydrolysis of ε-glycyllysine and ε-alanyllysine were very low.

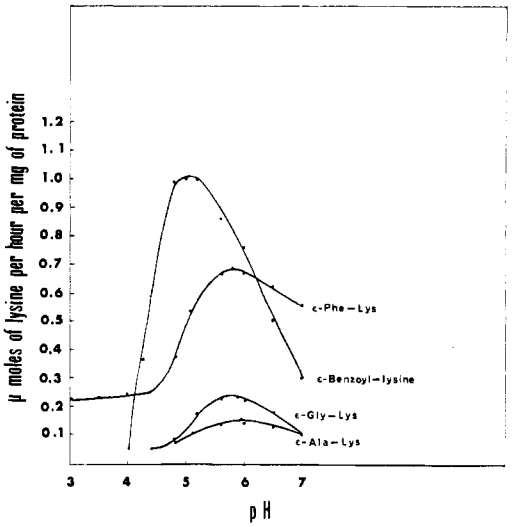

FIGURE 1: pH effects on the ε-acylase and ε-peptidase activities of the purified enzyme from *A. pestifer* EA.

The action of ε-peptidase from the extracts of the kidneys of hog and rats and from cells of *A. pestifer* EA is shown in Table I. ε-Peptides of lysine were hydrolyzed by the kidney extracts; α-peptides were also hydrolyzed by the crude tissue extracts. The bacterial extract hydrolyzed ε-alanyllysine to a measurable degree but not ε-leucyllysine; purified leucine aminopeptidase hy-

TABLE I: ε-Peptidase Activity.[a]

Substrate	Extract of Kidneys of Hogs[b]	Extract of Kidneys of Rats[c]	Extract of Cells *of A. pestifer* EA[d]
ε-Gly-Lys	0.043	0.027	0.231
ε-Ala-Lys	0.238	0.233	0.142
ε-Phe-Lys	0.592	0.319	0.36
ε-Leu-Lys	0.542	0.353	0.034
ε-(α-Asp-Lys)	0.113	0.144	−0.025

[a] Absorbancies at 340 mμ due to the hydrolysis of ε-peptides of lysine. [b] Mixture of 0.5 ml of enzyme, 0.5 ml of 0.005 M $Mn(OAc)_2$ (both in 0.1 M Tris–chloride buffer, pH 8), and 0.5 ml of substrate (3 μmoles/ml in water) was incubated at 37° for 3 hr. [c] Mixture of 0.5 ml of enzyme, 0.5 ml of 0.005 M $Mn(OAc)_2$, and 0.5 ml of substrate (3 μmoles/ml)—all in 0.067 M phosphate buffer, pH 7.2—was incubated at 42° for 3 hr. [d] Mixture of 0.5 ml of enzyme, 0.5 ml of substrate (3 μmoles/ml), and 0.5 ml of 0.1 M NaOAc buffer, pH 5, was incubated at 42° for 10 min.

[2] A culture of *Achromobacter pestifer* EA was a generous gift from Dr. I. Chibata, Osaka Research Laboratory, Tanabe Seiyaku Co., Ltd., Osaka, Japan.

[3] Bovine growth hormone was supplied by the Endocrinology Study Section, National Institutes of Health, Bethesda, Md., Lot No. NIH-GH-B8.

[4] Polyglycyl and polyglutamyl serum albumin were gifts from Dr. M. A. Stahmann, University of Wisconsin, Madison, Wis.

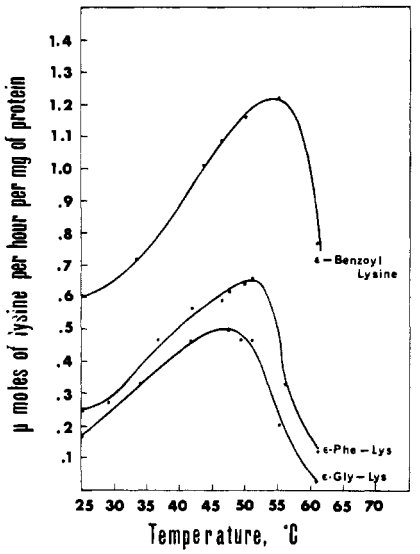

FIGURE 2: Temperature of inactivation of the ε-acylase and ε-peptidase activities of the purified enzyme from *A. pestifer* EA.

drolyzed ε-leucyllysine but not ε-alanyllysine. Therefore these substrates could be used to distinguish ε-peptidase from leucine aminopeptidase. The extracts of cells of *A. pestifer* EA hydrolyzed only ε-peptides; the α-peptides were not hydrolyzed.

The purified bacterial ε-peptidase did not hydrolyze α-peptide bonds in casein and bovine serum albumin, or α- or ε-peptide bonds in bacitracin A, collagen, or polyglutamyl bovine serum albumin. There was some increase in absorbancy by enzymatic action on bovine growth hormone and polyglycyl serum albumin.

The optimal pH values of the enzyme as an ε-peptidase and as an ε-acylase were studied using ε-peptides of lysine and ε-benzoyl-L-lysine as substrates, respectively, with 0.1 M sodium acetate buffers of pH 3–7. ε-Benzoyllysine and ε-phenylalanyllysine were assayed at substrate concentrations of 6 μmoles/ml; the other peptides at 3 μmoles/ml. The enzyme concentration was adjusted for the various substrates as follows: ε-benzoyllysine—12 μg of protein/ml, ε-phenylalanyllysine and ε-glycyllysine—30 μg of protein/ml, and ε-alanyllysine—60 μg of protein/ml. The substrate solution in water (0.2 ml), the appropriate enzyme solution in 0.1 M Na_2SO_4 solution (0.2 ml), and the desired buffer (0.2 ml) were incubated at 42° for 10 min and the liberated lysine was assayed; the results are shown in Figure 1. The optimal pH as an ε-acylase (ε-benzoyllysine as substrate) was 5.1 and as an ε-peptidase (ε-glycyl-ε-alanyl-, and ε-phenylalanyllysine as substrates) was 5.8.

The temperature of inactivation of ε-peptidase–acylase was studied by employing ε-peptides (ε-phenylalanyl- and ε-glycyllysines) and ε-benzoyllysine, respectively, as substrates with 0.1 M sodium acetate buffer, pH 5.8. The enzymatic activity was determined by measuring the lysine liberated at different temperatures; the data are shown in Figure 2. The temperature of inactivation as an ε-acylase (ε-benzoyllysine as substrate) was 54° and as an ε-peptidase (ε-phenylalanyllysine as the substrate) was 51°. The differences in temperatures of inactivation may be a function of protection from denaturation by substrate.

Ishikawa *et al.* (1962b) reported that ε-acylase was inhibited by mercuric ions. To test further whether ε-acylase and ε-peptidase are a single protein, inhibition of enzymatic activity by mercuric ions was tested. Both activities were completely inhibited by 3.33×10^{-4} M Hg^{2+}.

ε-Acylase of *A. pestifer* EA is also inhibited by oxalate ions (Ishikawa *et al.*, 1962b). The inhibition of the two enzyme activities was tested at two concentrations of substrate and several concentrations of oxalate. The inhibitor constants (K_i) were determined by plotting $1/v$ *vs.* [i] (Dixon, 1953) where v is the velocity of hydrolysis (μmoles of lysine released in 10 min) and [i] is the concentration of the inhibitor. K_i values for ε-benzoyllysine and ε-phenylalanyllysine are 6.15×10^{-2} M and 2.23×10^{-2} M, respectively.

Among ions tested, sulfate was the best activator of ε-acylase activity (Ishikawa *et al.*, 1962b). ε-Benzoyllysine and ε-phenylalanyllysine were employed as substrates to test the effect of sulfate ions on ε-acylase and ε-peptidase activities, respectively. Buffer (1 M) was used to diminish any effects of ionic strength on the enzymatic activity. There was a slight activation of both ε-peptidase and ε-acylase activities. The velocity decreased as the concentration of sulfate ions increased beyond 40×10^{-3} M.

To ascertain if a single enzyme is acting on two substrates, it is necessary to determine the velocities of reaction of the individual substrates (ε-benzoyllysine, ε-phenylalanyllysine, and ε-glycyllysine) and then on suitable mixtures (ε-benzoyllysine and ε-peptide) (Dixon and Webb, 1964). The analysis was made at pH 5 and pH 5.8 which are the respective pH optima for ε-acylase and ε-peptidase activities.

From Lineweaver and Burke (1934) plots using substrate concentrations of 0.5 to 4×10^{-3} M, V_m (maximum velocity) and K_m (Michaelis constant) were determined and are given in Table II.

TABLE II: Maximum Velocity (V_m) and Michaelis Constant (K_m) of ε-Peptidase/Acylase at pH 5.0 and 5.8.

pH	Substrate	$V_m \times 10^3$ M[a]	$K_m \times 10^3$ M
5.0	ε-Benzoyllysine	1.67	1.80
	ε-Phe-Lys	0.60	1.12
5.8	ε-Benzoyllysine	6.35	7.4
	ε-Phe-Lys	3.33	3.67
	ε-Gly-Lys	1.59	1.33

[a] Calculated concentration of lysine in the incubation mixture after 10 min at 42°.

TABLE III: Experimental and Calculated Velocities of a Mixture of Two Substrates.[a]

Substrates (μmole)	At pH 5		At pH 5.8			
	ε-Benzoyllysine + ε-Phe-Lys		ε-Benzoyllysine + ε-Phe-Lys		ε-Benzoyllysine + ε-Gly-Lys	
	v-expt	v-calcd	v-expt	v-calcd	v-expt	v-calcd
0.15	0.160	0.175	0.265	0.240	0.25	0.252
0.30	0.285	0.254	0.425	0.442	0.44	0.427
0.40	0.340	0.297	0.555	0.556	0.52	0.514
0.60	0.378	0.375	0.72	0.758	0.71	0.654
0.80	0.400	0.399	0.87	0.915	0.83	0.749

[a] v is expressed in μmole of lysine liberated in the incubation mixture in 10 min.

The velocities of hydrolysis of the mixture of two substrates were calculated based on one enzyme acting on two competing substrates using the equation (Dixon and Webb, 1964)

$$V_t = V_a \frac{a}{K_a} + V_b \frac{b}{K_b} \Big/ 1 + \frac{a}{K_a} + \frac{b}{K_b}$$

where V_a is the maximum velocity for substrate A of concentration a, V_b is the maximum velocity for substrate B of concentration b, K_a and K_b are the dissociation constants of the complex between the enzyme and substrates A and B, respectively.

Table III shows the experimental and calculated velocities of the mixture of two substrates at pH 5 and 5.8. The data suggest that a single enzyme catalyzes both activities.

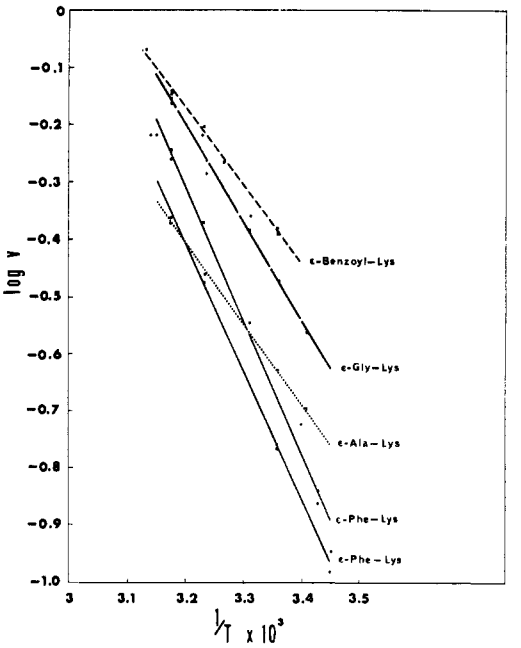

FIGURE 3: Arrhenius plots for the enzymatic hydrolysis of ε-peptides.

TABLE IV: Energies of Activation of Enzymatic and Chemical Hydrolysis of α- and ε-Peptides of Lysine.[a]

Substrate	Activation Energy in kcal/mole		
	Enzymatic Hydrolysis	Chemical Hydrolysis 6 N HCl	Chemical Hydrolysis 6 N NaOH
α-Gly-Lys		10.7	
α-Ala-Lys		11.9	15.4
α-(α-Asp)-Lys		11.9	
ε-Gly-Lys	7.7	10.0	
ε-Ala-Lys	6.4	13.6	9.6
ε-Phe-Lys	10.0 and 10.5		
ε-(α-Asp)-Lys		15.5	
ε-Benzoyllysine	7.9		

[a] Energies of activation for enzymatic hydrolysis are calculated from Arrhenius plots (Figure 3) and for chemical hydrolysis are calculated from the equation

$$\log k_2/k_1 = E(T_2 - T_1)/2.303T_1T_2$$

k_1 and k_2 are the rate constants at temperatures T_1 and T_2.

The velocities of hydrolysis (μmoles of lysine liberated/10 min) of ε-benzoyllysine and ε-peptides of lysine were determined at different temperatures using purified ε-peptidase–acylase from *A. pestifer* EA. For a zero-order reaction the velocity of the reaction is independent of the substrate concentration. Hence v is proportional to k, where v is the velocity of hydrolysis and k is the rate constant. Arrhenius plots of log v *vs.* $1/T$ are shown in Figure 3. The slope of the line is $-E/2.303R$ where E is the energy of activation and R is molar gas constant. Table IV shows the calculated activation energies of enzymatic and chemical hy-

drolysis. The activation energies, as expected, are lower for enzymatic hydrolysis.

Discussion

Because of the presence of interfering peptidases in extracts of rat and hog kidneys, the ε-peptidase from *A. pestifer* EA was employed for the studies on enzymatic hydrolysis of ε-peptides. This enzyme is stereospecific in its hydrolytic action as an ε-acylase (Chibata *et al.*, 1964). The enzyme acts as an ε-peptidase in that it hydrolyzes ε-peptides but not α-peptides of lysine synthesized from L-amino acids. The rates of hydrolysis decrease in the order ε-phenylalanyl, glycyl, alanyl, leucyl, and aspartyllysine. The carbon chain length, bulkiness, steric properties, and electronegativity of the amino acids in ε-peptide bond with lysine show significant effects on the enzyme–substrate complex formation.

Chibata *et al.* (1964) have reported that ε-acyl derivatives of ε-aminocaproic acid are not hydrolyzed by the acylase as shown by the acidic colorimetric ninhydrin method and paper chromatography. Since the acidic ninhydrin method employed for the assay of diaminocarboxylic acid requires two amino groups with one in the α-position (Work, 1957), these studies are not conclusive on the involvement of the free α-amino group in the enzymatic action. Preliminary experiments on the enzymatic hydrolysis of cobalt complexes of ε-benzoyllysine and ε-phenylalanyllysine showed no interference in the rates of hydrolysis, which would suggest that free α-amino and carboxylic groups are not necessary for the action of this enzyme.

ε-Peptidase is inactive toward α-peptide bonds in casein and bovine serum albumin. The ε-peptide bonds in collagen (ε-(α-glutamyl)lysine), bacitracin A (ε-(α-aspartyl)lysine), and polyglutamyl serum albumin are not hydrolyzed by this enzyme. This is not unexpected since the rate of hydrolysis of ε-(α-aspartyl)lysine is almost zero. There is some evidence for the hydrolysis of ε-peptide bonds in polyglycyl serum albumin and bovine growth hormone.

There are differences in pH optima and temperature of inactivation of the enzyme with respect to different substrates. The differences in affinities of the enzyme with the substrates or the differences in the charges of the substrates cause these variations. Since large excesses of substrates were employed to saturate the enzyme, differences in affinities for the enzyme between different substrates may not be important.

Both ε-peptidase and ε-acylase activities are inhibited by mercuric ions. Hence the enzyme may contain an active −SH group. Oxalate ions inhibit ε-acylase (Ishikawa *et al.*, 1962b) and ε-peptidase activities. Plots of the reciprocal of velocity *vs.* the inhibitor concentration according to Dixon (1953) show that oxalate is a competitive inhibitor for the hydrolysis of ε-benzoyllysine and ε-phenylalanyllysine. The velocities of hydrolysis of mixed substrates (ε-benzoyllysine and an ε-peptide) at different concentrations are always lower than the sum of the velocities of the single substrates. All of these data support the concept that the enzyme functions in two capacities, as an ε-acylase and as an ε-peptidase. Moreover, the observed velocities of hydrolysis of a mixture of ε-benzoyllysine and an ε-peptide are within experimental error of the calculated values for the hydrolysis of the mixture based on a single enzyme acting on two competing substrates.

The energy of activation calculated by Arrhenius plots of log *v vs.* $1/T$ will be accurate only if true values of the maximum velocities are obtained at each temperature. Gibson (1953) has pointed out that if the velocities are measured with the same substrate concentration at different temperatures, the energy of activation should be corrected according to the equation

$$H_2 - H'_2 = K_m/K_m + [S] \times H'_1$$

where K_m is the Michaelis constant, [S] is the substrate concentration, H'_2 is the apparent heat of activation (uncorrected for changes in K_m values), H_2 is the heat of formation of the enzyme–substrate complex, H_1 is the apparent heat of combination, obtained from the variation of K_m with temperature. If $[S]/K_m$ is large or H_1 is small, the correction becomes negligible. K_m does not change appreciably with temperature because the molecules of product per minute per molecule of enzyme was increased less than twofold by a 10° rise in temperature. A molar excess of substrate of approximately 10^9 was used in the determination of the energy of activation; therefore the correction is negligible. It was found that the activation energies of the enzymatic hydrolysis of ε-peptides were lower than that of chemical hydrolysis.

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Metabolic Control and Structure of Glycolytic Enzymes. III. Dissociation and Subunit Structure of Rabbit Muscle Pyruvate Kinase*

M. A. Steinmetz† and W. C. Deal, Jr.

ABSTRACT: Pyruvate kinase (mol wt 237,000) has been found to be a tetramer, each polypeptide chain having mol wt 57,200. The data further suggest that pyruvate kinase consists of two identical catalytic particles, or protomers (mol wt 115,000 each), with each protomer in turn consisting of two polypeptide chains (mol wt 57,000 each). With only one site each for Mn^{2+} and phosphoenolpyruvate per protomer, the two polypeptide chains within a protomer probably are not identical, despite their similarity in mass. Structural studies as a function of urea concentration revealed intermediates with $s^{0,6\%}_{20,w}$ = 7.3 S in 1.5 M urea, and 3.6 S in 3 M urea.

Complete dissociation ($s^{0,6\%}_{20,w}$ = 1.8 S) of the enzyme occurred in 4 M urea alone, indicating noncovalent subunit bonding. Characterization of the subunits in 7.4 M urea yielded values of $s^0_{20,w}$ = 2.01 S, $D^0_{20,w}$ = 3.46 × 10^{-7} cm^2/sec, and $M^0_w(s/D)$ = 56,300. Detailed subunit molecular weight analyses utilized sedimentation equilibrium in three separate dissociating systems; namely, (1) 7.4 M urea, (2) 7.4 M urea–0.12 M β-mercaptoethanol, and (3) 6.8 M guanidine–0.12 M β-mercaptoethanol. In the latter two systems, the subunits were more stable and M^0_w and M^0_z were essentially the same, indicating that the subunits had approximately equal molecular weights.

Recent years have seen the accumulation of a considerable amount of evidence indicating the presence of several polypeptide chains in the structure of many enzymes, especially in those enzymes with mol wt 65,000 or greater. Although most of the glycolytic enzymes of rabbit skeletal muscle are large enough that they would be expected to consist of several subunits, direct physical measurements of the subunits of only one such enzyme had been reported (Deal and Van Holde, 1962; Deal *et al.*, 1963b; Stellwagen and Schachman, 1962) when this study was undertaken. It seemed clear that a knowledge of subunit structure was a prerequisite for elucidation of the relationship between structure and function of these glycolytic enzymes. Because glycolysis plays such a central role in metabolism and because so little was known about the subunit structure of the glycolytic enzymes, this laboratory embarked on a program designed to ultimately provide an analysis of the subunit structure and control of all the glycolytic enzymes from a single source. Since enzymes are known to often differ from species to species and from tissue to tissue, such a restriction seemed desirable in order to be able to correlate all the results into a complete picture of glycolysis. We chose to focus mainly on the enzymes from rabbit skeletal muscle.

Our attention was drawn to pyruvate kinase because of its large size [mol wt 237,000 (Warner, 1958)] and because the enzyme had been reported to bind 2 moles of PEP[1]/mole of the native enzyme (Reynard *et al.*, 1961). Recently, it has also been shown that 2 Mn^{2+} ions are bound/mole of enzyme (Mildvan and Cohn,

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† Predoctoral Fellow (5-F1-GM-22, 851-02) of the Division of General Medical Sciences, National Institutes of Health.

[1] Abbreviations: for $D^0_{20,w}$, $s^0_{20,w}$, M^0_w, M^0_z, and $M^0_w(s/D)$, the superscript, ^{0}, indicates that the quantity has been extrapolated to zero protein concentration; $M_w(s/D)$, weight-average molecular weight determined from the Svedberg equation; M_w, weight-average molecular weight; M_z, z-average molecular weight; PEP, phosphoenolpyruvate; C_0, initial protein concentration; $(C_m + C_b)/2$, average of the protein concentration at the meniscus, and bottom of the solution; rtic, rotor temperature indicator and control; ATP, adenosine triphosphate.