tested, requiring only 10^{-7} M to completely inhibit human plasma angiotensin I converting enzyme activity. This supports the observation of Ondetti et al. (1977) using SQ-14,225 in rats and with the rabbit lung angiotensin I converting enzyme. Our qualitative results show that human plasma angiotensin I converting enzyme also is capable of cleaving bradykinin either with or without chloride in the assay system.

Thus, the angiotensin I converting enzyme from human plasma appears quite similar in physical properties to those enzymes isolated from other species. The procedure for purification of the angiotensin I converting enzyme from human plasma offers a convenient route for isolation and subsequent study of this enzyme.

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Rabbit Liver Transglutaminase: Physical, Chemical, and Catalytic Properties†

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ABSTRACT: Transglutaminase (R-glutaminyl-peptide:amine α-glutamyl-yltransferase [EC 2.3.2.13]) has been purified to apparent homogeneity from extracts of rabbit liver. The enzyme is a single polypeptide chain of approximately 80 000 molecular weight containing one catalytic site per molecule. That the isolated enzyme is the rabbit counterpart of the well-characterized guinea pig liver transglutaminase is evidenced by the similarities in their amino acid compositions and in their enzymic activities toward several substrates, together with the fact that the isolated rabbit enzyme is immunologically distinct from both rabbit plasma and rabbit platelet blood coagulation factor XIII. A striking difference between the catalytic activities of the rabbit and guinea pig enzymes is the low activity of rabbit transglutaminase for hydroxylamine incorporation into benzyloxycarbonyl-L-glutaminylglycine, a reaction for which the guinea pig enzyme shows a high reactivity. This finding reveals the cause of error in an earlier report (Tyler, H. M., and Laki, K. (1967) *Biochemistry 6,* 3259) that rabbit liver contains little, if any, of the enzyme. Preparation of, and analytical data on, several glutamine-containing peptide derivatives used in this study are reported here.

Early investigators reported enzymic activity responsible for incorporation of amines into proteins in the livers of a number of mammals (Sarkar et al., 1957) as well as in other tissues (Clarke et al., 1959; review: Waelsch, 1962). Species tested included guinea pig, rat, mouse, rabbit, and calf. Because guinea pig liver was particularly rich in this transglutaminase activity (Clarke et al., 1959; Wajda et al., 1963), subsequent studies centered on the enzyme from this species, and little attention was given to the transglutaminases from other mammals.

A combination of specific substrates, chromatographic techniques, and antiserum to the purified liver enzyme was

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TABLE I: Analytical Data on Benzyloxycarbonyl Peptides and Benzyloxycarbonyl Peptide *tert*-Butyl Esters.[a]

Peptide derivative[b,c]	Yield (%)	Formula (mol wt)	Mp (°C)	Calcd C	Calcd H	Calcd N	Found C	Found H	Found N	$[\alpha]_{289}^{27}$ γ in 0.1 N HCl[j]
Z-*Gln*-Gly-t-Bu	58	$C_{19}H_{27}N_3O_6$ (393.43)	172–174[d]	58.00	6.92	10.68	57.79	7.10	10.58	
Z-Gly-Gly-*Gln*-Gly-t-Bu	68	$C_{23}H_{33}N_5O_8$ (507.53)	180–182[e]	54.43	6.55	13.80	54.10	6.65	13.48	
Z-Gly-Gly-*Gln*-Gly	75	$C_{19}H_{25}N_5O_8 \cdot ½H_2O$ (460.44)	180 dec[f]	49.56	5.69	15.21	49.41	5.74	15.08	−101
Z-*Gln*-*Gln*-Gly-t-Bu	90	$C_{24}H_{35}N_5O_8 \cdot ½H_2O$ (530.57)	208 dec[g]	54.33	6.84	13.20	54.58	6.93	13.00	
Z-*Gln*-*Gln*-Gly	80	$C_{20}H_{27}N_5O_8 \cdot ½H_2O$ (474.46)	221–222[h]	50.63	5.95	14.76	50.71	6.02	14.66	−259
Z-Gly-*Gln*-*Gln*-Gly-t-Bu	66	$C_{26}H_{38}N_6O_9$ (578.61)	208–209 dec[g]	53.97	6.62	14.53	53.82	6.40	14.35	
Z-Glu-γ-t-Bu-Gly-*Gln*-*Gln*-Gly-t-Bu	94	$C_{35}H_{53}N_7O_{12}$ (763.83)	212–215 dec[d]	55.03	6.99	12.84	55.01	7.29	12.63	
Z-Glu-Gly-*Gln*-*Gln*-Gly	80	$C_{27}H_{37}N_7O_{12} \cdot ½H_2O$ (660.63)	240 dec[i]	49.08	5.80	14.84	49.10	5.81	14.49	−244

[a] The optically active amino acids are of the L configuration. [b] Z = benzyloxycarbonyl ($C_7H_7CO_2$). [c] t-Bu = *tert*-butyl ester (OC_4H_9). [d] Crystallized from ethanol-water. [e] Crystallized from ethanol-ether. [f] Crystallized from methanol. [g] Crystallized from absolute ethanol. [h] Crystallized from ethyl acetate. [i] Crystallized from water-acetone. [j] Optical rotations were measured with a Cary Model 60 spectropolarimeter using a 0.1-dm light path cell and concentrations of Z peptides of approximately 10 mM. Specific rotations [α] are calculated on the basis of concentrations determined from amino acid analyses.

employed to define an enzyme found in other organs and tissues, including erythrocytes, of the guinea pig as indistinguishable from that of liver (Chung, 1972, 1975; Chung and Folk, 1975). Similar means were used to identify and localize those transglutaminases that exist in zymogen form (protransglutaminases) and that are collectively referred to as blood coagulation factor XIII (Chung, 1972, 1975).

Recent studies in this laboratory have focused on the biosynthesis, catabolism, and function of the transglutaminases. The rabbit was chosen as a model for these investigations, and isolation and characterization of coagulation factor XIII from both the blood plasma and platelets of this animal have been accomplished (Lee and Chung, 1976).[1] This communication reports purification of rabbit liver transglutaminase together with documentation of some of its physical, chemical, and immunological properties and comparison of its catalytic features with those of the well-characterized guinea pig liver enzyme.

There is a report that rabbit liver contains very low levels of transglutaminase (Tyler and Laki, 1967). It is apparent from the findings presented here that this is not the case and it appears that the source of the error resides in a difference in catalytic specificity between the enzymes from the livers of rabbits and guinea pigs.

Materials and Methods

Guinea pig liver transglutaminase was prepared by a published procedure (Connellan et al., 1971a). The purified enzyme exhibited 95 ± 5% of the reported specific activity when assayed by hydroxamate formation with the specific substrate Z[2]-glutaminylglycine (Folk and Cole, 1966a). Enzyme concentration was determined by the use of the extinction coefficient of $E_{280}^{1\%} = 15.8$ and a molecular weight of 90 000 (Folk and Cole, 1966a).

[^{14}C]Methylamine (52 Ci/mol) and [^{14}C]iodoacetamide (33 Ci/mol) were obtained from New England Nuclear and were used without further purification after dilution with nonradioactive materials. *p*-Nitrophenyl acetate was obtained from Sigma. β-Casein, *A* variant, was isolated from the milk of a cow homozygous for this variant of β-casein (Aschaffenburg, 1963). Z-L-Glutaminylglycine was prepared by a procedure given for the D-peptide derivative (Folk and Cole, 1966b). DEAE-Cellulose paper (DE 81, ~3.5 μequiv/cm^2) was obtained from Whatman; Ampholine (pH 3.5–10) was from LKB Instruments. α-Bromo-4-hydroxy-3-nitroacetophenone was synthesized by a published method (Sipos and Szabo, 1961).

The *p*-nitrophenyl esters of Z-L-glutamine (Bodansky and duVigneaud, 1959), Z-L-glutaminyl-L-glutamine (Stewart, 1967), Z-glycine (Benoiton, 1963), Z-glycylglycine (Stewart, 1965), and Z-L-glutamic acid-γ-*tert*-butyl ester (Hofmann et al., 1965) were prepared by the cited methods, as was glycine *tert*-butyl ester·H_3PO_3 (Anderson and Callahan, 1960). Active ester couplings were carried out in minimum volumes of dimethylformamide (dried over molecular sieves [type 5A, Grace Davison Chemical]). Reactions were allowed to proceed for 4 to 24 h and the Z-peptide-*tert*-butyl esters were isolated after addition of water, ethanol, or ethyl acetate. Blocked peptides were dissolved in methanol and hydrogenated for 2 to 4 h at 40 lb of pressure in the presence of palladium black catalyst. The catalyst was removed by filtration and the solvent was removed under vacuum at 40 °C. The resulting oily esters were dissolved in dimethylformamide and were used immediately for coupling. Z-peptide-*tert*-butyl esters were converted to Z-peptides by treatment for 2 h at 0 °C with trifluoroacetic acid (99+%, Pierce). The analytical data on the Z-peptides and the Z-peptide esters are given in Table I. The minimum number of coupling steps was used in each case, e.g., preparation of Z-L-glutaminyl-L-glutaminylglycine-*tert*-butyl ester was made by reaction of the Z-dipeptide active ester with glycine-*tert*-butyl ester. Each of the compounds of Table I showed a single spot upon treatment with chlorine-toluidine (Natecki and Goodman, 1966) after thin-layer chromatography on silica gel in *n*-butyl alcohol-acetic acid-water (4:1:1) and in *sec*-butyl alcohol-3% NH_4OH (100:44). Evidence for stereochemical purity of the three Z-peptides used here as transglutaminase substrates was obtained by removing the Z group of small samples of each and examining the digestibility of the resulting peptide by aminopeptidase M (Boehringer Mannehim). In each case, chromatography of the digests in

[1] S. Y. Lee and S. I. Chung, manuscript in preparation.

[2] Abbreviation used: Z, benzyloxycarbonyl.

the above systems showed complete disappearance of the peptide and appearance of the expected amino acids.

Other materials and reagents have been described in previous publications (Folk and Cole, 1966a,b; Connellan et al., 1971a; Chung and Folk, 1972a).

The assay used during purification of rabbit liver transglutaminase is based on putrescine incorporation into Hammersten casein. It was conducted essentially as outlined earlier (Chung and Folk, 1972b), except that the [^{14}C]putrescine (51.8 Ci/mol) was used at a level of 9.65 μM. Protein was estimated by the method of Lowry et al. (1951). Purification of the enzyme from livers of New Zealand White rabbits was carried out by a procedure described for preparation of guinea pig liver transglutaminase (Connellan et al., 1971a), except with the following modifications: (a) the first DEAE-cellulose chromatography step was carried out by mixing with the supernatant fluid from 110 000g centrifugation sufficient equilibrated DEAE-cellulose to remove essentially all of the enzymic activity. The charged adsorbent was recovered by suction filtration, suspended in 0.15 M NaCl in Tris-Cl buffer, pH 7.5, containing 2 mM EDTA, and transferred into a chromatographic column. The adsorbent was washed with the NaCl-containing buffer solution until the effluent became colorless. Elution of the enzyme was carried out with a linear gradient of 0.15 to 1.0 M NaCl in the same buffer solution. (b) The partially purified enzyme obtained by chromatography on 10% agarose was subjected to a final (2nd) DEAE-cellulose chromatography step. Apparently homogeneous enzyme preparations were obtained by elution with a linear gradient of 0 to 0.6 M NaCl in the Tris buffer solution.

Polyacrylamide electrophoresis was carried out in gels prepared with 7.5% acrylamide, 0.1% sodium dodecyl sulfate, and 6 M urea (Schwartz et al., 1971). Samples were prepared with and without 1% 2-mercaptoethanol.

Exclusion chromatography was conducted on a 2.5 × 88 cm column of Sephadex G-100 (Pharmacia) in Tris-Cl buffer, pH 7.5, containing 2 mM EDTA, and on a 1 × 99 cm column of 4% agarose (Sepharose 4B, Pharmacia) in the same buffer solution containing 6 M guanidine hydrochloride. Eluates from the Sephadex column were analyzed for protein and for enzymic activity by absorbancy at 280 mm and by the enzyme assay given above, respectively; those from the agarose column were analyzed for protein by a fluorescamine method (Udenfriend et al., 1972).

Reference standards for gel electrophoresis and for exclusion chromatography included bovine serum albumin (Pentex), pepsin (Sigma), β-galactosidase (Sigma), ovalbumin (Pharmacia), and aldolase (Pharmacia).

Isoelectric focusing was carried out on polyacrylamide gels using the Ampholyte technique (Maurer, 1971) with Ampholine (pH 3.5–10). Protein concentration was measured by absorbancy at 280 mm; pH by glass electrode.

Amino acid analyses were performed with an automatic analyzer on samples of enzyme protein after acid hydrolysis (6 N HCl, 110 °C) for 24, 48, and 72 h. Half-cystine was estimated as cysteic acid after performic acid oxidation (Moore, 1963); tryptophan was by a spectrophotometric procedure (Edelhoch, 1967).

Immunization of a goat with rabbit enzyme protein was carried out using a published schedule (Abe et al., 1975). Tests of the monospecificity of the antiserum were conducted by the double-diffusion technique.

For kinetic studies the following assay methods and conditions were used: (a) *Hydroxylamine incorporation* was carried out at 37 °C in 0.1 M Tris-acetate buffer, pH 6.5, containing 0.1 M hydroxylamine, 0.5 mM dithiothreitol, and 20 mM $CaCl_2$. Incorporation was measured by the use of the colorimetric $FeCl_3$ procedure described previously (Folk and Cole, 1966a). (b) *Esterase activity* toward *p*-nitrophenyl acetate was measured as change in absorbancy at 400 nm due to *p*-nitrophenol release. Data were collected within the first 20 to 40 s of hydrolysis. Reactions were carried out at 25 °C in 0.1 M Tris-acetate buffer, pH 7.0, containing 5% *n*-propyl alcohol, 1 mM EDTA, and 10 mM $CaCl_2$. (c) [^{14}C]*Methylamine incorporation* was conducted at 25 °C in 0.1 M Tris-HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 1 mM EDTA. For amine incorporation into β-casein the reactions were terminated and products precipitated by the addition of trichloroacetic acid solution and radioactivity was measured as outlined previously (Chung and Folk, 1972a). For amine incorporation into the Z-peptides, reactions were terminated in the incubation tubes by the addition of 0.1 volume of 20 mM iodoacetamide. Radioactive products were separated from radioactive amine by a paper strip ion-exchange procedure similar in principle to that outlined by Sherman (1963). Aliquots of reaction mixtures were applied 2.5 cm from the ends of 1.5 × 9 cm strips of DEAE-cellulose paper and strips were eluted with 1% pyridine. After drying, the portions of paper near the point of application which contained the radioactive products were removed and placed in counting vials containing 10 mL of Hydromix counting fluid (Yorktown), and the radioactivity was measured in a liquid scintillation spectrometer.

Conditions were adjusted such that no more than 10% of the substrate of the lowest concentration was consumed within the reaction periods employed. The nomenclature is, in general, that of Cleland (1963). For clarity, the definitions of certain kinetic constants that have been previously defined (Folk, 1969) are given in the footnotes to Table IV. The kinetic constant subscript i designates a dissociation constant; h, the Michaelis constant for hydrolysis; and t, the Michaelis constant for transfer.

Reciprocal velocities were plotted graphically against the reciprocals of substrate concentration. The data were fitted to eq 1

$$v = \frac{VA}{K + A} \tag{1}$$

assuming equal variance for the velocities. All fits were performed by means of an interactive curve-fitting program, MLAB, developed at the National Institutes of Health and running on a PDP-10 digital computer (Knott and Reece, 1971). Final estimates of kinetic constants for the incorporation of methylamine were made by fitting the data points to eq 2,

$$v = \frac{V_{ab}AB}{K_{ah}K_{bt} + K_{at}B + K_{bt}A + AB} \tag{2}$$

that for the initial velocity of amine incorporation in mechanism I (Folk, 1969). The kinetic behavior of hydrolysis and amine transfer for all transglutaminases conform to mechanism I (Chung and Folk, 1972a) in which an acyl-enzyme intermediate, F, is partitioned between water and a primary

MECHANISM I

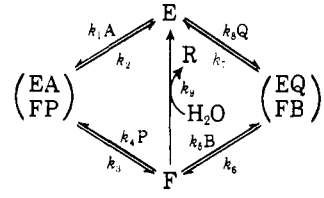

TABLE II: Purification of Transglutaminase from Rabbit Liver (Based on 1200 Grams of Liver).

Purification step	Total protein (g)	Total units[a]	Specific act. (units[a]/mg)	Yield (%)
Homogenate	160	1375	0.0086	
Supernatant fluid	60	1200	0.02	87
1st DEAE-cellulose chromatography	0.85	1020	1.20	74
Protamine extracts	0.088	550	6.25	40
Agarose chromatography	0.031	341	11.00	25
2nd DEAE-cellulose chromatography	0.012	273	22.75	20

[a] One unit is defined as the amount of enzyme that catalyzes incorporation of 1 nmol of putrescine into casein per min under the conditions given in the text.

TABLE III: Amino Acid Composition of Rabbit Liver Transglutaminase.

Amino acid	Amino acid residues per 80 000
Asp	70.8
Thr	33.8
Ser	47.0
Glu	94.6
Pro	27.7
Gly	52.3
Ala	44.3
½-cystine	27.5
Val	56.7
Met	11.0
Ile	28.3
Leu	62.4
Tyr	26.3
Phe	26.0
Lys	33.3
His	11.5
Arg	40.0
Trp	16.9

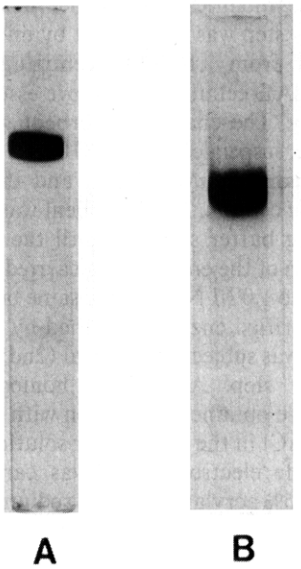

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (A) and isoelectric focusing (B) of purified rabbit liver transglutaminase. Experimental conditions are given in the text.

amine, B. In this mechanism, A is peptide-bound glutamine, P is ammonia, R is the peptide-bound glutamic acid product, and Q is the peptide-bound N-substituted glutamine product.

Results

Table II summarizes the purification of transglutaminase from rabbit liver. The enzyme protein purified through these steps showed a single band after polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figure 1A) and after isoelectric focusing (Figure 1B). A single peak of enzymic activity that corresponded to the single protein peak was observed upon chromatography on Sephadex G-100. Chromatography on agarose in 6 M guanidine revealed a single symmetrical peak of protein.

Goat antiserum elicited to the purified rabbit enzyme protein showed a single sharp precipitin line in diffusion experiments with purified enzyme (Figure 2), as well as with partially purified enzyme preparation. This antiserum also inhibited the catalytic activity of the enzyme in the casein–putrescine assay. Control serum gave no precipitin line and showed no inhibitory effect. Antiserum to the purified rabbit enzyme did not precipitate guinea pig liver transglutaminase (Figure 2) nor did it show any effect on the enzymic activity of this enzyme. Inversely, rabbit antiserum to purified guinea pig liver transglutaminase neither precipitated (Figure 2) nor inhibited the rabbit liver enzyme.

Apparent molecular weight values of 78 000, 85 000, and 87 000 for rabbit liver transglutaminase were obtained by polyacrylamide gel electrophoresis in denaturant with or without reducing agent, by exclusion chromatography in denaturant, and by exclusion chromatography without denaturant, respectively. An isoelectric point of 5.35 was estimated by isoelectric focusing.

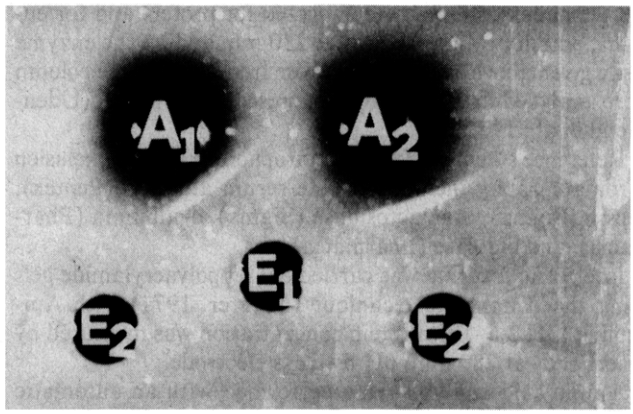

FIGURE 2: Immunological reactivity of transglutaminases from the livers of rabbit and guinea pig. Wells A1 and A2 contained goat antiserum to rabbit liver enzyme and rabbit antiserum to guinea pig liver enzyme, respectively; wells E1 and E2 contained purified rabbit liver enzyme and purified guinea pig liver enzyme, respectively.

The results of the amino acid analyses of the purified enzyme are summarized in Table III. The data are normalized to a molecular weight of 80 000. The values given are corrected for

TABLE IV: Kinetic Constants for Transglutaminases.[a]

Enzyme	Substrate	Constants[c]						
	Methylamine incorp reaction:	V_a (amide)[d]	V_{ab}	K_{ah} (mM)	K_{at} (mM)	K_{ibb}[e] (mM)	K_{bt} (mM)	$V/K \times 10^{-3}$ (M^{-1} min^{-1})
Rabbit liver	Z-L-*Gln*-Gly	136	1451 ± 243	5.7 ± 0.8	61 ± 12	0.26	2.8 ± 0.6	23.8
Guinea pig liver		54	214 ± 17	2.1 ± 0.5	8.5 ± 1.3	0.35	1.4 ± 0.2	25.2
Rabbit liver	Z-L-*Gln*-L-*Gln*-	*164*	*1273 ± 218*	*1.7 ± 0.4*	*12.8 ± 3.3*	*0.36*	*2.8 ± 0.6*	*99.5*
Guinea pig liver	Gly	200	1489 ± 273	1.1 ± 0.4	8.2 ± 2.4	0.33	2.4 ± 0.6	181.6
Rabbit liver	Z-Gly-Gly-L-	25	72 ± 11	0.48 ± 0.18	1.4 ± 0.5	0.54	1.6 ± 0.3	51.4
Guinea pig liver	*Gln*-Gly	106	190 ± 28	1.9 ± 0.5	3.3 ± 1.0	0.37	0.67 ± 0.15	57.6
Rabbit liver	Z-L-Glu-Gly-L-	422	322 ± 46	12.7 ± 1.5	9.7 ± 2.8	0.89	0.68 ± 0.14	33.2
Guinea pig liver	*Gln*-L-*Gln*-Gly	51	225 ± 48	0.72 ± 0.3	3.2 ± 0.9	0.16	0.69 ± 0.21	70.3
Rabbit liver	β-Casein	90	227 ± 36	0.04 ± 0.007	0.10 ± 0.02	0.34	0.85 ± 0.20	2270
Guinea pig liver		43	85 ± 5.7	0.02 ± 0.005	0.04 ± 0.006	0.02	0.39 ± 0.05	2125
	Hydroxylamine incorp reaction:[f]		V_{max}	K_m(app)				
Rabbit liver	Z-L-*Gln*-Gly		65.4 ± 1.0	68.6 ± 1.4				0.95
Guinea pig liver			3592 ± 646	49.4 ± 12.8				72.7
	Esterase reaction:		V_a(ester)	K_m(app)				
Rabbit liver	*p*-Nitrophenyl acetate		147 ± 15	1.0 ± 0.3				147
Guinea pig liver			171 ± 21	2.5 ± 0.6				68.4

[a] V_a, V_{ab}, and V_{max}, the maximum velocities for hydrolysis, for transfer with methylamine, and for transfer with hydroxylamine, respectively, are expressed in micromoles per minute (per μmol of enzyme[b]). [b] The molecular weights used were: 80 000 for rabbit liver enzyme; 90 000 for guinea pig liver enzyme. [c] The constants are assigned on the basis of mechanism I and are defined as (Folk, 1969): $K_{at} = [k_7(k_2 + k_3)]/[k_1(k_3 + k_7)]$, $K_{bt} = [(k_6 + k_7)(k_3 + k_9)]/[k_5(k_3 + k_7)]$, $V_a = (k_3k_9E_0)/(k_3 + k_9)$, $K_{ah} = [k_9(k_2 + k_3)]/[k_1(k_3 + k_9)]$, $K_{ibb} = [k_9(k_6 + k_7)]/(k_5k_7)$, $V_{ab} = (k_3k_7E_0)/(k_3 + k_7)$. V/K is defined as V_{ab}/K_{at} for methylamine incorporation, as V_{max}/K_m(app) for hydroxylamine incorporation, and as V_a(ester)/K_m(app) for *p*-nitrophenyl acetate hydrolysis and is the apparent first-order rate constant for reaction of enzyme and first substrate. [d] Estimated from the equality, $K_{at}V_a = K_{ah}V_{ab}$. [e] Estimated from the equality, $K_{at}K_{ibb} = K_{ah}K_{bt}$. [f] The concentration of hydroxylamine was 0.1 M.

destruction of serine and threonine and for rates of release of valine and isoleucine from timed hydrolysates. No amino sugars were detected in hydrolysates. A partial specific volume of 0.717 was calculated and an extinction coefficient of $E_{280}^{1\%} = 14.0$ was estimated on the basis of these data.

Following the final step in purification (second DEAE-cellulose chromatography) the enzyme rapidly lost catalytic activity with less than 50% of the initial activity remaining after 1 week storage at −40 °C and with little more than 10% of the initial activity remaining after 2 weeks. Attempts to stabilize enzymic activity by variations in the purification procedure, including ammonium sulfate precipitation steps, chromatography on other ion-exchange celluloses, and inclusion of various combinations of EDTA, $CaCl_2$, and dithiothreitol in the buffer solutions, proved of no value.

Active site titrations were performed on a preparation of purified enzyme protein that had lost 40% of its initial catalytic activity during storage. ^{14}C incorporation and remaining enzymic activity were measured after incubation of portions of this enzyme sample with various levels of [^{14}C]iodoacetamide (Folk and Cole, 1966a). Reactions were carried out for 10 min in 0.1 M Tris-acetate buffer, pH 6.3, containing 20 mM $CaCl_2$, 0.3 mM EDTA, and 0.2 M NaCl. Based on a molecular weight of 80 000, a value of 0.58 mol of active site per mol of protein was measured. This value is in close agreement with that of 0.6 mol per mol obtained by enzyme assay and qualifies as valid the specific activity given for purified enzyme in Table II. A similar result (0.59 mol of active site per mol of protein) was obtained upon titration of this enzyme sample with α-bromo-4-hydroxy-3-nitroacetophenone according to a published procedure (Folk and Gross, 1971). These findings are strong evidence that, like guinea pig liver transglutaminase (Folk and Cole, 1966a), the rabbit liver enzyme contains a single active site per molecule.

In contrast to the lability of the fully purified transglutaminase, enzyme purified through the agarose chromatography step was very stable showing essentially no loss in catalytic activity after 2 weeks storage at −40 °C. Enzyme prepared to this point showed about one-half the specific activity of fully purified material (Table II). Examination by polyacrylamide gel electrophoresis in sodium dodecyl sulfate revealed a major band that occupied the position of fully purified enzyme, together with one faster migrating and two slower migrating minor bands. Because of the very labile nature of the purified enzyme and the advantage of good stability of the material purified only through the agarose step, we chose to employ the latter for catalytic studies. The enzymic activity of the preparations was monitored frequently during these studies with the casein-putrescine assay and the values for maximum velocities recorded here are those extrapolated to full catalytic activity.

In Table IV are recorded kinetic constants for rabbit liver transglutaminase with a number of substrates. For comparative purposes, constants are also given for the guinea pig liver enzyme. With each substrate the values were estimated from data sets collected for both enzymes on the same or consecutive days. The constants obtained for methylamine incorporation and those for ester hydrolysis are not widely different for the

two enzymes. The similar specificities of the two enzymes for glutamine-containing substrates, as well as for *p*-nitrophenyl acetate, are reflected in the values for *V/K*, i.e., the apparent first-order rate constants for reaction of enzyme and first substrates. In contrast, however, a pronounced difference was found for hydroxylamine incorporation into Z-L-glutaminylglycine. The rabbit enzyme showed less than 2% of the maximum velocity displayed by the guinea pig liver enzyme in this reaction. This difference was confirmed using freshly prepared fully purified rabbit liver transglutaminase. That the level of hydroxylamine used (0.1 M) was saturating was evidenced by the fact that essentially the same values for V_{max} and K_m(app) as those recorded in Table IV were obtained at various concentrations of the amine between 5 and 200 mM. Preincubation of the enzyme with hydroxylamine under assay conditions caused essentially no loss in enzymic activity indicating no irreversible inhibition of the enzyme by this amine.

Discussion

The close agreement in values for molecular weight of rabbit liver transglutaminase obtained in reduced and unreduced form in denaturing solvents and in the native state provides evidence that this enzyme is a single-chain polypeptide of approximately 80 000 molecular weight. That it contains one catalytic site per molecule is established from the active site titrations. It is not surprising that the rabbit liver enzyme is similar in these features to the well-characterized guinea pig liver transglutaminase (reviews: Folk and Chung, 1973; Folk and Finlayson, 1977). Nor is it unexpected that there are only small differences in the amino acid compositions of the enzymes from the two animals, with the exception that the rabbit enzyme appears to contain a significantly larger quantity of half-cystine.

It is clear, however, that the two enzyme proteins are immunologically distinct. In addition to the lack of reactivity of the guinea pig enzyme with goat antiserum to the rabbit enzyme, it was found that rabbit transglutaminase fails to react with, and is not inhibited by, a rabbit antiserum elicited to the purified guinea pig liver enzyme (Figure 2).

The similarity in specificity of the two enzymes for methylamine incorporation into several synthetic glutamine peptide derivatives, as well as into β-casein, and their similarity in esterase activity (Table IV) suggests that, indeed, the enzyme described in this report is the rabbit counterpart of guinea pig liver transglutaminase. This is strongly supported by the fact that goat antiserum to purified rabbit plasma blood coagulation factor XIII and that to purified rabbit blood platelet factor XIII do not react with or inhibit the purified rabbit liver enzyme.[1]

An interesting feature of the enzymic specificity of rabbit transglutaminase is its relatively low activity for hydroxylamine incorporation into Z-L-glutaminylglycine (Table IV). This reaction is the basis of a simple and sensitive assay for guinea pig liver transglutaminase that has been used routinely in the purification of this enzyme (Folk and Cole, 1966a,b). For this reason, it was the assay chosen by Tyler and Laki (1967) for examination of rabbit liver extracts for an enzyme or enzymes capable of rendering fibrin insoluble in denaturants, i.e., stabilizing fibrin, presumably through covalent cross-link formation. Thus, it is understandable that these workers concluded that rabbit liver is very low in, or devoid of, transglutaminase as defined by the hydroxamate assay, and that the enzyme in rabbit liver responsible for catalyzing fibrin stabilization is distinct from transglutaminase. It seems likely that the enzyme described and partially purified by Tyler and Laki (1967) is the same enzyme described in detail here. It is relevant that fibrin stabilization by guinea pig liver transglutaminase through the formation of ϵ-(γ-glutamyl)lysine cross-links is well documented (reviews: Folk and Chung, 1973; Folk and Finlayson, 1977).

Another aspect of the rabbit liver transglutaminase-catalyzed hydroxylamine incorporation into Z-L-glutaminylglycine is the pronounced difference in the *V/K* value for this reaction and that obtained for methylamine incorporation into the same glutamine substrate. These values were found to be 0.95 and 23.8, respectively (Table IV). Since *V/K* values, as calculated here, are reflections of specificity of enzyme for first substrate (Bender and Kézdy, 1965), i.e., glutamine substrate, these values should be in reasonable agreement. The fact that they are not is a strong argument in favor of the validity of earlier suggestions as to the anomalous nature of some enzymic reactions that involve the use of the strong nucleophile, hydroxylamine (viz., Bender and Kézdy, 1965; Chung et al., 1970; Connellan et al., 1971b; Gross et al., 1975).

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Conversion of Skeletal Muscle Glycogen Synthase to Multiple Glucose 6-Phosphate Dependent Forms by Cyclic Adenosine Monophosphate Dependent and Independent Protein Kinases†

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ABSTRACT: Glycogen synthase was purified from rabbit skeletal muscle with a glucosamine 6-phosphate affinity column as the final step of purification. The product was primarily a trimer with a molecular weight of 269 000, contained less than 0.1 mol of alkali-labile phosphate/10^5 g of enzyme, had a −:+ glucose 6-phosphate (Glc-6-P) activity ratio of 0.7, and demonstrated positive cooperativity with respect to Glc-6-P. This activator produced a tenfold increase in the affinity of the nonphosphorylated enzyme for uridine diphosphoglucose with an $A_{0.5}$ for Glc-6-P of 15-25 μM. Phosphorylation of the purified glycogen synthase with the catalytic subunit of cAMP-dependent protein kinase resulted in incorporation of up to 2 mol of phosphate/10^5 g and increased the $A_{0.5}$ for Glc-6-P to ~250 μM. Incorporation of 1 mol of phosphate/10^5 g could also be achieved using a cAMP-independent synthase kinase purified from rabbit skeletal muscle. Glycogen synthase phosphorylated with the synthase kinase had a lower activity ratio and a higher $A_{0.5}$ for Glc-6-P (~500 μM) than glycogen synthase phosphorylated by the cAMP-dependent protein kinase. When glycogen synthase was phosphorylated with both the cAMP-dependent and cAMP-independent kinases, 3 mol of phosphate/10^5 g was incorporated, suggesting the existence of at least three distinct sites of phosphorylation. The $A_{0.5}$ for Glc-6-P increased progressively with phosphorylation to 3 mol/10^5 g ($A_{0.5} \sim 1800$ μM), while the −:+ Glc-6-P activity ratio reached a minimal value (0.02) after incorporation of less than 2 mol/10^5 g. These results indicate that the apparent affinity of muscle glycogen synthase for Glc-6-P is a more sensitive and accurate indicator of regulation of the enzyme by phosphorylation than is the −:+ Glc-6-P activity ratio. Phosphorylation of the synthase by a cAMP-independent protein kinase in combination with the cAMP-dependent protein kinase yields a Glc-6-P-dependent form of glycogen synthase that would probably be completely inactive at physiological concentrations of Glc-6-P.

Glycogen synthase from skeletal muscle is converted during solubilization and purification to a form that contains little alkali-labile phosphate and that is active in the absence of added Glc-6-P (Soderling et al., 1970; Roach et al., 1976). This Glc-6-P independent form of the enzyme ("I" form) can be phosphorylated in the presence of ATP and Mg^{2+} to a form that is dependent on Glc-6-P ("D" form) for activity (Friedman and Larner, 1963).

Conversion of glycogen synthase from the I to the D form has generally been determined by measuring decreases in the −:+ Glc-6-P activity ratio. Phosphorylation of glycogen synthase with cAMP[1]-dependent protein kinase results in a de-

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[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; BSA, crystalline bovine serum albumin; Tes, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Glc-6-P, glucose 6-phosphate; CM, carboxymethyl; DEAE, diethylaminoethyl; NADH, nicotinamide adenine dinucleotide reduced; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.