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The Specificity of Dipeptidyl Transferase*

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ABSTRACT: The kinetics of the hydrolytic action of highly purified beef spleen dipeptidyl transferase on a series of dipeptide esters have been determined by automatic titration. The results confirm and extend earlier conclusions regarding the specificity of the enzyme. Glycyl-(β-phenyl)L-lactic acid methyl ester was synthesized and was found to be cleaved by the enzyme to yield the depsipeptide, showing that the amide bond of suitable dipeptide esters (*e.g.*, Gly-Phe-OMe) can be replaced by an ester linkage in substrates of dipeptidyl transferase. It was also found that diazoacetylglycine ethyl ester is resistant to enzymic hydrolysis, whereas Gly-Gly-OEt is readily cleaved. This diazo compound, as well as diazoacetyl-L-phenylalanine ethyl ester (and its D isomer), do not inactivate the enzyme under the conditions of these studies.

Dipeptidyl transferase (formerly termed cathepsin C), an enzyme abundant in extracts of animal tissues, exhibits unusual specificity in its catalysis of reactions at amide and ester bonds. From earlier studies in this laboratory (Wiggans *et al.*, 1954; Izumiya and Fruton, 1956; Fruton and Mycek, 1956) and those of other investigators (Planta *et al.*, 1964), it became evident that the enzyme is specific for the activation of the terminal carbonyl group of a dipeptidyl derivative, provided that the dipeptidyl unit has a free α-amino (or α-imino) group and is composed of L-α-amino acid (or glycyl) residues. In its action on such dipeptide amides or esters, the enzyme showed preference for substrates in which the activated carbonyl group was contributed by an L-amino acid bearing a hydrophobic side chain (Phe, Tyr, Trp, Leu), although dipeptide esters such as Gly-Gly-OEt were hydrolyzed at an appreciable rate. The amino-terminal amino acid residue could be varied considerably; substrates having glycyl, L-prolyl, or various α-L-amino acid residues (but not lysyl) as amino-terminal components were cleaved readily. It was suggested (Izumiya and Fruton, 1956; Fruton, 1957) that, in the interaction of a substrate with the enzyme, the backbone of the dipeptidyl group assumes a conformation approximating a hexagonal structure. This hypothesis is consistent with the observed similarity in the rates of cleavage of pairs of substrates such as the ethyl esters of D-alanyl-L-tyrosine and α-aminoisobutyryl-L-tyrosine.

As has been shown previously (Jones *et al.*, 1952; Fruton *et al.*, 1953; Würz *et al.*, 1962; Nilsson and Fruton, 1964), dipeptidyl transferase is an exceptionally efficient catalyst of reactions in which the dipeptidyl group of a suitable substrate is transferred to an amine. With nucleophiles such as hydroxylamine, hydroxamic acids are formed; this reaction has provided a convenient method for the rapid assay of enzyme preparations (de la Haba *et al.*, 1959; Metrione *et al.*, 1966). Of more general interest is the fact that the enzyme readily catalyzes polymerization reactions in which dipeptidyl units are added to a growing oligopeptide chain to form products whose chain length is determined by their solubility in aqueous media. Since the relative extent of reaction of the activated dipeptidyl unit with either

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water (to cause hydrolysis) or with the amino group of another substrate (to form polymers) depends on the pK_a of the corresponding ammonium group and on the pH of the solution, it is understandable that at pH values at which the α-amino group of the substrate is largely protonated, the partition between hydrolysis and polymerization is strongly in favor of the former process. On the other hand, the efficiency of dipeptidyl transferase as a catalyst of transfer to suitable amine nucleophiles is so great that, at pH values in the range 7–8, where the conjugate acids of the substrates have their pK_a values, the predominant reaction is polymerization in spite of the considerable disparity in the concentrations of water and the amine. For this reason, the hydrolytic activity of dipeptidyl transferase is conveniently studied in the pH range 4–6, where the α-amino group is fully protonated. More acidic pH values (below 3.5) are unsuitable because the enzyme is rapidly and irreversibly inactivated under such conditions. For the study of the specificity of dipeptidyl transferase as a hydrolase, therefore, it has been the practice to determine the kinetics of enzyme action at pH values near 5, except in the special case of dipeptide derivatives having an amino-terminal prolyl residue, which do not undergo polymerization reactions in the pH range 6–8 (Fruton and Mycek, 1956).

Dipeptidyl transferase requires, for full activity, the addition of a sulfhydryl compound to the assay mixture; among the SH compounds tested, β-mercaptoalkylamines have proved to be the most effective (Fruton and Mycek, 1956). Experiments by Drs. R. M. Metrione and Y. Okuda in this laboratory indicate that the enzyme contains 8 sulfhydryl groups/unit of 210,000, the particle weight of the purified protein (Metrione *et al.*, 1966). It appears likely, therefore, that dipeptidyl transferase belongs to the group of enzymes among which papain and ficin are the best known members. It may be suggested that dipeptidyl transferase, like papain (Stockell and Smith, 1957; Lowe and Williams, 1965; Kirsch and Igelström, 1966; Brubacher and Bender, 1966), acts on its substrates with the intermediate formation of a thiol ester linking the activated carbonyl group of the dipeptidyl unit to a reactive sulfhydryl group of the enzyme. Such thiol esters may be expected to be effective acylating agents for amines in aqueous media, thus accounting for the efficient partitioning of the activated acyl group in favor of transfer to amine acceptors rather than to water (Dowmont and Fruton, 1952; Durell and Fruton, 1954; Mycek and Fruton, 1957).

In earlier studies of the kinetics of the hydrolytic action of dipeptidyl transferase (Fruton and Mycek, 1956), the rate of cleavage of dipeptide esters was estimated by determining the residual ester with the hydroxamic acid method of Hestrin (1949). In the present study, the pH-Stat method was employed in order to obtain more precise rate data. Also, new compounds have been synthesized for the examination of several unanswered questions relating to the specificity of the enzyme.

Experimental Section

Enzyme Experiments. The preparation of beef spleen dipeptidyl transferase (Metrione *et al.*, 1966) used in these studies was kindly provided by Dr. R. M. Metrione of this laboratory, and has a specific activity of 30.0 units/mg of protein. The rate of hydrolysis of dipeptide esters was determined by automatic titration of the liberated acid with *ca.* 0.01 N NaOH, at pH 5.0 and 37°, using a Radiometer TTT1 meter in conjunction with a TTA 31 titration assembly and a SBR2 titrigraph. Except where otherwise stated, the assay solution contained 4 mM β-mercaptoethylamine hydrochloride; no buffer was added. As the pK of the carboxyl group of dipeptides is near 3, no correction for partial dissociation of the hydrolytic product is needed at pH 5. The enzyme concentrations employed were in the range 0.5–1.0 unit/ml. The initial rates (up to 10–20% hydrolysis) were determined at six to eight values of substrate concentration (in the range 0.15–75 mM), and were used for $1/v$ *vs.* $1/S$ plots. In all cases, satisfactory Michaelis–Menten kinetics were observed, permitting the determination of $K_{M\,app}$ and of k_{cat} by the method of least squares. These terms are defined by the expression $v = k_{cat}ES/(K_M + S)$, where v is the initial velocity, S is the initial substrate concentration, and E is the enzyme concentration in units per milliliter. For the enzyme preparation used, 1 enzyme unit (as defined by Metrione *et al.*, 1966) corresponds to 0.033 mg of protein; if the particle weight of 210,000 is taken to represent 1 mole equiv, an enzyme solution of 1 unit/ml is 0.00016 mM.

Chromatography. Examination of the homogeneity of the peptides prepared in this work, and of cleavage products released upon enzymic hydrolysis, was performed by thin-layer chromatography, with silica gel G as the supporting phase (Eastman chromatogram sheets K301R). The following solvent systems were used: (A) 1-butanol–acetic acid–water (3:1:1, v/v); (B) methanol–benzene (15:85, v/v).

Gly-Pla-OMe Hydrobromide.[1] Z-Gly (2.1 g, 10 mmoles) and β-phenyl-L-lactic acid methyl ester (Pierce Chemical Co., 1.8 g, 10 mmoles) were coupled in the presence of methanesulfonyl chloride (0.76 ml, 10 mmoles), with dry pyridine (30 ml) as the solvent. After 90 min at −5°, the reaction mixture was kept at room temperature for 90 min and poured into ice water (100 ml). The resulting mixture was extracted three times with ether, and the combined ethereal extract was washed with ice water (twice), cold 5% citric acid (three times), and cold 3% $NaHCO_3$ (three times), and dried over Na_2SO_4. Removal of the solvent *in vacuo* gave a product that was recrystallized from ether–petroleum ether (bp 30–60°); yield, 2.0 g (53%); mp 61–63°. Thin-layer chromatography (solvent B) gave a single spot of R_F 0.74 (iodine vapor). *Anal.* Calcd for $C_{20}H_{21}NO_6$ (371.4): C, 64.7; H, 5.7; N, 3.8. Found: C, 65.0; H, 5.9; N, 3.8.

Z-Gly-Pla-OMe (0.52 g, 1.4 mmoles), prepared in the manner described above, was treated with 2 M HBr in glacial acetic acid (2 ml). Ether (100 ml) was added and

[1] Abbreviations used: Pla, β-phenyl-L-lactyl; Sar, sarcosyl; Z, benzyloxycarbonyl; (*N*-Me)Phe, *N*-methyl-L-phenylalanyl; DCC, dicyclohexylcarbodiimide.

the resulting oil was brought to crystallization with acetone; yield, 0.39 g (88%); mp 80–82°; $[\alpha]_D^{24}$ −10.6° (*c* 1, water). Thin-layer chromatography (solvent A) gave a single ninhydrin-positive (yellow) spot of R_F 0.71. *Anal.* Calcd for $C_{12}H_{16}BrNO_4$ (318.2): C, 45.4; H, 5.1; N, 4.4. Found: C, 45.3; H, 5.0; N, 4.4.

After incubation of the depsipeptide ester hydrobromide (10 mM) with dipeptidyl transferase (0.71 unit/ml) for 20 min under the conditions described above, samples were examined by thin-layer chromatography (solvent A). In addition to the ninhydrin-positive spot (R_F 0.74) given by residual substrate, there appeared a yellow spot (R_F 0.65) corresponding to glycylphenyllactic acid and a faint purple spot (R_F 0.27) corresponding to glycine. The intensity of the spot for glycine was the same as in a control experiment in which the enzyme had been omitted from the incubation mixture. It may be concluded therefore that the enzymic action was restricted to cleavage of the Pla–OMe bond of the depsipeptide ester, and that the appearance of glycine was a consequence of inherent instability of the depsipeptide bond in aqueous solution at pH 5.

Gly-Phe-OMe Hydrochloride. Z-Gly (1.05 g, 5 mmoles) and Phe-OMe (1.08 g, 5 mmoles) were coupled in the usual manner in the presence of DCC (1.03 g, 5 mmoles) and tributylamine (1.2 ml, 5 mmoles), with CH_2Cl_2 (20 ml) as the solvent. The resulting Z-Gly-Phe-OMe was an oil which was subjected to hydrogenolysis (palladium black) in the presence of methanol (20 ml) and concentrated HCl (0.5 ml). Upon removal of the solvent, 1.1 g (81%) of the dipeptide ester hydrochloride (mp 170–171°) was obtained. Thin-layer chromatography (solvent A) gave a single iodine-positive spot of R_F 0.69. *Anal.* Calcd for $C_{12}H_{17}ClN_2O_3$ (272.8): N, 10.3. Found: N, 10.0.

Diazoacetyl-L-phenylalanine Ethyl Ester. Gly-Phe-OEt hydrochloride (Wiggans *et al.*, 1954) (1.44 g, 5 mmoles; $[\alpha]_D^{24}$ +7.1° (*c* 2, water)) was dissolved in 2 M sodium acetate (4 ml). To the chilled solution, sodium nitrite (0.5 g, 7.2 mmoles) in water (1 ml) was added, followed by glacial acetic acid (0.2 ml). After 18 hr at 0°, the resulting crystalline precipitate was collected and washed successively with water, 3% $NaHCO_3$, and water; yield, 0.6 g (46%). After recrystallization from CCl_4, it melted at 120–121°; $[\alpha]_D^{24}$ +30.6° (*c* 1, ethanol). *Anal.* Calcd for $C_{13}H_{16}N_3O_3$ (262.3): C, 59.5; H, 6.1; N, 16.0. Found: C, 59.5; H, 5.9; N, 15.9.

Diazoacetyl-D-phenylalanine Ethyl Ester. This compound was prepared in the manner described above from Gly-D-Phe-OEt hydrochloride ($[\alpha]_D^{24}$ −7.2° (*c* 2, water)), which had been obtained by the route described previously for the L compound (Wiggans *et al.*, 1954); yield, 0.8 g (57%); mp 120–121°; $[\alpha]_D^{24}$ −31.6° (*c* 1, ethanol). *Anal.* Calcd for $C_{13}H_{16}N_3O_3$ (262.3): C, 59.5; H, 6.1; N, 16.0. Found: C, 59.7; H, 6.0; N, 15.8.

Other Peptide Derivatives. Diazoacetylglycine ethyl ester (mp 107°) was prepared from Gly-Gly-OEt hydrochloride according to the procedure of Curtius and Darapsky (1906). The hydrochlorides of Gly-Tyr-OEt, Sar-Phe-OEt, and Gly-(N-Me)Phe-OEt were prepared in the manner described by Izumiya and Fruton (1956). Gly-Trp-OMe *p*-toluenesulfonate was prepared according to Theodoropoulos and Fruton (1962). Gly-Phe-OBu^t hydrochloride was generously provided by Dr. G. M. Anderson, Lederle Laboratories, Pearl River, N. Y.

Action of Diazoacetyl Compounds on Dipeptidyl Transferase. The enzyme (1.2 units/ml) was incubated at 37° and pH 5.6 with 0.04 mM diazoacetyl compound and 0.1 mM $CuCl_2$. Because of the insolubility of diazoacetylphenylalanine ethyl ester in water, ethanol (5%) was present in the incubation mixture in all experiments. Control experiments, in which either the diazo compound or $CuCl_2$, or both, were omitted, were run in parallel. Samples (0.2 ml) of the incubation mixture were withdrawn at 10 and 30 min and tested for their activity toward Gly-Phe-OEt (10 mM) in the manner described above.

Results

In Table I are given values of $K_{M\,app}$ and k_{cat} for the hydrolysis of various dipeptide esters at pH 5.0 and 37°. In general, the values of $K_{M\,app}$ reported in Table I for the ethyl esters are in satisfactory agreement with earlier data (Fruton and Mycek, 1956) obtained with a crude enzyme preparation and by means of the hydroxamic acid method. Also, as found previously, the k_{cat} values for Gly-Phe-OEt, Gly-Tyr-OEt, and Gly-Leu-OEt and

TABLE I: Kinetics of Hydrolysis of Synthetic Substrates by Dipeptidyl Transferase.[a]

Substrate[b]	$K_{M\,app}$ (mM)	k_{cat}[c] (sec^{-1})	$k_{cat}/K_{M\,app}$ (mM^{-1} sec^{-1})
Gly-Pla-OMe (10–75)	19 ± 1	210 ± 12	11
Gly-Phe-OMe (1–10)	1.3 ± 0.1	61 ± 4	47
Gly-Phe-OEt (1–10)	1.5 ± 0.1	98 ± 6	65
Gly-Tyr-OEt (0.2–5)	0.63 ± 0.05	90 ± 5	143
Gly-Trp-OMe (0.15–10)	1.5 ± 0.1	293 ± 13	195
Gly-Leu-OEt (0.4–10)	4.0 ± 0.2	126 ± 9	30
Gly-Gly-OEt (1–10)	5.3 ± 0.4	74 ± 6	14
Sar-Phe-OEt (6–75)	61 ± 4	157 ± 11	2.6

[a] pH 5.0; 37° (for details, see Experimental Section). [b] The numbers in parentheses denote the range of substrate concentration (millimolar) employed. [c] Calculated from maximum velocity data on the assumption that 1 enzyme unit/ml corresponds to 0.00016 mM solution of dipeptidyl transferase (molecular weight 210,000).

Sar-Phe-OEt were similar, despite considerable differences in their $K_{M\,app}$ values. We consider the data presented in Table I more reliable than the earlier values because of the use of a more highly purified enzyme preparation and of the pH-Stat method.

In previous studies on the specificity of dipeptidyl transferase (Izumiya and Fruton, 1956) it was shown that substitution of the NH hydrogen in the interior peptide bond of a substrate by a methyl group (as in glycyl-*N*-methyl-L-phenylalanine ethyl ester) rendered the ester linkage resistant to enzymic hydrolysis. This finding has been confirmed in the present work, as no H^+ liberation was observed in the pH-Stat when the above *N*-methyl compound was tested under conditions (0.05 M substrate, 0.71 unit of enzyme/ml, pH 5.0, 37°, 20 min) that gave rapid hydrolysis of Gly-Phe-OEt. To determine whether the interior NH group of a substrate is an obligatory site of interaction with the enzyme, the depsipeptide glycyl-(β-phenyl)-L-lactic acid methyl ester (Gly-Pla-OMe) was synthesized by the methanesulfonyl chloride method (for details, see Experimental Section). This compound is readily cleaved by dipeptidyl transferase at the Pla–OMe bond (Table I), indicating that replacement of the interior NH group by oxygen does not render the terminal ester bond resistant to enzymic attack. The resistance of Gly-(N-Me)Phe-OEt may be attributed, therefore, to steric hindrance by the *N*-methyl group in the enzyme–substrate interaction.

In further confirmation of earlier views on the specificity and mechanism of dipeptidyl transferase, it was found in the present work that Gly-Phe-OBu^t is resistant to enzymic attack under conditions (0.05 M substrate, 0.71 unit of enzyme/ml, pH 5.3, 37°, 60 min) that gave rapid hydrolysis of Gly-Phe-OEt. This result is concordant with the known resistance of tertiary butyl esters to the attack of bases at the carbonyl carbon (Anderson and Callahan, 1960). Not only are Gly-(*N*-Me)Phe-OEt and Gly-Phe-OBu^t resistant to cleavage by the enzyme, but in the concentration range 1–5 mM neither compound inhibited the hydrolysis of Gly-Phe-OEt (1.5 mM) to a measurable extent under the conditions of this study.

It seemed of interest to examine the possibility that diazoacetylamino acid esters, because of their structural resemblance to substrates of dipeptidyl transferase, might be effective specific inhibitors of the enzyme. Accordingly, diazoacetylglycine ethyl ester, diazoacetyl-L-phenylalanine ethyl ester, and diazoacetyl-D-phenylalanine ethyl ester were prepared by the method of Curtius and Darapsky (1906); the modifications of this procedure used by Riehm and Scheraga (1965) proved to be unsatisfactory for the synthesis of these compounds. Of the three diazoacetyl compounds, only diazoacetylglycine ethyl ester was sufficiently soluble in water to be tested as a potential substrate. As expected, because of the absence of a free α-amino group, the diazo compound was found to be resistant under conditions (0.01 M substrate, 0.71 unit of enzyme/ml, pH 5.4, 37°, 10 min) that gave rapid hydrolysis of Gly-Gly-OEt.

No evidence was found for the inhibition of dipeptidyl transferase by any of the diazoacetyl compounds tested under the conditions given in the Experimental Section. Previous studies on the inhibition of pepsin by diazoacetylamino acid esters (Delpierre and Fruton, 1966; Rajagopalan *et al.*, 1966) had shown that these reagents are relatively ineffective in the absence of an added metallic catalyst, such as cupric ion. With dipeptidyl transferase, however, the inhibition caused by the mixture of diazoacetyl compound (all three were tested) and $CuCl_2$ was no greater than that produced by $CuCl_2$ alone. It was found that incubation of the enzyme (1.2 units/ml) with 0.1 mM $CuCl_2$ for 10 min at 37° and pH 5 led to a decrease in the activity of the enzyme to about 50% of the control value. In view of the failure of the diazo compound to cause greater inactivation, efforts were not made to reduce the extent of inhibition by Cu(II) by increasing the concentration of β-mercaptoethylamine in the assay mixture.

Discussion

The data presented in Table I indicate that the value of $K_{M\,app}$ for Gly-Pla-OMe is considerably higher than that for Gly-Phe-OMe. Although the NH group of the interior peptide bond may in fact contribute to the enzyme–substrate interaction, it would not be appropriate to conclude from this difference in the values of $K_{M\,app}$ that the affinity of the enzyme for the depsipeptide ester is much less than that for the peptide ester. The reason for this uncertainty is the fact that the nature of the rate-limiting steps in the hydrolytic process remain to be determined. If the over-all process is written as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\qquad\quad P_1$$

where k_2 and k_3 are the rate constants for acylation and deacylation of the enzyme, then $K_{M\,app} = K_S = k_{-1}/k_1$ only when $k_3 \gg k_2$, since $K_{M\,app} = k_3K_S/(k_2 + k_3)$. Under these circumstances, $k_{cat} = k_2k_3/(k_2 + k_3)$ reduces to $k_{cat} = k_2$. On the other hand, when $k_2 \gg k_3$, $K_{M\,app} = k_3K_S/k_2$ and $k_{cat} = k_3$. These considerations have been applied to several proteinases (for a review, see Bender and Kézdy, 1965). In the case of dipeptidyl transferase, the finding that the k_{cat} values for Gly-Phe-OEt and Gly-Phe-NH_2 are nearly the same, whereas those for Gly-Gly-OEt and Gly-Gly-NH_2 are very different (Fruton and Mycek, 1956), suggests that the ratio k_2/k_3 may differ widely for substrates of this enzyme. Further studies on the kinetics and mechanism of dipeptidyl transferase are needed, therefore, to determine the relationship between $K_{M\,app}$ and K_S when the hydrolysis of different substrates is compared.

The relative values of the $k_{cat}/K_{M\,app}$ ratios are in accord with the previous conclusions about the specificity of dipeptidyl transferase as regards preference for the presence of a hydrophobic side chain in the amino acid residue contributing the reactive carbonyl group. From the data in Table I it may be seen that the order of preference is Trp > Tyr > Phe > Leu > Gly. This order is

qualitatively similar to that noted for the action of chymotrypsin on a series of acetylamino acid methyl esters (Knowles, 1965). In confirmation of earlier data, the hydrolysis of Sar-Phe-OEt is characterized by a relatively high $K_{M\,app}$ value, suggesting that the *N*-methyl group of the sarcosyl residue is exerting steric hindrance in the enzyme–substrate interaction. The relatively low $k_{cat}/K_{M\,app}$ ratio for this substrate may be contrasted with the fact that the kinetic behavior of Pro-Phe-OEt and Gly-Phe-OEt are similar (Fruton and Mycek, 1956), suggesting that the rigid conformation of the pyrrolidine ring of the L-prolyl residue permits effective interaction of the dipeptidyl unit with the active center of the enzyme.

The available data are consistent with the view that, in the action of dipeptidyl transferase on its substrates, a dipeptidyl-enzyme is formed as an intermediate. In the hydrolytic process, which predominates near pH 5, this acyl-enzyme is cleaved by water. In the polymerase action of the enzyme, the propagation of the chain appears to depend on the interaction of dipeptidyl units derived from the dipeptidyl-enzyme. For example, the product formed from glycyl-DL-phenylalaninamide is almost exclusively the L octapeptide (Fruton *et al.*, 1953), indicating that the nucleophilic amino group of Gly-Phe-NH_2 can participate in the propagation of the chain only after the dipeptidyl unit has met the specificity requirements for the formation of the acyl-enzyme. In view of the oligomeric nature of dipeptidyl transferase (Metrione *et al.*, 1966), the possibility exists that its polymerase activity involves the cooperative action of catalytic sites on separate subunits, and that dipeptidyl units on adjacent subunits react during the chain propagation process. It may further be supposed that, at any stage in the polymerization, the reaction can be terminated by reaction with some of the substrate. If the product of the chain-termination reaction is insoluble, it is removed from the reaction. If dipeptidyl-enzyme reacts with the substrate to form a soluble product, as in the case of tetrapeptide amides (Fruton and Knappenberger, 1962), it is recycled *via* dipeptidyl-enzyme intermediates to insoluble polymer.

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