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The Action of Dipeptidyl Transferase as a Polymerase*

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ABSTRACT: The electrophoretic separation of the components of reaction mixtures in the action of dipeptidyl transferase on Gly-Tyr-NH_2 or Ala-Phe-NH_2 has permitted the analytical determination of the products that are formed. When ^{14}C-labeled dipeptide amides are used as substrates at pH 7.5, appreciable quantities of labeled tetrapeptide amide (Gly-Tyr-Gly-Tyr-NH_2 or Ala-Phe-Ala-Phe-NH_2) or hexapeptide amide (Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2) appear, in addition to the insoluble octapeptide amide derived from Gly-Tyr-NH_2 or the insoluble hexapeptide amide derived from Ala-Phe-NH_2. The data indicate that, with increasing substrate concentration and at constant enzyme concentration, the proportion of dipeptidyl units going to free dipeptide (by hydrolysis) decreases to a small value (*ca.* 10%), but the total fraction of dipeptidyl units going to free dipeptide and tetrapeptide amide remains relatively constant. This finding suggests the formation of an intermediate dipeptidyl–enzyme that reacts either with water or with dipeptide amide. With increasing enzyme concentration and at constant substrate concentration, the partition of dipeptidyl units shifts in favor of higher oligopeptides (hexapeptide amide and octapeptide amide), suggesting the possibility that the chain-propagation reaction involves the cooperative interaction of separate enzyme molecules bearing activated dipeptidyl units. The available data are inconsistent with chain elongation by the addition of dipeptidyl units to the α-amino group of oligopeptide amides, but favor rather a mechanism in which oligopeptidyl–enzyme intermediates are formed.

Dipeptidyl transferase, an enzyme purified from beef spleen (Metrione *et al.*, 1966), catalyzes the transfer of dipeptidyl units from suitable peptides, dipeptide amides, and dipeptide esters to acceptor nucleophiles (H_2O, NH_2OH, and NH_2R). The specificity of the enzyme with respect to the structure of the dipeptidyl unit has been defined in previous studies (Voynick and Fruton, 1968, and earlier papers cited therein). The present communication deals with the ability of dipeptidyl transferase to catalyze the transfer of one dipeptidyl unit acting as an acyl donor to another dipeptidyl unit acting as an acceptor amine. As reported in earlier papers from this laboratory (Jones *et al.*, 1952; Fruton *et al.*, 1953; Würz *et al.*, 1962; Nilsson and Fruton, 1964), such transfer reactions are involved in the polymerization of suitable dipeptide amides to yield sparingly soluble products, shown to be higher oligopeptides. The average chain length of the insoluble product derived from Gly-Tyr-NH_2 (as well as from

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Gly-Trp-NH_2 and Gly-Phe-NH_2) was found to be that of an octapeptide amide, whereas the insoluble product from Ala-Phe-NH_2 was predominantly the hexapeptide amide (Nilsson and Fruton, 1964).

Previous studies on the mechanism of the polymerization reaction had shown that, in the reaction with Gly-Tyr-NH_2, the addition of the tetrapeptide amide Gly-Tyr-Gly-Tyr-NH_2 did not accelerate the process, and that the tetrapeptide amide was cleaved to dipeptidyl units in its utilization for polymer formation (Fruton and Knappenberger, 1962). It was suggested, therefore, that the polymerization occurs as a single-chain process without the formation of free intermediates. One of the objectives of the present investigation was to examine this hypothesis.

A limitation of earlier work on this problem was the lack of a method for the separation and analytical determination of the components present in the reaction mixture after the action of dipeptidyl transferase on suitable dipeptide amides. In the present work, high-voltage paper electrophoresis in 20% acetic acid was found to effect a satisfactory separation of a series of peptides and peptide derivatives such as Gly-Tyr-NH_2, Gly-Tyr, Gly-Tyr-Gly-Tyr-NH_2, Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2, and Gly-Tyr-Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2. These oligopeptides were synthesized by an unequivocal route so as to provide authentic samples of the expected products in the enzymic polymerization of Gly-Tyr-NH_2. Similarly, Ala-Phe-Ala-Phe-NH_2 and Ala-Phe-Ala-Phe-Ala-Phe-NH_2 were synthesized to check the electrophoretic separation of the components formed in the enzymic polymerization of Ala-Phe-NH_2. The electrophoretic procedure was used for the separation of the radioactive components present in an incubation mixture after the action of dipeptidyl transferase on ^{14}C-labeled Gly-Tyr-NH_2 or Ala-Phe-NH_2, and permitted a study of the effect of changes in the initial substrate concentration and in enzyme concentration on the relative amounts of the products formed during the enzymic polymerization of these two dipeptide amides.

Experimental Section

Gly-Tyr-NH_2 Acetate. This compound was prepared as described previously (Fruton and Bergmann, 1942; Würz *et al.*, 1962). The labeled dipeptide amide was derived from uniformly labeled L-[^{14}C]tyrosine (Nuclear-Chicago), and had a specific radioactivity of 5640 cpm/μmole. Thin-layer chromatography (see later) gave a single spot of R_F 0.58 (ninhydrin).

Gly-Tyr-Gly-Tyr-NH_2 Acetate. This compound was prepared previously (Würz *et al.*, 1962). Thin-layer chromatography gave a single spot of R_F 0.61 (ninhydrin).

Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2 Hydrobromide. Z-Gly-Tyr (1.24 g, 3.33 mmoles) and Gly-Tyr-Gly-Tyr-OEt hydrochloride (1.74 g, 3.33 mmoles; obtained by hydrogenolysis of the Z-tetrapeptide ester (Würz *et al.*, 1962) in the presence of HCl) were coupled by the mixed-anhydride method (0.46 ml of isobutyl chloroformate) with *N*-methylmorpholine as the base and tetrahydrofuran (100 ml) as the solvent (Anderson *et al.*, 1967). The product (1.9 g, 68%) separated from the reaction mixture; after recrystallization from acetonitrile, it melted at 197–199°. Thin-layer chromatography gave a single spot of R_F 0.86 (iodine). *Anal.* Calcd for $C_{43}H_{48}N_6O_{12}$ (840.9): N, 10.0. Found: N, 9.8.

Ammonolysis of the Z-hexapeptide ester (0.84 g, 1 mmole) in methanol (50 ml) under the usual conditions required 3 weeks for complete reaction; yield, 0.68 g (84%). The amide was recrystallized from DMF[1]–water. Thin-layer chromatography gave a single spot of R_F 0.83 (iodine). *Anal.* Calcd for $C_{41}H_{45}N_7O_{11}$ (811.8): N, 12.1. Found: N, 12.0.

Treatment of the Z-hexapeptide amide (1 mmole) with 2 M HBr in glacial acetic acid (1 ml) in the usual manner gave the hexapeptide amide hydrobromide, which was recrystallized from DMF–ethanol; yield 0.59 g (78%). Thin-layer chromatography gave a single spot of R_F 0.79 (ninhydrin). *Anal.* Calcd for $C_{33}H_{40}BrN_7O_9$ (758.6): N, 12.9. Found: N, 13.0.

Gly-Tyr-Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2 Hydrobromide. Z-Gly-Tyr (0.74 g, 2 mmoles) and Gly-Tyr-Gly-Tyr-Gly-Tyr-OEt hydrochloride (1.5 g, 2 mmoles; derived from the Z-hexapeptide ester by hydrogenolysis in the presence of HCl) were coupled by the mixed-anhydride method in the manner described above. The product was recrystallized from DMF–water; yield 1.1 g (52%), mp 246–250°. Thin-layer chromatography gave a single spot of R_F 0.87 (iodine). *Anal.* Calcd for $C_{54}H_{60}N_8O_{15}$ (1061.1): N, 10.6. Found: N, 10.4.

Ammonolysis of the Z-octapeptide ester (1.06 g, 1 mmole) in methanol (80 ml) for 24 days yielded 0.98 g (95%) of the amide, mp 242–244° dec. *Anal.* Calcd for $C_{52}H_{57}N_9O_{14}$ (1032.1): N, 12.2. Found: N, 12.1.

Treatment of the Z-octapeptide amide (0.1 mmole) with 2 M HBr in glacial acetic acid (0.5 ml) in the usual manner gave the octapeptide amide hydrobromide, which was recrystallized from DMF–ethanol; yield 70 mg (71%). Thin-layer chromatography gave a single spot of R_F 0.76 (iodine). *Anal.* Calcd for $C_{44}H_{52}BrN_9O_{12}$ (978.9): N, 12.9. Found: N, 13.0.

Ala-Phe-NH_2 Acetate. Z-Ala-Phe-OEt was prepared from Z-Ala and Phe-OEt by the mixed-anhydride method, using isobutyl chloroformate, with *N*-methylmorpholine as the base and tetrahydrofuran as the solvent. The coupling product was obtained in 90% yield, and had the same properties as those described previously (Fruton and Bergmann, 1942). It was converted into the dipeptide amide acetate in the manner described by Fruton *et al.* (1953). The above procedure was also employed for the preparation of labeled Ala-Phe-NH_2 with uniformly labeled L-[^{14}C]-alanine (Nuclear-Chicago) to yield a product having a specific radioactivity of 13,240 cpm/μmole. Thin-layer

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; OSu, oxysuccinimido. The abbreviated designation of amino acid residues denotes the L form.

chromatography gave a single spot of R_F 0.61 (ninhydrin).

Ala-Phe-Ala-Phe-NH_2 Hydrobromide. Z-Ala-Phe (1.1 g, 3 mmoles) (Fu *et al.*, 1954) and *N*-hydroxysuccinimide (0.35 g, 3 mmoles) were coupled in the presence of DCC (0.62 g, mmoles) with acetonitrile (35 ml) as the solvent to yield Z-Ala-Phe-OSu (1.2 g, 86%), mp 138–139°. *Anal.* Calcd for $C_{24}H_{25}N_3O_7$ (467.6): N, 9.0. Found: N, 8.9.

The above active ester (0.47 g, 1 mmoles) was coupled with Ala-Phe-NH_2 acetate (0.325 g, 1.1 mmoles) in the presence of *N*-methylmorpholine (0.11 ml, 1.1 mmoles), with acetonitrile (60 ml) as the solvent. After working up the reaction mixture in the usual manner, the product was recrystallized from DMF–water; yield 0.3 g (52%), mp 279–281°. *Anal.* Calcd for $C_{32}H_{37}N_5O_6$ (587.7): N, 11.9. Found: N, 11.7.

Z-Ala-Phe-Ala-Phe-NH_2 was also prepared in 85% yield by ammonolysis of Z-Ala-Phe-Ala-Phe-OEt (mp 190–191°) obtained in 66% yield by coupling Z-Ala-Phe with Ala-Phe-OEt by the mixed-anhydride method (isobutyl chloroformate) with *N*-methylmorpholine as the base and tetrahydrofuran as the solvent.

Treatment of the above Z-tetrapeptide amide with 2 M HBr in glacial acetic acid (1 ml) in the usual manner gave the tetrapeptide amide hydrobromide in 94% yield. After recrystallization from ethanol–water, it melted at 303–304°. Thin-layer chromatography gave a single spot of R_F 0.70 (ninhydrin). *Anal.* Calcd for $C_{24}H_{32}BrN_5O_4$ (534.5): N, 13.1. Found: N, 13.0.

Ala-Phe-Ala-Phe-Ala-Phe-NH_2 Hydrobromide. Z-Ala-Phe-OSu (78 mg, 0.17 mmole) was coupled with Ala-Phe-Ala-Phe-NH_2 hydrobromide (98 mg, 0.18 mmole) in the presence of *N*-methylmorpholine (0.18 ml), with acetonitrile (30 ml) as the solvent. The product separated from the reaction mixture, and was recrystallized from DMF–water; yield 80 mg (60%), mp 282–284°. *Anal.* Calcd for $C_{44}H_{51}N_7O_8$ (805.9): N, 12.2. Found: N, 12.0.

Treatment of the above Z-hexapeptide amide with 2 M HBr in glacial acetic acid (0.4 ml) in the usual manner gave the hexapeptide amide hydrobromide in 90% yield. It was recrystallized from DMF–ethanol. Thin-layer chromatography gave a single spot of R_F 0.74 (ninhydrin). *Anal.* Calcd for $C_{36}H_{46}BrN_7O_6$ (752.7): N, 13.0. Found: N, 13.0.

Chromatography. Examination of the homogeneity of the peptides prepared in this work was performed by thin-layer chromatography, with silica gel G as the supporting phase (Eastman chromatogram sheets K301R) and 1-butanol–acetic acid–water (3:1:1, v/v) as the solvent.

Enzyme Experiments. The preparation of beef spleen dipeptidyl transferase used in these experiments had a specific activity of 28 units/mg of protein (Metrione *et al.*, 1966). The incubation mixture (0.5 ml) contained a solution of the substrate (previously adjusted to pH 7.5 with 0.1 N NaOH), dithiothreitol (0.002 M) as enzyme activator, and the enzyme; in all cases, the temperature was 37.5°. Ammonia liberation during the course of enzymic action was measured by the microdiffusion method of Seligson and Seligson (1951). For electrophoretic separation of the components of an incubation mixture, the entire 0.5-ml sample was diluted with an equal volume of a 1:1 mixture of glacial acetic acid and DMF (in some experiments, trifluoroethanol was used in place of DMF). This stopped the enzyme reaction and dissolved the polymeric products that had separated from the aqueous solution.

Paper Electrophoresis. In all experiments, a cooled flat plate assembly, with a Savant power supply, was employed. We are indebted to Dr. F. M. Richards for the availability of this instrument. The samples were applied with micropipets near one edge of Whatman No. 3MM paper (25 × 58 cm), and a voltage of 80 V/cm (45 mA) was used for 2.5 hr, with 20% acetic acid as the liquid phase. The paper was then dried at 50° for 30 min. Under these conditions, authentic samples of the peptides of interest in this study migrated the following distances toward the cathode (mean of ten determinations, in centimeters): Gly-Tyr-NH_2, 35.3 ± 0.7; Ala-Phe-NH_2, 35.8 ± 0.9; Gly-Tyr, 32.1 ± 0.8; Ala-Phe, 30.8 ± 0.9; Gly-Tyr-Gly-Tyr-NH_2, 24.7 ± 0.3; Ala-Phe-Ala-Phe-NH_2, 24.1 ± 0.6; Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2, 19.5 ± 0.2; Ala-Phe-Ala-Phe-Ala-Phe-NH_2, 19.1 ± 0.6; and Gly-Tyr-Gly-Tyr-Gly-Tyr-Gly-Tyr-NH_2, 15.7 ± 0.3. The electrophoretic mobility of the ethyl esters of the above peptides was found to be the same as that for the corresponding amides.

For the electrophoretic separation of the radioactive components of an incubation mixture, four samples were applied (5 cm apart) and, after electrophoresis, the dried paper was cut lengthwise into four 5-cm wide pieces, each of which was further cut into 5 × 1 cm strips; usually, 45 such strips were prepared for measurement of their radioactivity. As a control, a sample of the incubation mixture was not subjected to electrophoresis and applied to a 5 × 1 cm strip (blank strip) for counting.

Radioactivity Determinations. The 5 × 1 cm paper strips, prepared as described above, were placed in glass counting vials, scintillation liquid (0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene) was added, and the radioactivity was determined in a Model 3375 Packard scintillation spectrometer. The total number of counts for all the 5 × 1 cm strips from a single electrophoretic separation was 90–95% of the measured radioactivity on the blank strip, and was in the range 4000–8000 cpm. The values for the specific radioactivity of the labeled Gly-Tyr-NH_2 and Ala-Phe-NH_2 were determined by applying known amounts of these substances to 5 × 1 cm paper strips, which were handled as described above. To facilitate comparison of the data, all the radioactivity measurements have been converted into micromoles of Gly-Tyr or Ala-Phe units per milliliters of original incubation mixture.

Results

Electrophoretic Separation of Reaction Components. The electrophoretic method used in this work permitted the separation of the series of compounds (dipeptide

amide, free dipeptide, tetrapeptide amide, hexapeptide amide, and octapeptide amide) expected as components in the reaction mixture upon the action of dipeptidyl transferase on Gly-Tyr-NH_2 or Ala-Phe-NH_2. Under the conditions given in the Experimental Section (20% acetic acid, 80 V/cm, 2.5 hr), the electrophoretic migration of authentic samples of these compounds was sufficiently different to permit estimation of the amount of these components in an incubation mixture. As will be noted from the Experimental Section, the distance traveled by members of the Ala-Phe series were similar to those for the corresponding members of the Gly-Tyr series. In the case of the octapeptide amide derived from Gly-Tyr-NH_2 (or the hexapeptide amide derived from Ala-Phe-NH_2), its sparing solubility limited the amount that moved out from the origin during electrophoresis. Experiments with authentic samples of these higher oligopeptides showed that when quantities of the Gly-Tyr octapeptide amide less than about 0.08 μmole (or of the Ala-Phe hexapeptide amide less than 0.04 μmole) were subjected to electrophoresis, all the ninhydrin-reactive material migrated in the electric field. When larger amounts were applied, however, a significant proportion of ninhydrin-reactive material remained at the origin.

A representative electrophoretic separation of the radioactive components present in an incubation mixture after the action of dipeptidyl transferase on Gly-[^{14}C]Tyr-NH_2 (Figure 1) shows that considerable radioactivity has remained at the origin. In the present work, it has been assumed that this stationary radioactivity is associated with octapeptide amide. This assumption appears justified on the basis of the analytical studies reported previously (Nilsson and Fruton, 1964), and is consistent with the behavior of the authentic octapeptide amide, but the possibility remains that small amounts of higher oligopeptides (*e.g.*, decapeptide amide) are formed in the enzymic polymerization. Similarly, the analytical data for the insoluble product derived from Ala-Phe-NH_2 show it to be a hexapeptide amide (Nilsson and Fruton, 1964), and in the present work it was assumed that the stationary radioactivity in electropherograms was associated with this product.

It will be noted from Figure 1 that the separation of the hexapeptide amide and tetrapeptide amide from the other components of the Gly-Tyr series was fairly satisfactory. The overlap between the peak for Gly-Tyr and Gly-Tyr-NH_2 introduces some uncertainty in the assignment of radioactivity to these two components, and the line of division between them was arbitrarily set at the minimum between the two peaks.

Action of Dipeptidyl Transferase on Gly-Tyr-NH_2. The data in Table I report the results of experiments at three initial concentrations of the dipeptide amide. For each point, a parallel determination was performed of the amount of NH_3 liberated; it will be noted that the measured value is usually fairly close to that calculated from the composition of the incubation mixture. For this calculation, it was assumed that in the formation of the products, the NH_3 liberation (in micromoles per (1:1) mole of Gly-Tyr unit in product) was 1.0

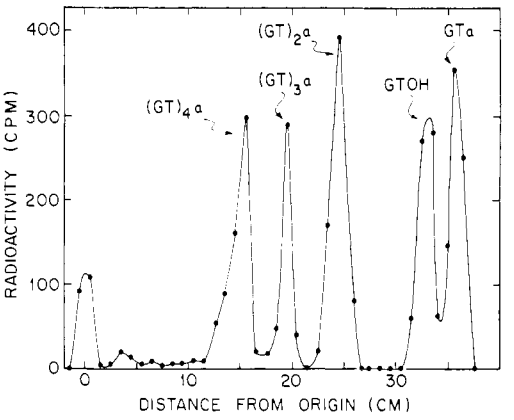

FIGURE 1: Representative electrophoretic separation of labeled components in a reaction mixture after the action of dipeptidyl transferase on glycyl-L-[^{14}C]tyrosinamide. Initial substrate concentration, 50 mM; enzyme concentration, 2 units/ml of incubation mixture; pH 7.5; 37.5°; incubation period, 2 hr. After the addition of acetic acid and DMF, a sample (50 μl) was subjected to electrophoresis as described in the Experimental Section. The radioactivity of the 5 × 1 cm strips obtained from the electropherogram is plotted against the distance from the origin toward the cathode. In the calculation of the amounts of the components in the original reaction mixture, the total radioactivity in the region 35–38 cm was assigned to dipeptide amide, 30–34 cm to free dipeptide, 22–27 cm to tetrapeptide amide, 17–21 cm to hexapeptide amide, and 1–16 cm to octapeptide amide. Abbreviations: GTa, dipeptide amide; GTOH, free dipeptide; $(GT)_2a$, tetrapeptide amide; $(GT)_3a$, hexapeptide amide; $(GT)_4a$, octapeptide amide.

for free dipeptide, 0.5 for tetrapeptide amide, 0.67 for hexapeptide amide, and 0.75 for octapeptide amide. In view of the limitations of the analytical method, the agreement may be considered satisfactory.

A striking new observation that emerges from the data in Table I is the fact that the tetrapeptide amide and hexapeptide amide are major products in the reaction mixture. It is evident that with increasing substrate concentration, the tetrapeptide amide becomes the predominant product in the reaction, and the amount of hydrolysis to Gly-Tyr becomes progressively less. Indeed, if the partition of Gly-Tyr units among the products of the reaction is expressed as in Table II, the sum of the percentages for Gly-Tyr and Gly-Tyr-Gly-Tyr-NH_2 is relatively constant (*ca.* 72%), and is independent of initial substrate concentration and of extent of reaction. At the lowest substrate concentration (12.5 mM), as the substrate becomes depleted, the proportion going to free dipeptide by hydrolysis rises, and that going to tetrapeptide amide falls; this finding is consistent with the earlier observation that Gly-Tyr-Gly-Tyr-NH_2 is hydrolyzed by dipeptidyl transferase to yield Gly-Tyr (Fruton and Knappenberger, 1962). At the highest substrate concentration tested (50 mM), during the time when only about 31% of the substrate has disappeared, the proportions going to Gly-Tyr and to Gly-Tyr-Gly-Tyr-NH_2 remain relatively constant.

Examination of the data in Tables I and II for the hexapeptide amide and octapeptide amide (which contain about 28% of the total Gly-Tyr units con-

TABLE I: Action of Dipeptidyl Transferase on Glycyl-L-[^{14}C]tyrosinamide.[a]

Time (min)	NH_3 Liberation[b] (μmoles/ml)	GTa Reacted[c] (μmoles/ml)	Products Formed (μmoles of GT units/ml)[d]			
			GTOH	$(GT)_2a$	$(GT)_3a$	$(GT)_4a$
		$[S_0]$ = 12.5 mM				
10	2.3 (2.6)	3.6	1.1	1.5	0.33	0.67
20	3.6 (3.5)	4.7	1.5	2.0	0.52	0.84
30	5.0 (5.1)	6.8	2.6	2.3	0.85	1.1
40	6.0 (6.0)	7.7	3.1	2.4	1.0	1.3
60	7.5 (7.3)	9.2	4.2	2.4	1.1	1.5
120	9.0 (9.0)	10.9	5.7	2.3	1.2	1.9
		$[S_0]$ = 25 mM				
5	1.9 (2.1)	3.1	0.56	1.7	0.15	0.73
10	3.2 (3.4)	5.0	1.0	2.7	0.45	1.0
20	5.3 (5.5)	8.2	1.7	4.1	1.2	1.3
30	7.0 (7.0)	9.9	2.4	4.7	1.7	1.5
40	7.5 (7.9)	12.4	2.7	4.8	1.9	2.1
60	9.7 (9.8)	13.9	3.6	5.2	2.3	2.8
120	10.0 (10.3)	14.5	3.9	5.2	2.6	2.8
		$[S_0]$ = 50 mM				
5	2.2 (2.5)	4.0	0.54	2.2	0.14	1.0
10	4.3 (4.5)	7.1	1.0	4.4	0.55	1.2
20	6.5 (7.0)	11.5	1.4	7.1	1.5	1.4
30	9.4 (9.0)	14.4	1.8	8.4	2.5	1.8
40	10.6 (10.3)	15.7	2.3	8.9	3.0	2.1
60	13.4 (12.5)	19.4	2.7	10.0	4.0	2.8
120	15.7 (15.1)	23.0	3.1	10.7	4.3	4.9

[a] pH 7.5; 37.5°; 1.0 enzyme unit/ml of incubation mixture; in all experiments, a precipitate appeared after about 60-min incubation. [b] The numbers in parentheses denote the calculated NH_3 liberation to be expected from the amounts of the products given in the last four columns. [c] $[S_0]$ minus amount of Gly-Tyr-NH_2 (GTa) found. [d] Abbreviations: GTOH, free dipeptide; $(GT)_2a$, tetrapeptide amide; $(GT)_3a$, hexapeptide amide; $(GT)_4a$, octapeptide amide (plus higher polymers, if present).

TABLE II: Partition of Gly-Tyr Units during Action of Dipeptidyl Transferase on Glycyl-L-tyrosinamide.[a]

Initial Concn of GTa (μmoles/ml)	Time (min)	GTa Found (μmoles/ml)	Product Formed × 100/GTa Reacted[b]			
			GTOH	$(GT)_2a$	$(GT)_3a$	$(GT)_4a$
12.5	10	8.9	30	42	10	18
	30	5.7	38	34	13	16
	60	3.3	45	26	13	16
	120	1.6	52	21	11	17
25.0	5	21.9	18	55	5	23
	10	20.0	20	54	9	20
	30	15.1	24	47	17	15
	60	11.1	26	37	17	20
50.0	5	46.0	14	55	4	25
	10	42.9	14	62	7	17
	20	38.5	12	62	13	12
	40	34.3	15	56	19	13

[a] The calculations in this table are based on the experimental data presented in Table I. [b] Abbreviations are the same as those in Table I. The fact that the total may differ from 100 is a consequence of the limitations of the analytical technique employed.

TABLE III: Action of Dipeptidyl Transferase on Glycyl-L-[^{14}C]tyrosinamide.[a]

Enzyme Concn (units/ml)	Time (min)	GTa Reacted[b] (μmoles/ml)	Products Formed (μmoles of GT units/ml)[c]			
			GTOH	$(GT)_2a$	$(GT)_3a$	$(GT)_4a$
0.5[d]	20	4.3	0.7 (16)	2.2 (51)	0.2 (5)	1.1 (25)
	40	6.1	1.2 (20)	3.0 (49)	0.6 (10)	1.6 (26)
	60	7.2	1.7 (24)	3.6 (50)	0.9 (13)	1.1 (15)
1.0[e]	20	8.4	1.6 (19)	4.0 (48)	1.2 (14)	1.6 (19)
	40	12.3	3.0 (24)	4.9 (40)	2.0 (16)	2.3 (19)
	60	14.0	3.6 (26)	5.3 (38)	2.3 (16)	2.8 (20)
2.0[f]	20	16.4	4.7 (29)	5.1 (31)	2.5 (15)	4.1 (25)
	40	18.2	5.5 (30)	5.0 (27)	2.6 (14)	5.2 (29)
	60	18.7	5.7 (31)	5.0 (27)	2.5(13)	5.5 (29)

[a] Initial concentration, [S_0], of Gly-Tyr-NH_2, 25 μmoles/ml of incubation mixture; pH 7.5; 37.5°. [b] [S_0] minus amount of Gly-Tyr-NH_2 (GTa) found. [c] The numbers in parentheses denote the ratio: product formed × 100/GTa reacted. The abbreviations are the same as those in Table I. [d] No precipitate during the 60-min incubation period. [e] Precipitate appeared after about 57-min incubation. [f] Precipitate appeared after about 15-min incubation.

TABLE IV: Action of Dipeptidyl Transferase on L-[^{14}C]Alanyl-L-phenylalaninamide.[a]

Initial Concn of APa (μmoles/ml)	Time (min)	APa Reacted[b] (μmoles/ml)	Products Formed (μmoles of AP units/ml)[c]		
			APOH	$(AP)_2a$	$(AP)_3a$
5	20	2.75	0.92 (34)	0.51 (19)	1.3 (47)
	60	3.4	1.0 (30)	0.39 (11)	2.0 (59)
12.5	20	5.6	0.68 (12)	1.3 (23)	3.6 (64)
	60	6.0	0.73 (12)	1.2 (20)	4.1 (68)
25	20	7.1	0.40 (6)	2.7 (38)	4.0 (56)
	60	8.3	0.62 (7)	2.9 (35)	4.8 (58)

[a] pH 7.5; 37.5°; 1.0 enzyme unit/ml of incubation mixture; in all experiments, a precipitate appeared within 3–4 min after the start of the incubation. [b] Initial concentration of Ala-Phe-NH_2 (APa) minus amount of APa found. [c] The numbers in parentheses denote the ratio: product formed × 100/APa reacted. Abbreviations: APOH, free dipeptide; $(AP)_2a$, tetrapeptide amide; $(AP)_3a$, hexapeptide amide (plus higher polymers, if present).

verted) shows that they are formed in relatively constant proportion at the lowest substrate concentration (12.5 mM), but that at higher substrate concentrations, the depletion of the substrate shifts the balance in favor of the hexapeptide amide.

In Table III, data are presented for the yield of products from Gly-Tyr-NH_2 (25 mM) at three concentrations of enzyme. As reported previously (Nilsson and Fruton, 1964), the time of appearance of insoluble precipitate is inversely related to the enzyme concentration, and the data in Table III indicate that the octapeptide amide separates from the solution when it has attained a concentration of about 0.75 mM (3 μmoles of Gly-Tyr units/ml of incubation mixture). At the lowest enzyme concentration tested, where no insoluble product was evident during 60-min incubation, the data suggest that the octapeptide amide was cleaved and that the resulting Gly-Tyr units were partitioned among other products.

It will also be noted from Table III that although the initial rate of the disappearance of Gly-Tyr-NH_2 is roughly proportional to the enzyme concentration, there are only slight changes in the proportion of Gly-Tyr units going to free dipeptide and to octapeptide amide. The most significant shift in the partition of Gly-Tyr units with increasing enzyme concentration is between the tetrapeptide amide and the hexapeptide amide, especially during the first 20-min period.

Action of Dipeptidyl Transferase on Ala-Phe-NH_2. The data in Table IV show that the general conclusions drawn above for the enzymic polymerization of Gly-Tyr-NH_2 also apply to the transformation of Ala-Phe-

TABLE V: Action of Dipeptidyl Transferase on L-[^{14}C]Alanyl-L-phenylalaninamide.[a]

Initial Concn of APa (μmoles/ml)	Enzyme Concn (units/ml)	APa Reacted[b] (μmoles/ml)	Products Formed (μmoles of AP units/ml)[c]		
			APOH	$(AP)_2a$	$(AP)_3a$
5	2	5.0	2.7 (54)	0.1 (2)	2.2 (44)
12.5	2	9.7	1.6 (17)	0.5 (5)	7.8 (80)
25	1	8.8	0.6 (7)	2.6 (30)	5.6 (64)
	2	17.3	1.5 (9)	1.5 (9)	14.3 (83)
	4	22.4	2.3 (10)	0.7 (3)	19.4 (87)
50	2[d]	21.2	1.2 (6)	5.2 (25)	14.7 (69)
	2	34.7	2.2 (6)	3.1 (9)	29.2 (84)

[a] pH 7.5; 37.5°; incubation period, 2 hr (unless otherwise stated); precipitate present in all incubation mixtures within 1–5 min. [b] Initial concentration of Ala-Phe-NH_2 (APa) minus amount of APa found. [c] The numbers in parentheses denote the ratio: product formed × 100/APa reacted. The abbreviations are the same as those in Table IV. [d] Time of incubation, 10 min.

TABLE VI: Effect of Urea on the Action of Dipeptidyl Transferase on L-Alanyl-L-phenylalaninamide.[a]

Enzyme Concn (units/ml)	Urea (M)	APa Reacted[b] (μmoles/ml)	Products Formed × 100/APa Reacted[c]		
			APOH	$(AP)_2a$	$(AP)_3a$
1.0	0	8.8	7	30	64
	1.0	7.1	10	39	49
2.0	0	17.3	9	9	83
	0.5	9.1	11	31	60
	1.0	8.5	11	33	57
	2.0	5.7	14	46	42
4.0	0	22.4	10	3	87
	1.0	16.4	11	12	75

[a] Initial concentration, [S_0], of Ala-Phe-NH_2, 25 μmoles/ml of incubation mixture; pH 7.5; 37.5°; incubation period, 2 hr. [b] [S_0] minus amount of Ala-Phe-NH_2 (APa) found. [c] Abbreviations are the same as those in Table IV. The fact that the total may differ from100 is a consequence of the limitations of the analytical technique employed.

NH_2. With increasing substrate concentration, the proportion of Ala-Phe units going to free dipeptide is markedly decreased, and that going to tetrapeptide amide is markedly enhanced, with a relatively constant proportion going to the hexapeptide amide. Furthermore, there is evidence that the tetrapeptide amide initially formed is partly converted into other components of the system (see also Table V, data for 50 mM Ala-Phe-NH_2).

The significant difference between the action of a given amount of enzyme on Gly-Tyr-NH_2 and on Ala-Phe-NH_2 is that, in the polymerization of the latter compound, the proportion of Ala-Phe units going to free dipeptide plus tetrapeptide amide is much smaller and that going to higher oligopeptide is much greater than with Gly-Tyr-NH_2. This finding confirms the earlier observation that the enzymic polymerization of Ala-Phe-NH_2 is much more efficient than that of Gly-Tyr-NH_2 (Nilsson and Fruton, 1964). As will be noted from Table V, the proportion of Ala-Phe units appearing in the hexapeptide amide may approach 90% at relatively high enzyme concentrations. Under these conditions, the tetrapeptide amide initially formed is largely converted into other components of the reaction mixture. It is of special interest that at the highest substrate concentration tested (50 mM), the extent of hydrolysis to free dipeptide is less than at the lowest substrate concentration (5 mM). This suggests that most of the tetrapeptide amide which disappears during the course of the reaction contributes its Ala-Phe units to the formation of hexapeptide amide.

Because of the earlier suggestion that the polymerase activity of dipeptidyl transferase may be related to its oligomeric nature (Metrione *et al.*, 1966), it seemed

TABLE VII: Action of Dipeptidyl Transferase on Glycyl-L-[^{14}C]tyrosinamide in the Presence of Unlabeled L-Alanyl-L-phenylalaninamide.[a]

Initial Concn of APa (μmoles/ml)	Time (min)	GTa Reacted[b] (μmoles/ml)	Products Formed (μmoles of GT units/ml)[c]			
			GTOH	$(XY)_2a$	$(XY)_3a$	$(XY)_4a$
0	20	8.4	1.9 (23)	4.1 (49)	1.2 (14)	1.2 (14)
	60	13.8	2.3 (23)	5.4 (39)	2.6 (19)	2.5 (18)
5[d]	20	5.7	0.80 (14)	3.1 (54)	0.80 (14)	1.0 (18)
	60	7.6	1.0 (13)	4.0 (53)	1.2 (16)	1.5 (20)
12.5[e]	20	3.5	0.28 (8)	1.9 (54)	0.37 (11)	0.93 (27)
	60	4.9	0.43 (9)	2.6 (53)	0.75 (15)	1.1 (23)
25[f]	20	2.6	0.15 (6)	1.5 (58)	0.23 (9)	0.66 (25)
	60	4.0	0.35 (9)	2.2 (55)	0.51 (13)	0.91 (23)

[a] Initial concentration of Gly-Tyr NH_2 (GTa), 25 μmoles/ml of incubation mixture; pH 7.5; 37.5°; 1.0 enzyme unit/ml of incubation mixture. [b] Initial concentration of GTa minus amount of GTa found. [c] Abbreviations: GTOH, free dipeptide; $(XY)_2a$, tetrapeptide amide; $(XY)_3a$, hexapeptide amide; $(XY)_4a$, octapeptide amide (plus higher polymers, if present). The numbers in parentheses denote the ratio: product formed × 100/GTa reacted. [d] Precipitate at 21 min. [e] Precipitate at 8.5 min. [f] Precipitate at 4.5 min.

of interest to examine the effect of urea on the enzymic polymerization of Ala-Phe-NH_2. It will be noted from Table VI that the effect of urea (0.5–2.0 M) is to reduce the amount of dipeptide amide that had reacted in 2 hr, and to shift the proportion of Ala-Phe units in favor of tetrapeptide amide, at the expense of hexapeptide amide. The fraction of Ala-Phe units going to free dipeptide by hydrolysis does not appear to be markedly affected, however.

Action of Dipeptidyl Transferase on a Mixture of Gly-Tyr-NH_2 and Ala-Phe-NH_2. In Table VII are given data for experiments in which the enzyme was allowed to act on labeled Gly-Tyr-NH_2 (25 mM) in the presence of various concentrations of unlabeled Ala-Phe-NH_2. As only the radioactive components of the electropherograms were determined, the results denote the fate of the labeled Gly-Tyr units. It will be evident that Ala-Phe-NH_2 is an effective competitor for the enzyme, and exerts a marked inhibition in the proportion of Gly-Tyr units going to free dipeptide. Most of the Gly-Tyr units that had undergone reaction appeared in the tetrapeptide amide and octapeptide amide fractions, and it may be surmised that these oligopeptides represent copolypeptides containing both Ala-Phe and Gly-Tyr units. Efforts to separate such copolymers from the corresponding homopolymers have been unsuccessful thus far.

When dipeptidyl transferase was allowed to act on labeled Ala-Phe-NH_2 in the presence of unlabeled Gly-Tyr-NH_2 (Table VIII), the inhibition of the utilization of Ala-Phe units was found to be relatively slight. This observation is consonant with the greater efficiency of the enzyme in the polymerization of Ala-Phe-NH_2. Of special interest, however, is the marked decrease in the incorporation of Ala-Phe units into the hexapeptide amide fraction, with a corresponding increase in the proportion of Ala-Phe units in tetrapeptide amide. Presumably, copolymers containing both Gly-Tyr and Ala-Phe units are formed, in addition to homopolymers of the Ala-Phe series.

Discussion

Earlier studies on the action of dipeptidyl transferase as a polymerase had suggested that, in addition to the higher oligopeptides that separate from the solution, peptides of intermediate chain length were also formed in the process (Würz *et al.*, 1962; Nilsson and Fruton, 1964). The development of a suitable method for the separation and analytical estimation of these components has provided clear evidence in support of this conclusion.

The data presented in the present communication are consistent with the hypothesis that the enzyme–substrate complex formed by the interaction of dipeptidyl transferase and a suitable dipeptide amide (XYa) is converted into a dipeptidyl enzyme (reaction 1) which can react either with water to yield free dipeptide (reaction 2) or with the amino group of the dipeptide amide to form tetrapeptide amide (reaction 3).

$$E + XYa \rightleftharpoons E[XYa] \longrightarrow XY\text{-}E \quad (1)$$

$$XY\text{-}E + H_2O \longrightarrow XYOH + E \quad (2)$$

$$XY\text{-}E + XYa \longrightarrow XYXYa + E \quad (3)$$

This view is supported by the relative constancy in the total fraction of reacted Gly-Tyr units going to Gly-Tyr plus Gly-Tyr-Gly-Tyr-NH_2 (Table II) for a given enzyme concentration. A similar relationship

TABLE VIII: Action of Dipeptidyl Transferase on L-[^{14}C]Alanyl-L-phenylalaninamide in the Presence of Unlabeled Glycyl-L-tyrosinamide.[a]

Initial Concn of GTa (μmoles/ml)	APa Reacted[b] (μmoles/ml)	Products Formed (μmoles of AP units/ml)[c]		
		APOH	$(XY)_2a$	$(XY)_3a$
0	7.1	0.40 (6)	2.7 (38)	4.0 (56)
12.5[d]	6.7	0.39 (6)	3.3 (49)	3.0 (45)
25[e]	6.2	0.27 (4)	3.3 (53)	2.7 (43)
50[f]	6.1	0.28 (5)	3.7 (61)	2.2 (36)

[a] Initial concentration of Ala-Phe-NH_2 (APa), 25 μmoles/ml of incubation mixture; pH 7.5; 37.5°; incubation period, 20 min; 1.0 enzyme unit/ml of incubation mixture. [b] Initial concentration of APa minus amount of APa found. [c] Abbreviations: APOH, free dipeptide; $(XY)_2a$, tetrapeptide amide; $(XY)_3a$, hexapeptide amide (plus higher polymers, if present). The numbers in parentheses denote the ratio: product formed × 100/APa reacted. [d] Precipitate at 2.5 min. [e] Precipitate at 5 min. [f] Precipitate at 7 min.

applies to the reaction of the enzyme on Ala-Phe-NH_2 (Table IV). As shown previously for Gly-Tyr-Gly-Tyr-NH_2, the tetrapeptide amide is a substrate (Fruton and Knappenberger, 1962), and may undergo the process described by reaction 4.

$$E + XYXYa \rightleftharpoons E[XYXYa] \longrightarrow XY\text{-}E + XYa \quad (4)$$

Examination of the data for the formation of the octapeptide amide from Gly-Tyr-NH_2, or of the hexapeptide amide from Ala-Phe-NH_2, suggests that this process begins at the very start of the reaction, and that the proportion of dipeptidyl units going to the higher oligopeptide is greater than that going to the next lower member of the series. As will be seen from Table II, it is only when the concentration of Gly-Tyr-NH_2 decreases that the proportion of Gly-Tyr units going to hexapeptide amide exceeds that going to octapeptide amide. This finding makes it unlikely that the hexapeptide amide is a direct intermediate in the formation of the octapeptide amide, and suggests instead that the higher oligopeptide amides are formed by a chain-terminating reaction of a peptidyl enzyme with dipeptide amide (reactions 5 and 6).

$$XYXY\text{-}E + XYa \longrightarrow XYXYXYa + E \quad (5)$$

$$XYXYXY\text{-}E + XYa \longrightarrow XYXYXYXYa + E \quad (6)$$

Reactions 3, 5, and 6 are all examples of a general transfer reaction in which a peptidyl enzyme reacts with the amino group of the substrate dipeptide amide (reaction 7).

$$(XY)_n\text{-}E + XYa \longrightarrow (XY)_{n+1}a + E \quad (7)$$

Under suitable conditions, these oligopeptide amides are susceptible to attack by free enzyme and removal of the amino-terminal dipeptide unit. Reaction 4, according to this view, is a special case of the general reaction 8.

$$E + (XY)_na \rightleftharpoons E[(XY)_na] \longrightarrow XY\text{-}E + (XY)_{n-1}a \quad (8)$$

The finding that the partition of dipeptidyl units between free dipeptide plus tetrapeptide amide, on the one hand, and oligopeptide amide, on the other, is dependent upon enzyme concentration (Tables III and V) raises the question whether the chain-propagation reaction involves the cooperative interaction of separate enzyme molecules bearing dipeptidyl units, possibly according to the mechanism suggested in reactions 9 and 10 (or 10a).

$$XY\text{-}E_1 + XY\text{-}E_2 \longrightarrow XYXY\text{-}E_2 + E_1 \quad (9)$$

$$XYXY\text{-}E_2 + XY\text{-}E_3 \longrightarrow XYXYXY\text{-}E_2 + E_3 \quad (10)$$

$$XYXY\text{-}E_2 + XY\text{-}E_3 \longrightarrow XYXYXY\text{-}E_3 + E_2 \quad (10a)$$

In view of the finding that dipeptidyl transferase is an oligomeric enzyme (Metrione *et al.*, 1966), the possibility presents itself that the E_1, E_2, E_3, etc., of reactions 9 and 10 may represent subunits of such an oligomeric protein. It is hoped that this question may be answered through studies on the relation of the catalytic activity of dipeptidyl transferase to its state of aggregation at the enzyme concentrations used in the present studies. A preliminary indication in support of the above conclusion is offered by the data in Table VI on the effect of urea on the partition of Ala-Phe units, but these data cannot be taken as decisive. The mechanism of polymerization proposed above bears a formal analogy to that postulated for the chain-propagation reaction in the polymerization of the aminoacyl units of aminoacyl-tRNA in the presence of suitable oligonucleotides and ribosomes (Moldave, 1965).

Mention should be made of the possibility that the electrophoretic fractions termed tetrapeptide amide, hexapeptide amide, or octapeptide amide may include unknown proportions of the corresponding free oligopeptides, whose electrophoretic mobility in 20% acetic acid is similar to that of the corresponding amides. Although the presence of small amounts of such free peptides cannot be excluded, it should be noted that they would arise by the reaction of an activated intermediate with free dipeptide (Gly-Tyr or Ala-Phe).

As will be noted from the data presented above, relatively high concentrations of these free dipeptides are only attained under conditions of low initial substrate concentration. An alternative mode of formation of free peptides larger than XYOH, through hydrolytic cleavage of $(XY)_n$-E, also may be considered a possibility. Earlier analytical data on the composition of the insoluble products derived from Gly-Tyr-NH_2 or Ala-Phe-NH_2, however, showed them to be oligopeptide amides (Nilsson and Fruton, 1964), and it is probable also that the soluble tetrapeptide and hexapeptide fractions represent largely the amides, rather than the free peptides.

The availability of the quantitative data presented in this communication invites efforts to formulate mathematical expressions based on the acyl-enzyme mechanism offered above. The large number of competing and consecutive reactions, at least under the conditions of the present study, make such a kinetic analysis extremely complex. It is hoped that future work will permit simplification of the problem and an approach to the mathematical formulation of the kinetics of the enzyme-catalyzed polymerization effected by dipeptidyl transferase. Such an analysis may have relevance to other enzymic polymerization reactions.

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