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Kinetic Investigation of the α-Chymotrypsin-Catalyzed Hydrolysis of Peptide Substrates

The Relationship between Peptide-Structure N-Terminal to the Cleaved Bond and Reactivity

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A number of peptide substrates of the general structure Ac-L_{xn}- . . . -L_{x2}-L_{x1}-Gly-NH_2 have been synthesized and their α-chymotrypsin-catalyzed hydrolyses studied. The acylation rate constants, k_{23} (= k_{cat}), and the dissociation constants of the enzyme-substrate complexes, K_{EA} (= K_m), have been determined using a modified pH-stat and a numerical method for the acquisition and processing of the data. On the basis of these constants a quantitative relationship between the peptide structure N-terminal to the cleaved bond and reactivity has been determined. The results are shown to be consistent with the enzyme-substrate interaction scheme proposed in 1971 by Segal *et al.* (*Biochemistry 10*, 3728). A comparison of the k_{23}/K_{EA} values indicates that the influence of a single structural change on the overall reactivity is virtually independent of the nature of the remainder of the substrate. In addition a comparison of the k_{23} and K_{EA} values shows that, in general, changes in substrate structure are mainly reflected by changes in k_{23} rather than in K_{EA}. A few exceptions have been found: K_{EA} or K_{EA} and k_{23} change when glycine is introduced at the $x2$ position, when this glycine is replaced by alanine or when alanine is introduced at the $x3$ position.

The observation that α-chymotrypsin has a marked side-chain specificity [1], in that it preferentially catalyzes the hydrolysis of peptide bonds C-terminal to aromatic amino-acid residues, led to the conclusion that the interactions between a single specific amino-acid residue and the active site of the enzyme are of primary importance in determining which peptide bond is to be cleaved. These interactions, which occur within what can be termed the primary interaction range, have therefore been investigated kinetically in detail using derivatives of single amino acids, mainly *N*-acylated esters, as model substrates [2–4]. The results of such studies have established, on a quantitative basis, the role of the large hydrophobic side chain [5] and the amido NH group [5,6] of the substrate in the stabilization of the enzyme-substrate complex and in the orientation of the substrate in the active site. The elucidation of the three-dimensional structure of α-chymotrypsin by X-ray diffraction methods [7] has allowed the information obtained by kinetic methods to be rationalized in structural terms.

Abbreviations. NMR, nuclear magnetic resonance. IUPAC/IUB rules for peptides are followed, see *Eur. J. Biochem.* (1972) *27*, 201–207.

Enzyme. α-Chymotrypsin (EC 3.4.4.5).

In the case of protein [8] and peptide [9] substrates, it appears that additional residues on both sides of the specific amino acid also interact with the active site. The importance of such additional interactions, which occur in the secondary interaction range, may be investigated by extending the kinetic studies to suitable peptide substrates. In order to obtain kinetic data with peptides which is as accurate as that for ester substrates, it is necessary that only one peptide bond is hydrolyzed. For this purpose substrates of the general structure

$$E_x\text{-}L_{xn}\text{-}\ldots\text{-}L_{x2}\text{-}L_{x1}\overset{\downarrow}{}\text{-}L_{y1}\text{-}L_{y2}\text{-}\ldots L_{ym}\text{-}E_y$$

have been synthesized, where only L_{x1} is a specific amino acid residue. Thus it is expected that only the peptide bond between L_{x1} and L_{y1} (indicated by the arrow) is cleaved during the measurements. In designing these substrates, it is important to select end groups E_x and E_y which do not give rise to disturbing interactions with the enzyme.

The pH-stat titration offers a simple method of analysis for ester hydrolysis. However, this method is technically more difficult to apply to peptide hydrolysis [10], since the formation of amine product

during the reaction leads to buffering capacities which affect the functioning of the pH-control unit of the titrator. This difficulty can be overcome by suitably modifying the commercially available instrument.

The work presented in this paper deals with the steady-state kinetic analysis of the α-chymotrypsin-catalyzed hydrolysis of the tetrapeptide Ac-Ala-Ala-Tyr-Gly-NH_2 and of the tripeptides Ac-L_{x2}-L_{x1}-Gly-NH_2, in which L_{x1} is tyrosine or phenylalanine and L_{x2} is glycine, alanine, valine, or proline. The dipeptide substrates Ac-Phe-Gly-NH_2 and Ac-Tyr-Gly-NH_2, whose kinetic analysis was previously reported [11], are included for comparison.

EXPERIMENTAL PROCEDURE

SUBSTRATES

General Remarks

Melting points were measured in open capillary tubes and are uncorrected. Optical rotations were measured with a Zeiss photoelectric polarimeter in a 1-dm cell and are estimated to be correct to ± 1 °. Thin-layer chromatograms were run on silica gel HF_{254} (Merck, Darmstadt, Germany) with the following solvents: (A, B, C) chloroform—methanol (A) (9:1, v/v), (B) (4:1) and (C) (2:1), and (D) carbon tetrachloride—1-butanol—water (7:2:1, v/v/v).

NMR spectra were recorded using a Varian A-60 or H-100 spectrometer and showed the expected signals in all cases; this data is available on request. Samples for elemental analysis were dried at room temperature and 0.001 Torr for 24—48 h. Most solvents, reagents, and amino acid derivatives were purchased from Fluka AG (Buchs, Switzerland). 1,2-Dimethoxyethane was dried by distillation from sodium hydride. Hydrogen-chloride-saturated 1,2-dimethoxyethane and ammonia-saturated methanol were prepared by bubbling dry gas through stirred anhydrous solvent at 0 °C and subsequent equilibration to 25 °C. *N*-Acetoxysuccinimide, m.p. 129—130 °C (Lit. [12] m.p. 131—132 °C), was prepared by reacting *N*-hydroxysuccinimide with acetic anhydride in acetone and crystallizing from ethanol. 1-Acetoxybenzotriazole, m.p. 93—95 °C (Lit. [13] m.p. 98 °C) was prepared by acetylating 1-hydroxybenzotriazole, m.p. 155.5—157 °C (*cf.* [14]; Lit. [15] m.p. 157 °C) with acetic anhydride in ethyl methyl ketone and crystallizing from benzene—cyclohexane.

Standard Preparative Procedures

(A) *Coupling with* N-*Hydroxysuccinimide Esters of* t-*Butyloxycarbonyl Amino Acids.* The hydrochloride of the amino acid or peptide ethyl ester (1 equiv.) and triethylamine or *N*-methylmorpholine (1 equiv.) were dissolved in 1,2-dimethoxyethane—water (10:1, v/v, 5 ml/mmol). The *N*-hydroxysuccinimide ester of *t*-butyloxycarbonyl amino acid (1 equiv.) was then added and the solution stirred at room temperature for 2—4 h. The reaction mixture was reduced to one-half volume, diluted with water, and extracted several times with ethyl acetate. The combined ethyl acetate phases were washed with 1 N hydrochloric acid, 1 M sodium hydrogen carbonate, and water, dried over sodium sulphate and evaporated to dryness under reduced pressure at a temperature not exceeding 40 °C.

(B) *Removal of the* t-*Butyloxycarbonyl Amino-Protecting Group.* The solution or suspension of *t*-butyloxycarbonyl peptide ethyl ester (1 equiv.) in anhydrous 1,2-dimethoxyethane (2—3 ml/mmol) was treated with saturated anhydrous hydrogen chloride in 1,2-dimethoxyethane (15—25 equiv.) at 0 °C and stirred at room temperature for 1 h. The solvent was evaporated under reduced pressure, and the residue freed of hydrogen chloride by several cycles of addition and evaporation of anhydrous 1,2-dimethoxyethane and by drying at 0.001 Torr over potassium hydroxide.

(C) *Removal of the Benzyloxycarbonyl Amino-Protecting Group.* The benzyloxycarbonyl peptide ethyl ester (1 equiv.) was dissolved in anhydrous ethanol (10 ml/mmol) containing dry hydrogen chloride (4—5 equiv.) and hydrogenated at normal pressure over palladium on charcoal (10% Pd, 250 mg/mmol) for 5 h. The catalyst was removed by filtration and the filtrate evaporated to dryness. The residue was freed of hydrogen chloride by addition and evaporation of anhydrous ethanol and by drying at 0.001 Torr over potassium hydroxide.

(D) N-*Acetylation with* N-*Acetoxysuccinimide.* The hydrochloride of the peptide ethyl ester (1 equiv.) was dissolved in water (1 ml/mmol) and neutralized with triethylamine or *N*-methylmorpholine (1 equiv.). After dilution with 5—10 volumes of 1,2-dimethoxyethane, *N*-acetoxysuccinimide (1 equiv.) was added. The reaction mixture was stirred at room temperature for 4—6 h and worked up as described in procedure (A).

(E) *Ammonolysis of the C-Terminal Ester Group.* A solution of the acetyl peptide ethyl ester (1 equiv.) in a small volume of anhydrous methanol was treated with saturated ammonia in methanol (40—50 equiv.) at 0 °C and set aside at room temperature for 1—10 days. The solvent was evaporated and the residue dried at 0.001 Torr.

Synthesis of Ac-Tyr-Gly-NH_2(I)

Z-Tyr-Gly-OEt (X). To a stirred solution of Z-Tyr-OH (18.92 g, 60.0 mmol), H-Gly-OEt · HCl (8.50 g, 60.8 mmol), triethylamine (6.13 g, 60.5 mmol) and *N*-hydroxysuccinimide (7.03 g, 61.0 mmol

in dioxane—water (1:1, v/v) 120 ml, a solution of dicyclohexylcarbodiimide (12.28 g, 60.0 mmol) in dioxane (60 ml) was added at 0 °C. The reaction mixture was stirred at 5 °C for 15 h. The crystalline dicyclohexyl urea formed was removed by filtration and the filtrate reduced to half its volume, diluted with ethanol (1 volume) and ethyl acetate (20 volumes), extracted with 1 N HCl, 1 M $NaHCO_3$, and water, dried over Na_2SO_4 and evaporated to dryness. The residue was crystallized from methanol: yield, 18.14 g (76%), m.p. 173.5—174.5 °C, R_F 0.56 (B), $[\alpha]_D^{25} = -8.9°$ ($c = 1.0$, dioxane). $C_{21}H_{24}N_2O_6$: calc. C 62.99, H 6.04, N 7.00; found C 62.96, H 6.08, N 7.08.

Ac-Tyr-Gly-OEt (XI). The dipeptide ethyl ester hydrochloride obtained from X (8.61 g, 21.5 mmol) by hydrogenolysis (procedure C) was acetylated with *N*-acetoxysuccinimide (3.30 g, 21.0 mmol) in the presence of triethylamine (2.14 g, 21.1 mmol) under standard conditions (procedure D). The crude product was crystallized from water: yield, 3.60 g (56%), m.p. 134—136. 5°, R_F 0.49 (B), $[\alpha]_D^{27} = +6.0°$ ($c = 1.0$, 96% ethanol). $C_{15}H_{20}N_2O_5$: calc. C 58.43, H 6.54, N 9.09; found C 58.30, H 6.50, N 9.08. The literature [16] records m.p. 133—135 °C for a product obtained by the azide method.

Ac-Tyr-Gly-NH_2 (I). The dipeptide ethyl ester XI (1.213 g, 3.93 mmol) was treated with saturated ammonia in methanol as described in procedure E. The product crystallized on scratching after the reaction mixture had been concentrated to one-half volume. Yield, 957 mg (87%), m.p. 225.5—226.5 °C, R_F 0.17 (B), $[\alpha]_D^{23} = +33.5°$ ($c = 4.0$, acetone—water 1:1). $C_{13}H_{17}N_3O_4$: calc. C 55.90, H 6.14, N 15.05; found C 55.81, H 6.19, N 15.15. The literature [16] records m.p. 225—226 °C, $[\alpha]_D^{24} = +35.0°$ ($c = 4$, acetone—water 1:1).

Synthesis of Ac-Gly-Tyr-Gly-NH_2 (II)

Boc-Gly-Tyr-Gly-OEt (XII). The dipeptide ethyl ester hydrochloride obtained from X (4.73 g, 11.8 mmol) by hydrogenolysis (procedure C) was coupled with Boc-Gly-ONSu (3.13 g, 11.5 mmol) in the presence of triethylamine (1.17 g, 11.5 mmol) under standard conditions (procedure A). The chromatographically pure product, R_F 0.66 (C), was not crystallized. Yield, 4.66 g (96%).

Ac-Gly-Tyr-Gly-OEt (XIII). The tripeptide ethyl ester hydrochloride prepared from XII (4.62 g, 10.9 mmol) by procedure B was neutralized with triethylamine (1.09 g, 10.8 mmol) and acetylated with *N*-acetoxysuccinimide (1.67 g, 10.6 mmol) as described in procedure D. The crude product was crystallized from acetone—ethyl acetate and from acetone: yield, 1.25 g (32%), m.p. 152.5—154.5 °C, R_F 0.40 (B), $[\alpha]_D^{23} = -8.0°$ ($c = 1.0$, 96% ethanol). $C_{17}H_{23}N_3O_6$: calc. C 55.88, H 6.35, N 11.50; found C 55.94, H 6.26, N 11.35.

Ac-Gly-Tyr-Gly-NH_2 (II). The crude product obtained by ammonolysis (procedure E) from XIII (744 mg, 2.04 mmol) was crystallized from 2-propanol and from 96% ethanol to afford 633 mg (88%) of the monohydrate, m.p. 139—140 °C, R_F 0.39 (C), $[\alpha]_D^{26} = -15.0°$ ($c = 1.0$, dimethylformamide). $C_{15}H_{20}N_4O_5 \cdot H_2O$: calc. C 50.84, H 6.26, N 15.81; found C 50.71, H 6.29, N 15.96.

Synthesis of Ac-Ala-Tyr-Gly-NH_2 (III)

Boc-Ala-Tyr-Gly-OEt (XIV). The dipeptide ethyl ester hydrochloride obtained from X (5.69 g, 14.2 mmol) by hydrogenolysis (procedure C) was neutralized with triethylamine (1.43 g, 14.1 mmol) and coupled with Boc-Ala-ONSu (4.05 g, 14.1 mmol) under standard conditions (procedure A). The product was crystallized from ethanol—ethyl acetate and from ethyl methyl ketone; yield, 5.35 g (86%), m.p. 168.5—170.5 °C, $[\alpha]_D^{24} = -38°$ ($c = 1.0$, 96% ethanol). $C_{21}H_{31}N_3O_7$: calc. C 57.65, H 7.12, N 9.61; found C 57.64, H 7.19, N 9.71.

Ac-Ala-Tyr-Gly-OEt (XV). The tripeptide ethyl ester hydrochloride prepared from XIV (3.14 g, 7.19 mmol) by procedure B was dissolved in water (5 ml) and neutralized with solid sodium hydrogen carbonate (604 mg, 7.19 mmol). The solution was diluted with 1,2-dimethoxyethane (35 ml) and *N*-acetoxysuccinimide (1129 mg, 7.19 mmol) was added. The reaction mixture was stirred at room temperature for 5 h, concentrated to one-half volume, and worked up as described in procedure D. The residue was crystallized from water: yield, 1.94 g (62%) of material which lost water at 81—82 °C and melted at 177—178 °C, R_F 0.65 (B), $[\alpha]_D^{24} = -47°$ ($c = 1.0$, 96% ethanol). Elemental analysis indicated the presence of 3 mol water. $C_{18}H_{25}N_3O_6 \cdot 3\,H_2O$: calc. C 49.87, H 7.21, N 9.70; found C 49.76, H 7.06, N 9.70.

Ac-Ala-Tyr-Gly-NH_2 (III). The tripeptide ethyl ester XV (1.55 g, 4.08 mmol) was treated with saturated ammonia in methanol according to procedure E. The crude product was crystallized from methanol—96% ethanol—chloroform: yield, 1335 mg (84%), m.p. 199—200 °C, R_F 0.15 (B), $[\alpha]_D^{24} = -62°$ ($c = 1.0$, 96% ethanol). Elemental analysis and NMR spectrum indicated the presence of 1/3 mol chloroform. $C_{16}H_{22}N_4O_5 \cdot 1/3\ CHCl_3$: calc. C 50.27, H 5.77, N 14.36; found C 50.31, H 5.79, N 14.54.

Synthesis of Ac-Val-Tyr-Gly-NH_2 (IV)

Boc-Val-Tyr-Gly-OEt (XVI). The protected dipeptide X (4.56 g, 11.4 mmol) was hydrogenolyzed according to procedure C; the dipeptide ethyl ester hydrochloride thus obtained was dissolved in water (12 ml) and neutralized with *N*-methylmorpholine

(1.03 g, 10.2 mmol). After dilution with 1,2-dimethoxyethane (60 ml) Boc-Val-ONSu (3.14 g, 10.0 mmol) was added. The reaction mixture was stirred at room temperature for 5 h, concentrated to about 20 ml, and diluted with water (30 ml). The crystalline product was collected and recrystallized from ethanol-water; yield, 2.67 g (57%), m.p. 206.5—208.5 °C, R_F 0.57 (B), $[\alpha]_D^{25} = -30°$ ($c = 1.0$, dioxane). $C_{23}H_{35}N_3O_7$: calc. C 59.34, H 7.58, N 9.03; found C 59.40, H 7.57, N 9.11.

Ac-Val-Tyr-Gly-OEt (XVII). The tripeptide ethyl ester hydrochloride prepared from XVI (2.41 g, 5.18 mmol) by procedure B was acetylated with *N*-acetoxysuccinimide (794 mg, 5.05 mmol) in the presence of *N*-methylmorpholine (515 mg, 5.09 mmol) under standard conditions (procedure D). The product was recrystallized twice from ethanol—water: yield, 1.70 g (82%), m.p. 231—232°C, R_F 0.50 (B), $[\alpha]_D^{25} = -46°$ ($c = 1.0$, 96% ethanol). $C_{20}H_{29}N_3O_6$: calc. C 58.95, H 7.17, N 10.31; found C 59.05, H 7.18, N 10.33.

Ac-Val-Tyr-Gly-NH$_2$ (IV). The tripeptide ethyl ester XVII (765 mg, 1.88 mmol) was dissolved in formamide (2.5 ml) and methanol (4.5 ml) and treated with 8 N ammonia in methanol (12.0 ml). After standing at room temperature for 30 h, the solution was concentrated to about 5 ml and diluted with ethylmethyl ketone. The crystalline product was collected and recrystallized from methanol: yield, 497 mg (70%), m.p. 242—243 °C, R_F 0.20 (B), $[\alpha]_D^{22} = -40°$ ($c = 1.0$, water). $C_{18}H_{26}N_4O_5$: calc. C 57.13, H 6.93, N 14.81; found C 56.96, H 6.93, N 14.57.

Synthesis of Ac-Pro-Tyr-Gly-NH$_2$ (V)

Boc-Pro-Tyr-Gly-OEt (XVIII). The dipeptide ethyl ester hydrochloride obtained from X (4.44 g, 11.1 mmol) by hydrogenolysis according to procedure C was coupled with Boc-Pro-ONSu (3.30 g, 10.6 mmol) in the presence of *N*-methylmorpholine (1.07 g, 10.6 mmol) under standard conditions (procedure A) to afford 4.00 g (82%) of crude product which could not be crystallized.

Ac-Pro-Tyr-Gly-OEt (XIX). The tripeptide ethyl ester hydrochloride prepared from XVIII (4.00 g, 8.64 mmol) by procedure B was dissolved in water (10 ml) and neutralized with *N*-methylmorpholine (784 mg, 7.74 mmol). After dilution with 1,2-dimethoxyethane (70 ml) 1-acetoxybenzotriazole (1.37 g, 7.73 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and worked up as described in procedure D. The residue was recrystallized from ethanol-water and twice from wet ethyl methyl ketone to afford 836 mg (26%) of the monohydrate, m.p. 186.5—187.5 °C, R_F 0.49 (B), $[\alpha]_D^{20} = -66°$ ($c = 1.0$, 96% ethanol). $C_{20}H_{27}N_3O_6 \cdot H_2O$: calc. C 56.72, H 6.90, N 9.92; found C 56.64, H 6.87, N 9.82.

Ac-Pro-Tyr-Gly-NH$_2$ (V). The tripeptide ethyl ester XIX (680 mg, 1.68 mmol) was treated with ammonia in methanol according to procedure E. The crude product was crystallized from ethanol and twice from methanol—2-propanol. Yield, 449 mg (71%), m.p. 224—225 °C, R_F 0.18 (B), $[\alpha]_D^{22} = -75°$ ($c = 1.0$, 96% ethanol). $C_{18}H_{24}N_4O_5$: calc. C 57.43, H 6.43, N 14.89; found C 57.33, H 6.45, N 14.71.

Synthesis of Ac-Phe-Gly-NH$_2$ (VI)

Boc-Phe-Gly-OEt (XXII). H-Gly-OEt · HCl (1.40 g, 10.0 mmol) was coupled with Boc-Phe-ONSu (3.21 g, 8.9 mmol) in the presence of triethylamine (1.01 g, 10.0 mmol) according to procedure A. The chromatographically homogeneous crude product, R_F 0.64 (B), was used without further purification for the next reaction; yield, 3.07 g (99%). A sample was recrystallized from benzene—hexane to afford material of m.p. 87—88 °C, $[\alpha]_D^{25} = -4.3°$ ($c = 2.0$, 96% ethanol), $C_{18}H_{26}N_2O_5$: calc. C 61.70, H 7.48, N 8.00; found C 61.90, H 7.53, N 7.95. The literature [17] records m.p. 89.5—90 °C, $[\alpha]_D^{25} = -4.3°$ ($c = 2$, ethanol).

Ac-Phe-Gly-OEt (XXIII). The dipeptide ethyl ester hydrochloride obtained from XXII (2.38 g, 6.80 mmol) by procedure B was neutralized with triethylamine (688 mg, 6.80 mmol) and acetylated with *N*-acetoxysuccinimide (1.068 g, 6.80 mmol) under standard conditions (procedure D). The crude product was recrystallized twice from water: yield, 899 mg (45%), m.p. 133—135 °C, R_F 0.51 (B), $[\alpha]_D^{25} = -3.1°$ ($c = 1.0$, 96% ethanol). $C_{15}H_{20}N_2O_4$: calc. C 61.63, H 6.90, N 9.58; found C 61.50, H 6.83, N 9.52.

Ac-Phe-Gly-NH$_2$ (VI). The dipeptide ethyl ester XXIII (751 mg, 2.57 mmol) was treated with saturated ammonia in methanol according to procedure E. The crude product was crystallized from ethanol-ethyl methyl ketone. Yield, 574 mg (85%), m.p. 183—184 °C, R_F 0.32 (B), $[\alpha]_D^{25} = +32.0°$ ($c = 1.0$, 96% ethanol). $C_{13}H_{17}N_3O_3$: calc. C 59.30, H 6.51, N 15.96; found C 59.09, H 6.56, N 16.19.

Synthesis of Ac-Ala-Phe-Gly-NH$_2$ (VII)[1]

Boc-Ala-Phe-Gly-OEt (XXIV). The dipeptide ethyl ester hydrochloride obtained from XXII (4.70 g 13.4 mmol) by procedure B was coupled with Boc-Ala-ONSu (3.70 g, 12.9 mmol) in the presence of triethylamine (1.30 g, 12.9 mmol) under standard conditions (procedure A). The crude product was crystallized from ethyl acetate—hexane: yield, 4.11 g (80%), m.p. 151—152 °C, R_F 0.62 (D), $[\alpha]_D^{25} = -41.5°$ ($c = 1.0$, 96% ethanol).

[1] The syntheses of Ac-Ala-Phe-Gly-NH$_2$ and Ac-Pro-Phe-Gly-NH$_2$ were performed by H. R. Brunner.

Ac-Ala-Phe-Gly-OEt (XXV). The tripeptide ethyl ester hydrochloride prepared from XXIV (3.05 g, 7.23 mmol) according to procedure B was neutralized with triethylamine (715 mg, 7.10 mmol) and acetylated with *N*-acetoxysuccinimide (1.11 g, 7.07 mmol) under standard conditions (procedure D). The chromatographically homogeneous crude product, R_F 0.59 (C), was used without further purification for the next reaction; yield, 2.15 g (84%). A sample was crystallized from ethanol—ethyl acetate to afford material of m.p. 198—199 °C, $[\alpha]_D^{24} = -60°$ ($c = 1.0$, 96% ethanol).

Ac-Ala-Phe-Gly-NH$_2$ (VII). The crude product obtained by ammonolysis of XXV (1.72 g, 4.75 mmol) according to procedure E was crystallized twice from 96% ethanol; yield, 1.03 g (66%), m.p. 211—212 °C, R_F 0.50 (C), $[\alpha]_D^{24} = -75°$ ($c = 0.56$, 96% ethanol). $C_{16}H_{22}N_4O_4$: calc. C 56.47, H 6.63, N 16.74; found C 56.44, H 6.71, N 16.47.

Synthesis of Ac-Pro-Phe-Gly-NH$_2$ (VIII)[1]

Boc-Pro-Phe-Gly-OEt (XXVI). The dipeptide ethyl ester hydrochloride prepared from XXII (3.80 g, 10.85 mmol) by procedure B was coupled with Boc-Pro-ONSu (3.30 g, 10.55 mmol) in the presence of triethylamine (1.08 g, 10.69 mmol) under standard conditions (procedure A). The crude product was crystallized from ethyl acetate—hexane: yield, 3.10 g (66%), m.p. 108—109 °C, R_F 0.63 (D), $[\alpha]_D^{25} = -69°$ ($c = 1.0$, 96% ethanol).

Ac-Pro-Phe-Gly-OEt (XXVII). The tripeptide ethyl ester hydrochloride obtained from XXVI (2.30 g, 5.14 mmol) by procedure B was neutralized with triethylamine (515 mg, 5.10 mmol) and acetylated with 1-acetoxybenzotriazole (895 mg, 5.05 mmol) as described for Ac-Pro-Tyr-Gly-OEt (XIX). The product could not be crystallized; yield, 922 mg (47%), R_F 0.62 (C).

Ac-Pro-Phe-Gly-NH$_2$ (VIII). The tripeptide ethyl ester XXVII (798 mg, 2.05 mmol) was treated with ammonia in methanol according to procedure E. The crude product was purified chromatographically on a silica-gel column by elution with chloroform—methanol (2:1, v/v). The fractions containing pure product were combined. Recrystallization from methanol gave 575 mg (78%) of material, m.p. 203—205 °C, R_F 0.51 (B), $[\alpha]_D^{24} = -94°$ ($c = 0.54$, 96% ethanol). $C_{18}H_{24}N_4O_4$: calc. C 59.98, H 6.71, N 15.55; found C 59.94, H 6.74, N 15.47.

Synthesis of Ac-Ala-Ala-Tyr-Gly-NH$_2$ (IX)

Boc-Ala-Ala-Tyr-Gly-OEt (XX). The tripeptide ethyl ester hydrochloride prepared from XIV (3.50 g, 8.00 mmol) by procedure B was coupled with Boc-Ala-ONSu (2.18 g, 7.61 mmol) in the presence of *N*-methylmorpholine (800 mg, 7.91 mmol) under standard conditions (procedure A). The crude product was crystallized from 2-propanol: yield, 3.51 g (91%), m.p. 202—204 °C, R_F 0.79 (A), $[\alpha]_D^{23} = -23.5°$ ($c = 1.0$, dimethylformamide). $C_{24}H_{36}N_4O_8$: calc. C 56.68, H 7.14, N 11.02; found C 56.62, H 7.20, N 11.06.

Ac-Ala-Ala-Tyr-Gly-OEt (XXI). The tetrapeptide ethyl ester hydrochloride obtained from XX (2.85 g, 5.61 mmol) by procedure B was neutralized with *N*-methylmorpholine (557 mg, 5.51 mmol) and acetylated with *N*-acetoxysuccinimide (850 mg, 5.41 mmol) according to procedure D. The reaction mixture was concentrated to one-half volume and extracted with ethyl acetate—1-butanol (2:1, v/v). The organic phases were washed, dried and evaporated to dryness, and the residue was crystallized from methanol. Yield, 1.68 g (69%), m.p. 246.5—248 °C, R_F 0.36 (B), $[\alpha]_D^{24} = -29°$ ($c = 1.0$, dimethylformamide). $C_{21}H_{30}N_4O_7$: calc. C 55.99, H 6.71, N 12.44; found C 55.85, H 6.62, N 12.34.

Ac-Ala-Ala-Tyr-Gly-NH$_2$ (IX). A solution of XXI (1.37 g, 3.04 mmol) in formamide (2 ml) was diluted with methanol (8 ml), treated with 8 N ammonia in methanol (20.0 ml), and allowed to stand for 24 h at room temperature. The reaction mixture was evaporated to dryness and the residue recrystallized twice from methanol: yield, 891 mg (70%), m.p. 236.5—237.5 °C, R_F 0.27 (C), $[\alpha]_D^{26} = -59°$ ($c = 1.0$, methanol). $C_{19}H_{27}N_5O_6$: calc. C 54.14, H 6.46, N 16.62; found C 54.00, H 6.45, N 16.56.

APPARATUS AND METHODS

Apparatus

The course of peptide hydrolysis is followed by means of the data-collecting system described previously [18] except that the pH-stat has been modified so that a delayed time relay participates in the control of the addition of the titrating base solution (Fig. 1). This modification was made on the basis of the following considerations. In the standard pH-stat instrument, the titrator is equipped with a device which controls the base addition in such a way that in a small range close to the preset operational pH (proportional band) the mean velocity of base addition is proportional to the difference between the preset pH value and the actual pH of the reaction medium. Any change in reaction rate is therefore accompanied by a pH shift along the proportional band. The pH shift causes deviations in the registered progression curve which are negligible as long as the buffering capacity of the medium is low. However, when a buffering product is formed during the reaction, as in the case of peptide hydrolysis at pH 8, appreciable deviations occur. This has been verified by control experiments. The simplest way to avoid the disturbing effect would be to run the instrument with the proportional band switched off,

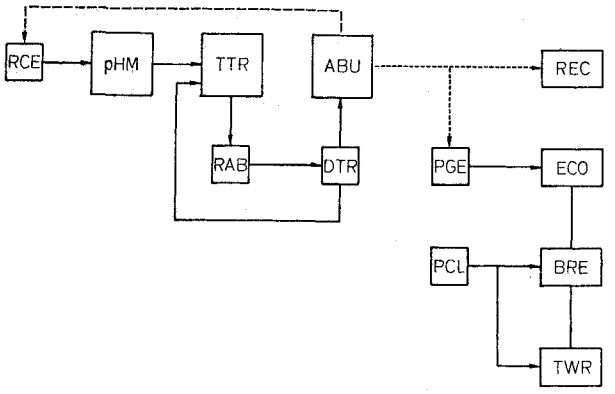


Fig. 1. *The main components of the modified pH-stat.* RCE = reaction cell with electrode, pHM = pH meter, TTR = titrator, ABU = automatic burette, RAB = relay of the latter, DTR = delayed time relay, REC = recorder, PGE = pulse generator, PCL = programmable clock, ECO = electronic counter, BRE = buffer register and TWR = teletype writer set. (——) Electrical connections, (----) mechanical connections, (— — —) base addition line

so that the base addition is controlled only by the comparator of the titrator. The resulting registered curve, although correct, would be in the form of steps which increase in size with increasing amount of buffering amine product P [19]. Such strongly stepped curves would still be of little value, particularly when the end point needs to be accurately determined. However, if the increase in the step size could be avoided, this would seem to be a reasonable approach.

The step size ΔN is related to the buffering capacity C by the equation

$$\Delta N = C\Delta \mathrm{pH_c} \qquad (1)$$

where $\Delta \mathrm{pH_c}$ represents the hysteresis amplitude characteristic of the comparator. The buffering capacity C is defined [19] by the equation

$$C = 2.3 \frac{10^{\mathrm{p}K_a(\mathrm{P}) - \mathrm{pH}}}{(1 + 10^{\mathrm{p}K_a(\mathrm{P}) - \mathrm{pH}})^2} \mathrm{P} \qquad (2)$$

where P is the amount of buffering product P and $\mathrm{p}K_a(\mathrm{P})$ its $\mathrm{p}K_a$. Thus when the pH equals $\mathrm{p}K_a(\mathrm{P})$ as is nearly the case for our measurements, the buffering capacity C is equal to $0.575 \times \mathrm{P}$, which represents the highest value to be reached.

It is evident from Eqn (1) that the pH-stat must be modified in such a way that the step size is no longer dependent on the buffer capacity and the hysteresis amplitude. The modification consists of the introduction of a delayed time relay which participates in the control of base addition. This is achieved by disconnecting the relay of the automatic burette from the drive unit and using it to actuate the delayed time relay. The latter operates two parallel switches, one of which now controls the drive unit, and the other clamps the emittor of the input transistor of the comparator to its base *via* a 5-kΩ resistor. Thus at the comparator input a voltage is generated which changes the functional state of the comparator by overcoming its threshold and hysteresis voltage.

The modified pH-stat operates in the following way (Fig. 2A). As long as the pH of the reaction mixture is higher than the preset operational pH, $\mathrm{pH_c}$, the comparator remains in state 0. When the pH falls below $\mathrm{pH_c}$, the state of the comparator changes from 0 to 1 (as in the non-modified system) and the delayed time relay is activated. During a preset period of time, Δt (0.2—2 s), when the delayed time relay is on, the drive unit of the burette is running and the comparator is reset to state 0. Thus base is added at constant amounts $\Delta N'$ determined by the rate at which the drive unit operates, the time interval Δt, and the concentration of the base solution. As a consequence of this mode of operation, the slowing down of the reaction is marked by increasing periods between successive base additions.

As the buffering capacity increases, the pH increment, $\Delta \mathrm{pH_i}$, resulting from the addition of fixed amounts of base, decreases. In order to achieve a regular sequence of base addition it is necessary that these increments do not become smaller than the inaccuracy $\Delta \mathrm{pH_c}'$ of the pH measurements (approx. 0.002 pH unit). Thus, for the part of the progression curve to be used for rate evaluation, $\Delta N'$ should be chosen such that the condition $\Delta \mathrm{pH_i} > \Delta \mathrm{pH_c}'$ is fulfilled. In the case (Fig. 2B) where the pH increment becomes smaller than this critical value ($\Delta \mathrm{pH_i} < \Delta \mathrm{pH_c}'$), for example when the buffering capacity is high, the burette will duplicate increments until $\Delta \mathrm{pH_c}'$ is exceeded.

Kinetic Analysis

The formation of carboxylic acid product Q is followed by measuring the amount of base required to maintain the solution at constant pH. The amount of base added, N, is expressed as a number of pulses, p, which are generated by the data-collecting system described previously [18]:

$$\mathrm{N} = fp\, c_{\mathrm{N}}$$

where c_{N} is the base concentration and f is the volume of base solution added per pulse. Since the amine product P is partially ionized at the pH where the measurements are made, the relationship between N and the amount of Q involves the $\mathrm{p}K_a$ of the product:

$$\mathrm{Q} = (1 + 10^{\mathrm{p}K_a(\mathrm{P}) - \mathrm{pH}})\,\mathrm{N}. \qquad (3)$$

The $\mathrm{p}K_a$ of the amine product (glycine amide for all substrates) was determined by titration under identical conditions to those in the kinetic studies and was found to be 8.07. Since this value is close

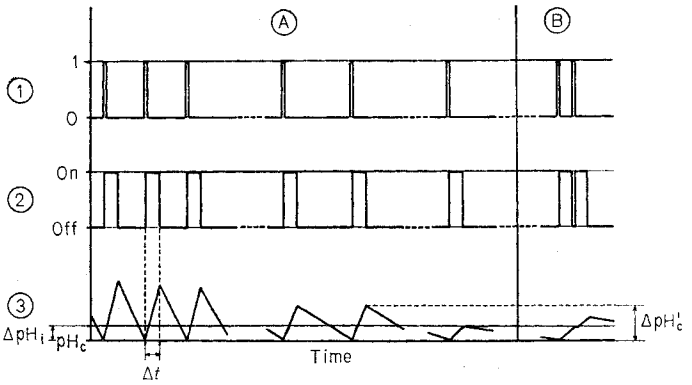


Fig. 2. *Functioning of the modified pH-stat.* The change occurring in the state of the comparator (①) during the reaction, and the corresponding changes in the operation of the burette (②) and in the pH of the reaction mixture (③) are indicated for $\Delta pH_i > \Delta pH_c'$ (Ⓐ) and for $\Delta pH_i < \Delta pH_c'$ (Ⓑ). Details are given in the text

to the operational pH, the N/Q ratio is approximately 0.5 which still allows accurate registration of the progression curve.

The data was processed as described previously [18]: the initial rates were determined numerically from Q values (data points) spaced at 5 to 10-s intervals along the progression curve. Second-order polynomials were fitted to the first 15—20 data points (corresponding to 10—20% of the complete progression curve) and the initial rates v_0 determined from the derivatives at zero time. The parameters k_{cat} and K_m of the Michaelis-Menten equation

$$v_0/[E] = \frac{k_{cat}}{1 + \frac{K_m}{[A]}} \qquad (4)$$

and their standard deviations were determined by iterative regression on Eqn (4) using first estimates of the parameters determined by linear regression on the reciprocal form (v_0 *vs* $v_0/[A]$) of Eqn (4).

Measurements

The concentration of α-chymotrypsin (three-times crystallized, salt-free preparation from Sigma, Lots 86B-0470 and 50C-2550) was determined by the *N*-cinnamoylimidazole method [20]. All reagents were of analytical grade, and the water was doubly distilled (quartz apparatus).

Peptide hydrolysis was carried out in a measuring cell containing 1 ml 5 mM potassium phosphate buffer of pH 7.9, 1 ml 2 M sodium chloride solution, 0.1—7.5 ml substrate solution in water, and water to a final volume of 9.5 ml. The base solution in the burette was 0.1—0.2 M sodium hydroxide. All solutions were pipetted using calibrated Hamilton syringes. These reaction mixtures devoid of enzyme and samples of the enzyme solution (stock solution stored at 4 °C) were thermostatted in a water bath at 25 °C. They were then placed in the titration apparatus and their pH adjusted manually to 7.90 by the addition of small amounts of base. The measurements at 25 °C were started by the addition of 0.5 ml of enzyme solution.

Table 1. *Stoichiometry of the reactions*

Substrate	Amount of substrate	
	subjected to hydrolysis	hydrolyzed
	μmol	μmol
Ac-Tyr-Gly-NH_2	49.0	48.5
Ac-Gly-Tyr-Gly-NH_2	101	98.4
Ac-Ala-Tyr-Gly-NH_2	236	224
Ac-Val-Tyr-Gly-NH_2	47.2	44.7
Ac-Pro-Tyr-Gly-NH_2	42.5	40.3
Ac-Phe-Gly-NH_2	75.2	75.7
Ac-Ala-Phe-Gly-NH_2	46.1	46.1
Ac-Pro-Phe-Gly-NH_2	39.9	40.7
Ac-Ala-Ala-Tyr-Gly-NH_2	45.5	45.3

RESULTS

The stoichiometry of the reactions (Table 1), determined by substituting the infinity readings of the amount of base added into Eqn (3), is consistent only with the hydrolysis of the single peptide bond between L_{x1} and L_{y1}. (For the peptides containing phenylalanine this was confirmed by full titration, up to pH 10.5, of the amine formed at the end of the reaction.) This, in addition to the fact that no biphasic reaction curve was observed, also confirms that the peptide substrates were optically pure, all the amino acid residues having the L-configuration.

For all substrates, the initial rates conformed to the Michaelis-Menten equation: the reciprocal plots for Ac-Ala-Tyr-Gly-NH_2 and Ac-Gly-Tyr-Gly-NH_2 are given in Fig. 3, as examples.

The kinetic constants k_{cat} and K_m calculated by the least-squares method from initial rates are

Table 2. *Steady-state kinetic constants for the peptide substrates measured in 0.2 M sodium chloride at pH 7.90 and 25 °C*

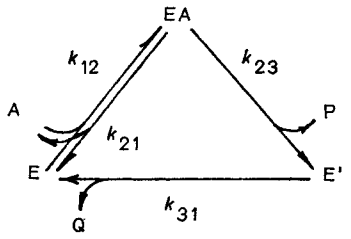
Substrate	Enzyme conc.	Range of initial substrate concn	k_{cat}	K_m	k_{cat}/K_m
	μM	mM	s^{-1}	mM	$s^{-1}\ M^{-1}$
Ac-Tyr-Gly-NH_2 [a]	22.0	9—37	0.64 ± 0.10	23.3 ± 2.8	27.5
Ac-Gly-Tyr-Gly-NH_2	18.7	3—16	0.48 ± 0.04	7.8 ± 0.5	61.5
Ac-Ala-Tyr-Gly-NH_2	15.0	4.8—57	1.41 ± 0.08	29.1 ± 1.2	48.4
Ac-Val-Tyr-Gly-NH_2	18.9	3.8—16.8	2.80 ± 0.15	14.7 ± 0.6	190
Ac-Pro-Tyr-Gly-NH_2	19.3	5.8—25.1	4.41 ± 0.14	31.5 ± 0.8	140
Ac-Phe-Gly-NH_2 [a]	38.0	4.9—31	0.140 ± 0.006	14.6 ± 0.3	9.6
Ac-Ala-Phe-Gly-NH_2	18.4	3.4—25.3	0.355 ± 0.024	23.2 ± 1.2	15.3
Ac-Pro-Phe-Gly-NH_2	18.3	2.3—14.6	0.765 ± 0.052	14.9 ± 0.7	51.3
Ac-Ala-Ala-Tyr-Gly-NH_2	1.43	1.2—7.6	10.3 ± 0.4	1.87 ± 0.05	5500

[a] Reported in a previous communication [11].

summarized in Table 2. The values obtained for Ac-Tyr-Gly-NH_2 are in good agreement with those reported by Foster and Niemann [21], determined by a different experimental technique (ninhydrin method).

DISCUSSION

It is generally accepted that the α-chymotrypsin-catalyzed hydrolysis of specific substrates in the steady-state may be appropriately described by the three-centre model:



Scheme 1

where EA represents the enzyme-substrate complex and E′ the acyl-enzyme intermediate. When initial rates are considered and no product is added the rate equation derived on the basis of this model has the form of a simple Michaelis-Menten equation. Assuming that the free enzyme and the enzyme-substrate complex are in rapid equilibrium ($k_{21} \gg k_{23}$), the steady-state kinetic constants k_{cat} and K_m are related to the rate constants of the single reaction steps by the following equations:

$$K_m = K_{EA}\frac{k'_{31}}{k_{23}+k'_{31}} \qquad k_{cat} = \frac{k_{23}k'_{31}}{k_{23}+k'_{31}}$$

where $K_{EA} = \frac{k_{21}}{k_{12}}$ and $k'_{31} = k_{31}[H_2O]$.

Since it is implicit in the three-centre model that peptide and peptide-ester substrates which have the same acyl moiety form identical acyl-enzyme intermediates, the deacylation rate constant k'_{31} for the peptides E_x-. . .-L_{x1}-Gly-NH_2 may be determined from measurements of the hydrolysis of the corresponding peptide esters E_x-. . .-L_{x1}-OMe. Such experiments, which will be reported in a later publication, showed that, for all the peptide substrates considered, k_{cat} is much smaller than k'_{31}; this indicates that acylation is rate-limiting ($k_{23} \ll k'_{31}$). Thus, the above expressions for K_m and k_{cat} become

$$K_m = K_{EA} \text{ and } k_{cat} = k_{23}.$$

The validity of these relationships has been demonstrated for the substrates Ac-Trp-NH_2 and Ac-Phe-NH_2 by stopped-flow measurements where the directly determined dissociation constant K_{EA} was shown to be equal to the steady-state constant

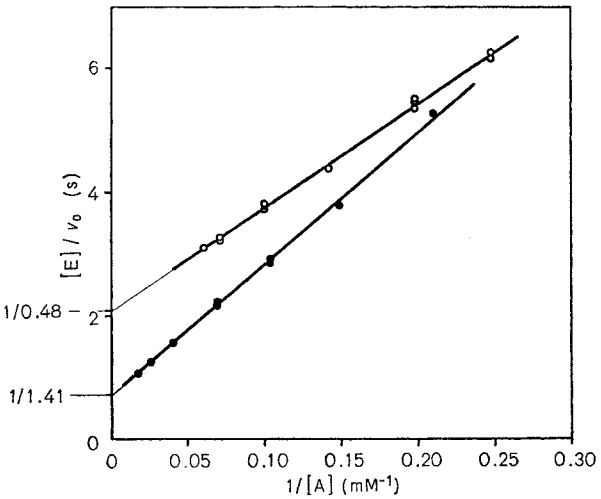


Fig. 3. *Kinetics of the hydrolysis of tripeptides.* Ac-Gly-Tyr-Gly-NH_2 (○) and Ac-Ala-Tyr-Gly-NH_2 (●) are given as examples

Table 3. *Relationship between the structural changes of the substrates, the presence or absence of a particular interaction, and the changes in the steady-state kinetic constants*
The occurrence of an interaction is denoted by + for $b(xn)$ interactions and by a letter (representative of the side chain involved) for $r(xn)$ interactions. For each of the substrate pairs considered the values given are the ratios of the lower to the upper values of the constants. The symbols for the interactions are defined in Fig. 4

Acyl of moiety the substrates	Interactions proposed on the basis of the interaction scheme					Ratios resulting from comparisons 1—7 of		
	$b_N(x3)$	$b_O(x3)$	$r(x2)$	$b_N(x1)$	$r(x1)$	k_{23}/K_{EA}	k_{23}	K_{EA}
						1 2 3 4 5 6 7	1 2 3 4 5 6 7	1 2 3 4 5 6 7
Ac-Phe-				+	*f*	2.9 2.2 0.8 3.2 1.6 1.8 3.3 3.9 2.7 2.9 114	4.6 0.8 2.9 4.0 2.5 2.2 2.1 2.0 5.8 3.1 7.3	1.6 0.3 3.7 1.3 1.6 1.2 0.6 0.5 2.1 1.1 0.06
Ac-Tyr-				+	*y*			
Ac-Gly-Tyr-		+		+	*y*			
Ac-Ala-Phe-		+	*a*	+	*f*			
Ac-Ala-Tyr-		+	*a*	+	*y*			
Ac-Val-Tyr-		+	*v*	+	*y*			
Ac-Pro-Phe-		+	*p*	+	*f*			
Ac-Pro-Tyr-		+	*p*	+	*y*			
Ac-Ala-Ala-Tyr-	+	+	*a*	+	*y*			

K_m [22]. For the peptide substrates therefore, the influences of structural changes in the substrate on the association-dissociation step and on the acylation step may be determined directly from measurements of K_m and k_{cat} respectively.

Since the reactivity of a substrate is a consequence of its spatial arrangement within the active site, structure-activity relationships may best be understood in terms of the interactions which determine this arrangement. Such an approach requires an interaction scheme which is valid for all the peptides considered. It has recently been proposed from crystallographic studies on the binding of peptidyl chloromethyl ketones to γ-chymotrypsin that the peptidyl moiety of the inhibitor forms an antiparallel β-structure with the Ser-214, Trp-215 and Gly-216 residues of the active site [23]. In discussing the reactivity of the peptide substrates it may be assumed that similar interactions also occur between the residues L_{x1}, L_{x2}, and L_{x3} of the substrate and the active site of α-chymotrypsin, as visualized in the interaction scheme given in Fig. 4. The ratio k_{23}/K_{EA}, which is indicative of overall reactivity, and the constants k_{23} and K_{EA} are then related to the nature and number of these interactions. A summary of the comparisons made for each of the substrates is presented in Table 3.

With reference to this table, it can be seen that the replacing of phenylalanine by tyrosine at the $x1$ position (comparison 1) leads to a three-fold increase in k_{23}/K_{EA} for all substrate pairs considered; a similar increase is observed for the model substrates Ac-Phe-NH_2 and Ac-Tyr-NH_2 [21]. On the basis of these observations it seems likely that a hydrogen bond is formed between the hydroxyl of tyrosine

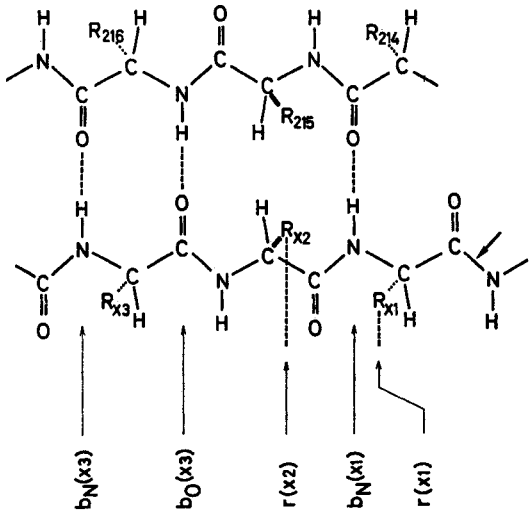


Fig. 4. *Interaction scheme.* The upper part represents the peptide chain of the enzyme active site and the lower part the peptide chain of the substrate. Two types of interactions are shown: the $b(xn)$ interactions which occur between the backbones, and the $r(xn)$ interactions which occur between the side chains. The positions at which these interactions are assumed to occur [23] are indicated by arrows

and a group of the active site, which strengthens the $r(x1)$ interaction.

Extending the peptide Ac-Tyr-Gly-NH_2 by a glycine residue on the x-side (comparison 2) results in a two-fold increase in k_{23}/K_{EA}. This would appear to be consistent with the formation of a $b_O(x3)$ hydrogen bond. However, introducing a side-chain methyl at the $x2$ position leads to no further increase in reactivity (comparison 3). This is in apparent contradiction to the scheme, which suggests that the interaction between the methyl and the hydrophobic side chain of Ile-99 [23], $r(x2)$, should further enhance the reactivity. A possible explanation for these effects is that the two-fold increase observed in comparison 2 results not from the formation of the $b_O(x3)$ hydrogen bond but from a non-specific interaction not considered in the interaction scheme. When a methyl is introduced at the $x2$ position the non-specific interaction is given up and the specific interaction $r(x2)$ is formed. Assuming the influence of these interactions on the overall reactivity to be comparable, then no increase in k_{23}/K_{EA} will be observed. This explanation is consistent with the changes in k_{23} and K_{EA} observed in these comparisons, as will be discussed below.

Since Ac-Gly-Tyr-Gly-NH_2 does not seem to behave according to the interaction scheme, the effect of introducing a side chain at the $x2$ position cannot be examined separately. Thus, the introduction of a whole amino acid residue has to be considered. Extending Ac-Tyr-Gly-NH_2 and Ac-Phe-Gly-NH_2 by an alanine residue (comparison 4) results in an increase in k_{23}/K_{EA} of two-fold in both cases. This reactivity increase can at most account for the formation of the $r(x2)$ interaction; the $b_O(x3)$ hydrogen bond seems not yet to be formed.

Replacing the alanine at the $x2$ position by valine and proline (comparisons 5 and 6) leads to increases in k_{23}/K_{EA} of four- and three-fold respectively. This is in accordance with the interaction scheme since an increase in the size of the hydrophobic side chain should result in a strengthening of the $r(x2)$ interaction with a corresponding increase in overall reactivity. Extending the peptide chain of Ac-Ala-Tyr-Gly-NH_2 by an alanine residue (comparison 7) leads to a very large increase in overall reactivity of 114-fold. This enhanced reactivity may be explained on the basis of the interaction scheme which indicates that it is possible to form two new hydrogen bonds, $b_O(x3)$ and $b_N(x3)$. It is uncertain whether the methyl of L_{x3} interacts with the active site, and if so to what extent this contributes to the observed reactivity.

From comparisons 1, 4, and 6 it may be seen that the same structural change in different substrate pairs leads to similar increases in k_{23}/K_{EA}. This suggests that the influence of a single structural change is virtually unaffected by the nature of the remainder of the substrate. A similar conclusion can be reached from comparisons of the acylation rates k_{23}, although here the similarities are less pronounced; the effects of structural changes on the dissociation constants K_{EA} are small, but are consistent with this conclusion.

The comparisons of the k_{23} and K_{EA} values for the different substrates indicate that, in general, changes in structure are reflected by changes in k_{23} rather than in K_{EA}: that is, structural changes appear to induce changes in the spatial arrangement of the reacting atoms rather than in the affinity. This, however, does not seem to be the case when the peptide chain of Ac-Tyr-Gly-NH_2 is extended by a glycine residue (comparison 2); the k_{23} value remains virtually unchanged while K_{EA} is decreased to one third. This indicates that the additional interactions resulting from the introduction of the glycine appear to have little effect on the spatial arrangement of the reacting atoms but contribute to the stability of the enzyme-substrate complex. When this glycine is replaced by alanine (comparison 3), k_{23} and K_{EA} increase by three- and four-fold respectively, reflecting the loss of the non-specific interaction and the formation of the specific $r(x2)$ interaction.

Another case in which K_{EA} changes with change in substrate structure is the introduction of alanine at position $x3$ (comparison 7): the increase in overall reactivity results from an increase in k_{23} coupled with a decrease in K_{EA}. One possible explanation for the change in both constants is that the interactions which occur further away from the peptide bond which is to be cleaved have a diminished effect on the spatial arrangement of the reacting atoms but an enhanced effect on the stability of the enzyme-substrate complex.

In conclusion, the kinetic results presented in this paper are shown to be consistent with the interaction scheme proposed from crystallographic studies [23]. On this basis it is possible to evaluate the contributions of individual interactions, in particular those occurring within the secondary interaction range, to the reactivity of peptide substrates. Such studies should lead to a greater understanding of the significance of these interactions in determining the specificity of α-chymotrypsin.

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