The kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-N-(acetylglycyl)-L-tyrosinhydrazide

The lack of unambiguous kinetic constants for the α-chymotrypsin-catalyzed hydrolysis of substrates of the type RCONHCHR'CONHCHR''COY, where –COY is the carboxyl derivative undergoing solvolysis, led us to investigate the kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-N-(acetylglycyl)-L-tyrosin-hydrazide in aqueous solutions at 25° and pH 7.80 and 0.02 *M* in the Tris component of a Tris–HCl buffer. This reaction system was selected for study because (a), information was needed for the case of R' = H before proceeding to substrates containing two or more centers of asymmetry; (b), it could be followed by a convenient and unambiguous analytical procedure[1,2], and (c), the kinetic constants could be compared with those evaluated earlier for a comparable system involving α-N-acetyl-L-tyrosinhydrazide[1,2], a closely related substrate of the type RCONHCHR'COY.

The selection of pH 7.80, as one of the reaction parameters, was not arbitrary but was based upon consideration of the data given in Table I. It was concluded that the optimum pH for the reaction system was 7.80 ± 0.05, a value identical, within the limits of error, with that determined for the comparable system containing α-N-acetyl-L-tyrosinhydrazide, *i.e.*, 7.95 ± 0.20 (see refs. 1 and 2).

TABLE I

DETERMINATION OF OPTIMUM pH FOR THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-(ACETYLGLYCYL)-L-TYROSINHYDRAZIDE*

pH	7.33	7.73	7.85	8.10	8.27
time (min)	*Relative extent of reaction***				
4	0.90	0.96	0.99	1.00	0.85
6	0.92	0.92	1.00	0.90	0.85
8	—	0.98	1.00	0.90	0.78
10	0.93	1.00	0.95	0.88	0.88
12	0.96	0.97	1.00	0.92	0.88
14	0.93	1.00	1.00	0.91	0.85
16	0.93	1.00	0.99	0.90	0.88
mean	0.93	0.98	0.99	0.92	0.85

* In aqueous solutions at 25° and 0.02 *M* in the Tris component of a Tris–HCl buffer with $[E]$ = 0.140 mg protein nitrogen/ml and $[S]_0 = 4.15 \cdot 10^{-4}$ *M*. Essentially the same results were obtained when $[S]_0 = 10.37 \cdot 10^{-4}$ *M*.

** Maximum extent of reaction for any given reaction time normalized to a value of 1.00. All other values in the same horizontal line refer to fractional extent of reaction relative to maximum.

The data from 26 experiments performed in order to evaluate the constants K_S and k_3 for the system of interest are summarized in Table II. Evaluation of these data, by the method of BOOMAN AND NIEMANN[3], using in this instance a DATATRON 205 digital computer for the actual computations, gave values of $K_S = (22 \pm 9) \cdot 10^{-3}$ *M* and $k_3 = (1.1 \pm 0.4) \cdot 10^{-3}$ *M*/min/mg protein nitrogen per ml for a rate equation of the form $dP/dt = k_3[E][S]/(K_S + [S])$. It will be recalled that $K_S = (29.5 \pm 6.0) \cdot 10^{-3}$ *M* and $k_3 = (1.1 \pm 0.2) \cdot 10^{-3}$ *M*/min/mg protein nitrogen per ml for the comparable system involving α-N-acetyl-L-tyrosinhydrazide[1,2].

Abbreviation: Tris, tris(hydroxymethyl)aminomethane.

It is evident that the constants K_S and k_3 for the systems α-chymotrypsin–α-N-(acetylglycyl)-L-tyrosinhydrazide and α-chymotrypsin–α-N-acetyl-L-tyrosinhydrazide, in aqueous solutions at 25° and pH 7.8 ± 0.2 and 0.02 *M* in the Tris component of a Tris–HCl buffer, are identical within the limits of experimental error. The implication that replacement of CH_3CONH– by $CH_3CONHCH_2CONH$– is without significant effect upon the course of the reaction is surprising in view of the marked variation in values of K_S and k_3 observed in the series, α-N-formyl-, α-N-acetyl-,

TABLE II

KINETICS OF THE α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-(ACETYLGLYCYL)-L-TYROSINHYDRAZIDE*

$[S]_o \times 10^4 M$	Δt min**	v_0*** $A/min \times 10^3$	P_n****
0.765	6	2.29 ± 0.05	2
0.84	2.5	2.44 ± 0.06	2
1.39	4	3.36 ± 0.07	1
1.95	3	5.59 ± 0.10	2
2.79	3	8.29 ± 0.26	2
2.79	2.5	7.19 ± 0.14	1
3.72	4	9.43 ± 0.29	1
5.57	2	15.64 ± 0.32	1
5.99	3	16.43 ± 0.36	2
8.36	2	22.81 ± 0.30	2
8.69	4	25.68 ± 0.67	2
11.15	1	31.50 ± 0.68	1
11.98	3	36.02 ± 0.69	3
12.41	4	34.77 ± 0.41	2
12.53	1	34.97 ± 0.70	1
13.93	1	36.12 ± 1.05	1
15.52	1	40.60 ± 0.56	1
16.72	1	45.08 ± 3.15	1
17.96	3	47.69 ± 0.97	2
18.11	1	56.33 ± 0.68	3
19.50	1	49.72 ± 1.73	1
20.34	3	53.00 ± 0.95	2
20.90	1	51.57 ± 1.80	1
22.29	1	55.70 ± 1.63	1
23.95	3	56.99 ± 0.19	3
24.82	4	60.48 ± 0.61	3

* In aqueous solutions at 25° and pH 7.80 and 0.02 *M* in the Tris component of a Tris–HCl buffer with $[E]$ = 0.140 mg protein nitrogen/ml.

** Time interval between successive determinations of amount of liberated hydrazine. For each experiment eight determinations were made including the one for $t = 0$.

*** The initial velocity, v_0, is given in units of absorbancy (A)/min.

* Degree of polynomial required to describe dP/dt^3.

α-N-dichloroacetyl- and α-N-trimethyl-acetyl-L-tyrosinhydrazide[1]. However, with the present demonstration of the kinetic equivalence, or near equivalence, of α-N-(acetylglycyl)- and α-N-acetyl-L-tyrosinhydrazide we may infer that the additional carboxamide function, present in the former substrate, does not influence the course of the reaction either by an added steric interaction or by a supplementary binding interaction unless one is faced with the rather improbable situation that both such interactions occur but that they kinetically nullify each other. Thus, it appears that α-N-(acetylglycyl)-L-tyrosinhydrazide is a reasonable point of departure for

the exploration of the kinetic properties of substrates of the type RCONHCHR'-CONHCHR''COY, where R = CH_3—, R' ≠ H, R'' = *p*-$CH_2C_6H_4OH$ and Y = –$NHNH_2$, *i.e.*, those substrates containing two centers of asymmetry.

Experimental

L-Tyrosine (10 g) was acylated with chloroacetyl chloride[4] to give crude α-N-chloroacetyl-L-tyrosine which was allowed to react for 72 h with 150 ml 28% aq. ammonia. The reaction mixture was evaporated to dryness and the residue recrystallized from ethanol to give glycyl-L-tyrosine hydrochloride. The dipeptide hydrochloride was suspended in 75 ml methanol, the mixture saturated with dry HCl, allowed to stand for 24 h at room temperature, the solvent and excess HCl removed *in vacuo* and the residue dried *in vacuo* over solid NaOH to give 7.4 g glycyl-L-tyrosine methyl ester hydrochloride. The latter product was taken up in 50 ml water containing 4.3 g anhyd. $NaHCO_3$ and covered with 100 ml ethyl acetate. Acetic anhydride, 2.6 g, was added in several portions and with vigorous shaking. The ethyl acetate phase was collected, dried over anhyd. Na_2SO_4 and evaporated to dryness. The syrupy residue was taken up in 25 ml anhyd. ethanol and the solution heated to its refluxing temperature prior to the dropwise addition of 2 ml of hydrazine hydrate. The reaction mixture was allowed to stand at room temperature overnight, the colorless precipitate collected, washed successively with absolute ethanol and ethyl ether, dried *in vacuo* and recrystallized twice from water to give 1.3 g α-N-(acetylglycyl)-L-tyrosinhydrazide, m.p. 244.0–245.0°, corr.

Anal. Calcd. for $C_{13}H_{18}O_4N_4$(294): C, 53.0; H, 6.2; N, 18.9. Found: C, 53.1; H, 6.2; N, 19.0.

The procedure employed for the kinetic studies was identical with that described previously[2]. The actual experimental parameters are given in Table II. The enzyme preparation was crystalline salt-free α-chymotrypsin, Armour Lot No. 00592.

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