Amines as Modifiers of the Tryptic Hydrolysis of Neutral Substrates*

François Seydoux,† Gérard Coutouly, and Jeannine Yon

ABSTRACT: The effect of 1- and 2-alkylamines on the tryptic hydrolysis of neutral substrates has been investigated. With acetylglycine *p*-nitrophenyl ester as substrate, 2-alkylamines have been found to be more efficient activators than the corresponding 1-alkylamines. In contrast, the latter amines are bound to the enzyme with greater affinity than the former. 2-Heptylamine is the most powerful activator of the tryptic hydrolysis of acetylglycine ethyl and *p*-nitrophenyl esters (activation up to 45-fold under conditions where [S] $< K_m$). The imidazole pK shift to the acidic region has been found to be larger with 2-heptylamine ($\Delta pK = -0.69$) than with ethylamine ($\Delta pK = -0.31$). In the ternary complex formed with enzyme, 2-heptylamine, and acetylglycine *p*-nitrophenyl ester, the catalytic activity is increased by at least two orders of magnitude and is close to that of specific substrates. All amines studied increase both k_{cat} and K_m for the hydrolysis of acetylglycine *p*-nitrophenyl ester. In the case of acetylglycine ethyl ester, K_m is significantly decreased in the presence of 2-heptylamine. Evidence is presented that activation by amines is greater for the deacylation step than for the acylation. The affinity of 2-heptylamine toward enzyme appears to be dependent on the type of enzyme intermediate. The order of decreasing stabilities for amine complexes in this case are Michaelis complex–2-heptylamine > free enzyme–2-heptylamine > acyl-enzyme–2-heptylamine. It is suggested that the active-site conformation is modified during the catalytic processes.

The hydrolysis of *p*-nitrophenyl acetate is only slightly activated by amines, which partially inhibit the hydrolysis of ethyl hippurate. These results are consistent with the existence of an auxiliary binding site for neutral substrates which can interact with a primary site occupied by amines.

The activating effect of some small chain alkylamines and alkylguanidines on the tryptic hydrolysis of Ac-Gly-OEt has been demonstrated by Inagami and coworkers (Inagami and Murachi, 1964; Inagami and York, 1968). On the other hand, the same derivatives have been found to inhibit competitively the tryptic hydrolysis of specific substrates like Bz-Arg-OEt[1] (Inagami, 1964).

Inagami's interpretation of this activation phenomenon envisions the formation of a ternary complex among substrate, effector, and enzyme. In this ternary complex, the enhancement of the catalytic activity results from a conformational change induced by the effectors, as with the alkylammonium or alkylguanidinium moiety of the specific substrates (Inagami and Murachi, 1964). This type of effector, structurally related to the normal substrates, has been called "autosteric" by Koshland and Neet (1968). The activating amine and Ac-Gly-OEt lay side by side at the binding site, forming a structure which is close to that of a specific substrate like α-*N*-acetyllysine ethyl ester. The resulting activation is only observed on k_{cat} and not on K_m. The observed inhibition by butylamine results from an overlapping between the binding sites of substrate and amine.

This attractive interpretation has been criticized by Erlanger and Castleman (1964) who showed that large amines, not structurally related to specific substrates, can activate the same reaction.

Since this activation phenomenon could provide some interesting information about the mechanism of the specificity of trypsin action, we have undertaken in the present work a systematic kinetic study on the effect of alkylamines as modifiers of the tryptic hydrolysis of several neutral substrates.

Experimental Section

Materials. Bovine trypsin, twice recrystallized from Worthington (batch TRJ7A), chymotrypsin free, was purified by 1 M NaCl as previously described (Seydoux and Yon, 1967). Determination of the total protein concentration was based on a $E_{278}^{1\%}$ of 15.9 (Yon, 1959). Active site titration with NPGB of this purified material (Chase and Shaw, 1967) indicates a purity of 87 ± 2% with respect to the total protein concentration.

Ac-Gly-OEt, purchased from Schuchardt, was redistilled under reduced pressure and recrystallized from ether, mp 46–47° (lit. (Inagami and Mitsuda, 1964) mp 47.5–48°).

Ac-Gly-ONP obtained from Cyclo Chemical Corporation was twice recrystallized in ethanol–ether mixtures, mp 126° (lit. (Ingles and Knowles, 1967) mp 126–128°).

Bz-Gly-OEt, Ac-Phe-OMe, and NPAc were purchased from Mann Research Laboratories and used without further purification. NPGB was a gift of E. Shaw which is gratefully acknowledged.

AMINES. Ethylamine, 1-butylamine (Eastman Kodak), 1-propylamine, 1-pentylamine, 1-hexylamine, 1-heptylamine, 1-octylamine, 2-propylamine, 2-butylamine, 2-octylamine (Fluka), cyclopropylamine, 2-pentylamine, 2-heptylamine (Aldrich), 3-heptylamine, and 2-hexylamine (K & K) were redistilled before use and neutralized with 2 N HCl in aqueous solution.

* From the Laboratoire d'Enzymologie Physico-chimique et Moléculaire, Faculté des Sciences, 91 Orsay, France. *Received October 12, 1970.* This work was supported by grants from the Centre National de la Recherche Scientifique (G.R. 13) and from the Délégation Générale à la Recherche Scientifique et Technique (G.R. 67 00 603 02).

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1968), are: Bz-Gly-OEt, ethyl hippurate; Ac-Gly-ONP, acetylglycine *p*-nitrophenyl ester; Ac-Phe-OMe, acetyl-L-phenylalanine methyl ester; Bz-Arg-OEt, benzoyl-L-arginine ethyl ester hydrochloride; NPAc, *p*-nitrophenyl acetate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; MES, 2-(*N*-morpholino)ethanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

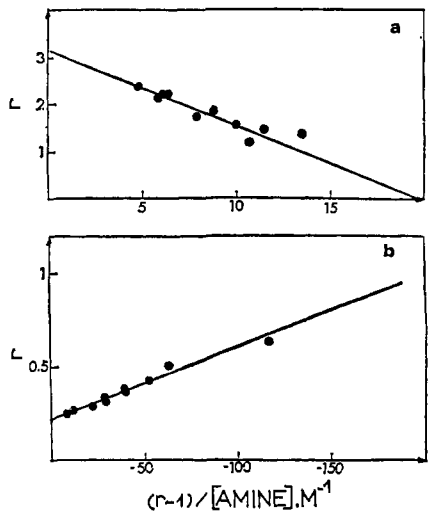

FIGURE 1: Tryptic hydrolysis of Ac-Gly-ONP; comparison of the effects of 2-butylamine (a) and 1-butylamine (b). Linear plot of the data according to eq 2 (see text). Conditions as given in Table I.

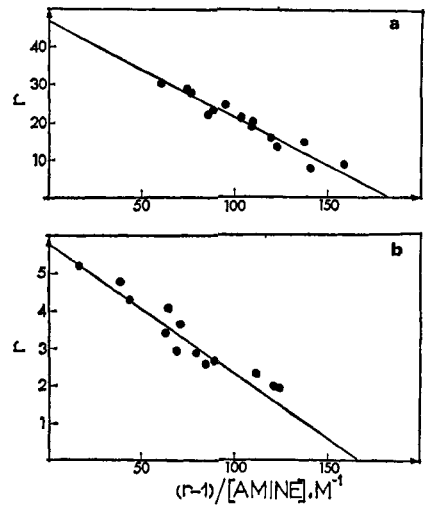

FIGURE 2: Tryptic hydrolysis of Ac-Gly-ONP; comparison of the effects of 2-heptylamine (a) and 1-heptylamine (b). Same representation as in Figure 1. For conditions, see Table I.

Rate Measurements. The initial rates of hydrolysis of Ac-Gly-OEt, Bz-Gly-OEt, and Ac-Phe-OMe have been determined using a pH-Stat arrangement as previously described (Seydoux and Yon, 1967).

In the case of Ac-Gly-ONP and NPAc, spectrophotometric measurements of the *p*-nitrophenol release were made with a Cary 16K spectrophotometer at 347.5 nm, using a difference in molar extinction between *p*-nitrophenol and ester of 5050 (OD M^{-1}).

All kinetic measurements were carried out at 25° and in the presence of 0.025 M $CaCl_2$. In the presence of alkylammonium ions, NaCl was added to maintain the ionic strength constant at the indicated value.

In the case of Ac-Gly-ONP, the kinetic measurements were carried out below pH 7 in order to limit the spontaneous hydrolysis and prevent a possible enzyme-catalyzed aminolysis of this substrate (Inward and Jencks, 1965). In all cases, the observed rates of hydrolysis were corrected for the non-enzymatic rates measured in the absence of enzyme.

Analysis of Experimental Data. The functional nonlinear relationships used in this work were fitted to the experimental data according to the iterative procedure described by Cleland (1967) and programmed for a Wang desk-top calculator.

Activation and partial inhibition phenomena were analyzed according to eq 1 in which k_{app} is the rate constant (= observed rate divided by enzyme concentration) in the presence of the effector A, $k_{0,app}$ is the rate constant without effector and K_A^*, K_A^{**} are two empirical parameters.

$$k_{app} = k_{0,app} \frac{1 + [A]/K_A^{**}}{1 + [A]/K_A^*} \quad (1)$$

According to Inagami and Murachi (1964) eq 1 can be rearranged as follows

$$r = -K_A^*(r - 1)/[A] + r_{max} \quad (2)$$

$r = k_{app}/k_{0,app}$ is defined as a relative rate, the parameter $r_{max} = K_A^*/K_A^{**}$ can be used to measure the magnitude of the activation ($r_{max} > 1$) or partial inhibition ($r_{max} < 1$); if a total inhibition of the reaction is observed, $r_{max} = 0$.

Active imidazole pK values were estimated from a plot k_{app} *vs.* $k_{app} \times$ (H) as discussed by Inagami and Murachi (1964).

Results

Reactions with Ac-Gly-ONP. For this substrate, the Michaelis parameters k_{cat} and K_m have been found to be 0.14 ± 0.01 sec^{-1} and 1.32 ± 0.15 × 10^{-3} M, respectively, at pH 6.31.

The effect of two series of 1-alkylamines [$CH_3(CH_2)_{n-1}NH_2$ (*n* varying from 1 to 8, series I)] and 2-alkylamines [*d,l*-CH_3-$(CH_2)_{n-3}CH(NH_2)CH_3$ (*n* varying from 3 to 8, series II)] was analyzed under conditions of [S] < K_m. No effect has been found with methylamine. All other amines behave as activators, except 1-propylamine, 1-butylamine, and 1-pentylamine, which are partial inhibitors under these conditions. 3-Heptylamine and cyclopropylamine are also activators. Rate *vs.* amine concentration are accurately described by eq 1 or its linear form (eq 2) as shown in Figures 1 and 2. Parameters of those equations obtained for several amines are given in Table I.

2-Heptylamine appears to be a better activator (r_{max} = 46.5) than ethylamine (r_{max} = 5.6). In order to separate the effect of these amines on the k_{cat} and K_m parameters, these parameters were determined in the presence of several amines. As shown on Table II, both k_{cat} and K_m increase for all amines studied. With 1-butylamine, K_m increases more rapidly than k_{cat} and this explains the partial inhibition observed in this case under conditions of [S] < K_m.

In addition, k_{cat} and K_m have been evaluated as a function of 2-heptylamine concentration. The results are given in Figure 3. A very large increase in k_{cat} is observed and simultaneously K_m increases more slowly with 2-heptylamine concentration.

The variations of k_{cat} *vs.* 2-heptylamine concentrations have been analyzed according to eq 1, r_{max} and K_A^* have been found to be 221 ± 59 and 0.55 ± 0.18 M, respectively, with $k_{0,cat}$ = 0.14 ± 0.01 sec^{-1} at pH 6.31, ionic strength 0.58. The value obtained for k_{cat} at high 2-heptylamine concentration is close to these obtained for specific substrates (Baines *et al.*, 1964).

pH Profile. The pK of active imidazole was measured as a function of ethylamine and 2-heptylamine concentrations.

TABLE I: Alkylamines as Modifiers of the Tryptic Hydrolysis of Ac-Gly-ONP.[a]

Amine	r_{max}	K_A* (M)	r_{max}/K_A* (M^{-1})
1-Ethylamine	5.58 ± 0.19	$1.00 \pm 0.08 \times 10^{-1}$	55 ± 4
1-Propylamine[b]	0.60	4×10^{-2}	16
1-Butylamine	0.22 ± 0.01	$4.0 \pm 0.4 \times 10^{-3}$	54 ± 6
1-Pentylamine[b]	0.75	1×10^{-2}	75
1-Hexylamine	4.40 ± 0.28	$4.84 \pm 0.76 \times 10^{-2}$	91 ± 13
1-Heptylamine	5.82 ± 0.40	$3.51 \pm 0.47 \times 10^{-2}$	166 ± 21
1-Octylamine	4.25 ± 0.12	$2.21 \pm 0.17 \times 10^{-2}$	192 ± 14
2-Propylamine	9.85 ± 1.15	$7.60 \pm 1.5 \times 10^{-1}$	13 ± 1.2
2-Butylamine	3.16 ± 0.12	$1.60 \pm 0.21 \times 10^{-1}$	20 ± 2.2
2-Pentylamine	10.65 ± 0.46	$1.51 \pm 0.13 \times 10^{-1}$	70 ± 4.9
2-Hexylamine	35.45 ± 1.45	$3.13 \pm 0.15 \times 10^{-1}$	113 ± 2.5
2-Heptylamine	46.50 ± 2.10	$2.55 \pm 0.20 \times 10^{-1}$	183 ± 10.5
2-Octylamine	31.20 ± 1.15	$1.35 \pm 0.07 \times 10^{-1}$	232 ± 8.5
3-Heptylamine	11.0 ± 0.55	$2.61 \pm 0.22 \times 10^{-1}$	42 ± 2.3
Cyclopropylamine[c]	11.5 ± 0.17	$4.17 \pm 0.12 \times 10^{-2}$	276 ± 6.2

[a] Conditions: pH 4.95 ± 0.05, 0.032 M acetate buffer, ionic strength 0.58, [S] = 4×10^{-4} M. [b] Values for these amines are obtained graphically. [c] pH 4.66.

TABLE II: Effect of Alkylamines on the Michaelis Parameters of the Tryptic Hydrolysis of Ac-Gly-ONP.[a]

Amine	Concn (M)	k_{cat} (sec^{-1})	K_m (M)
b		$9.6 \pm 0.3 \times 10^{-3}$	$1.43 \pm 0.10 \times 10^{-3}$
Ethylamine	9.4×10^{-2}	$1.29 \pm 0.12 \times 10^{-1}$	$6.04 \pm 0.80 \times 10^{-3}$
1-Butylamine	3.0×10^{-3}	$1.66 \pm 0.14 \times 10^{-2}$	$5.30 \pm 0.70 \times 10^{-3}$
	3.7×10^{-2}	$4.60 \pm 0.66 \times 10^{-2}$	$2.40 \pm 0.4 \times 10^{-2}$
2-Butylamine	1.15×10^{-1}	$9.10 \pm 1.5 \times 10^{-2}$	$8.0 \pm 1.8 \times 10^{-3}$
1-Heptylamine	7.0×10^{-2}	$3.59 \pm 0.97 \times 10^{-1}$	$1.43 \pm 0.46 \times 10^{-2}$
2-Heptylamine[c]	1.41×10^{-1}	$4.33 \pm 0.19 \times 10^{-1}$	$3.12 \pm 0.26 \times 10^{-3}$

[a] Conditions: pH 4.90, 0.032 M acetate buffer, ionic strength 0.25. [b] At pH 6.04, 0.032 M MES buffer, $k_{cat} = 9.40 \pm 0.17 \times 10^{-2}$ sec^{-1}, $K_m = 1.67 \pm 0.13 \times 10^{-3}$ M. [c] At pH 6.04, 0.032 M MES buffer, $k_{cat} = 0.307 \pm 0.033$ sec^{-1}, $K_m = 2.81 \pm 0.51 \times 10^{-3}$ M.

TABLE III: pH Profile of the Tryptic Hydrolysis of Ac-Gly-ONP in the Presence of Amines.[a]

Amine	Concn ($M \times 10^1$)	$pK_{H,app}$
2-Heptylamine	0	6.83 ± 0.04
	0.127	6.75 ± 0.03
	0.236	6.71 ± 0.03
	0.483	6.69 ± 0.01
	1.01	6.52 ± 0.03
	1.91	6.43 ± 0.02
	2.06	6.40 ± 0.03[b]
Ethylamine	0.290	6.75 ± 0.02
	0.575	6.67 ± 0.03
	1.15	6.63 ± 0.04

[a] Conditions: [S] = 10^{-4} M, MES or TES buffer 0.032 M. Ionic strength 0.58. [b] Ionic strength 0.31.

These amines decrease the pK values measured with Ac-Gly-ONP under the conditions [S] < K_m as shown in Figure 4. The dependency of the apparent pK ($pK_{H,app}$) on amine concentration, which is given in Table III is correctly described by eq 3,

$$K_{H,app} = K_{H,O} \frac{1 + [A]/K_A^{**}}{1 + [A]/K_A^{*}} \quad (3)$$

in which K_A* and K_A** are empirical parameters, respectively, equal to 0.25 and 0.051 M in the case of 2-heptylamine and 0.1 and 0.048 in the case of ethylamine. $pK_{H,O}$, the imidazole pK in the absence of amines, has been found to be 6.83 ± 0.04, in good agreement with the value reported by Inagami and York (1968).

The imidazole pK shift to the acidic region, extrapolated to saturation of amine, is larger with 2-heptylamine (ΔpK = −0.69) than with ethylamine (ΔpK = −0.32). This pK shift can only partially account for the large activation of the Ac-Gly-ONP hydrolysis obtained with these amines at acidic pH.

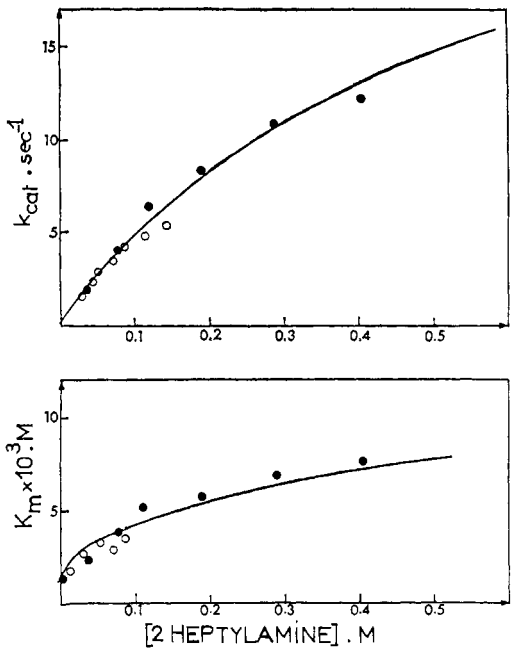

FIGURE 3: Tryptic hydrolysis of Ac-Gly-ONP. k_{cat} and K_m parameters *vs.* 2-heptylamine concentration. Conditions: 0.032 M MES buffer; (○) pH 6.26, ionic strength 0.25; (●) pH 6.31, ionic strength 0.58. The curve of k_{cat} *vs.* (A) is calculated as described in text. The curve of K_m *vs.* (A) is calculated according to eq 11 with $\beta/K_A'' = 400$, $\alpha/K_A' = 200$, $K_{A,app} = 0.1$, $K_{A,app}'' = 0.5$ (see Discussion).

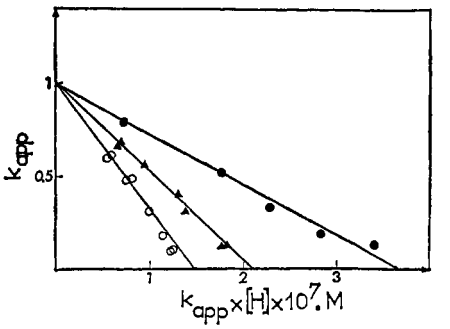

FIGURE 4: The pH profiles of Ac-Gly-ONP hydrolysis. (○) No amine; (▲) ethylamine, 5.75 10^{-2} M; (●) 2-heptylamine, 0.19 M. Linear representation of k_{app} *vs.* $k_{app} \times$ (H). k_{app} is expressed in arbitrary units. Other conditions as given in Table III.

Reactions with Ac-Gly-OEt. As previously reported by Inagami and Murachi (1964), ethylamine enhances the tryptic hydrolysis of Ac-Gly-OEt at neutral pH ($r_{max} = 9.2 \pm 0.5$, $K_A^* = 5.5 \pm 0.6 \times 10^{-2}$ M). A similar but more extensive activation is effected by 2-heptylamine ($r_{max} = 46 \pm 2$, $K_A^* = 9.2 \pm 0.6 \times 10^{-2}$ M), at [S] = 0.2 M.

The effect of 2-heptylamine on k_{cat} and K_m is shown in Table IV. In the presence of this amine, an increase in k_{cat} is observed and a small but significant decrease in K_m can be noted, as previously reported by Erlanger and Castleman (1964). No effect on K_m with ethylamine has been reported by Inagami and Murachi (1964).

Reactions with Other Neutral Substrates. With Bz-Gly-OEt as substrate, K_m and k_{cat} were found to be $5.6 \pm 1.2 \times 10^{-2}$ M and $5.05 \pm 0.84 \times 10^{-2}$ sec^{-1}, respectively, at pH 7.0. Ethylamine ($r_{max} = 0.25 \pm 0.07$, $K_A^* = 6.1 \pm 1.6 \times 10^{-2}$ M), 2-heptylamine ($r_{max} = 0.39 \pm 0.04$, $K_A^* = 7.8 \pm 2.5 \times 10^{-2}$ M), and 2-octylamine ($r_{max} = 0.39 \pm 0.01$, $K_A^* = 6.2 \pm 0.3 \times 10^{-2}$ M) partially inhibit the tryptic hydrolysis of this neutral substrate, at pH 7.0, ionic strength 0.425, 25°, $CaCl_2$ (0.025 M), and [substrate] = 1.65×10^{-2} M. In contrast, with 1-butylamine, a total inhibition is observed ($r_{max} = 0$, $K_A = 1.3 \times 10^{-3}$ M). Similarly, cyclopropylamine has been found to inhibit completely the hydrolysis of Ac-Phe-OMe. The pre-steady state hydrolysis of NPAc is slightly activated at pH 4.8 by ethylamine, cyclopropylamine, and 2-heptylamine, but these effects are smaller than with Ac-Gly-ONP under the same conditions and can be attributed in large part to the imidazole pK shift observed with these amines.

Discussion

Interpretation of the Kinetic Data. At saturation of substrate, Ac-Gly-ONP is hydrolyzed by trypsin 19 times faster than Ac-Gly-OEt at pH 7. This behavior strongly contrasts with that reported by Bender and Kézdy (1965a) in the case of carbobenzoxylysine esters derivatives, for which the maximum rates of hydrolysis are independent of the nature of the leaving group. On the other hand, K_m's for the former substrates differ at least by two orders of magnitude. Therefore, it seems reasonable to assume that the deacylation step is rate limiting for Ac-Gly-ONP and acylation for Ac-Gly-OEt, on the basis of the well-known kinetic scheme involving two intermediary complexes, the Michaelis complex and the acyl-enzyme (Bender and Kézdy, 1965b). In agreement with this assumption, a small presteady-state burst of *p*-nitrophenol has been observed during the tryptic hydrolysis of Ac-Gly-ONP with [S] $\gtrsim K_m$ at pH 4.8 and high-enzyme concentration.

Our kinetic data can be interpreted in terms of the kinetic scheme given on Figure 5.

In this scheme, K_A, K_A', and K_A'' are the dissociation constants for the amines, A, bound to the free enzyme, E, and to the two intermediary complexes, ES (Michaelis complex) and ES' (acyl-enzyme), respectively; K_H, K_H', K_H'' are the corresponding ionization constants of imidazole; K_S the dissociation constant of the Michaelis complex; α and β are the factors to account for any change in the rate of acylation and deacylation due to the amines in the ternary complexes ESA and ES'A.

From this scheme, the empirical parameters of eq 1 and 2 can be related to true equilibrium constants with respect to different limiting steps for each substrate.

In a further approximation, we may assume that in the absence of amine, the imidazole's pK has the same value in the free enzyme and in the intermediary complexes, or $K_H = K_H' = K_H''$; similarly, in the presence of amine, $K_{AH} = K_{AH}' = K_{AH}''$.

TABLE IV: Effect of 2-Heptylamine on the Michaelis Parameters of the Tryptic Hydrolysis of Ac-Gly-OEt.[a]

2-Heptylamine × 10^1 M	k_{cat} (sec^{-1})	K_m (M)
0	$1.90 \pm 0.10 \times 10^{-2}$	0.88 ± 0.08
0.51	$1.57 \pm 0.08 \times 10^{-1}$	0.44 ± 0.03
1.24	$2.35 \pm 0.15 \times 10^{-1}$	0.41 ± 0.04

[a] Conditions: pH 7.0, ionic strength 0.58.

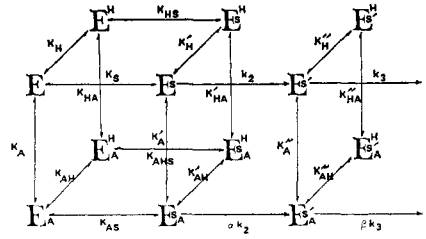

FIGURE 5: Kinetic scheme describing the tryptic hydrolysis of neutral substrate in the presence of amines as modifiers, and taking into account the ionization of active imidazole. Explanations are given in the text.

In the case of Ac-Gly-OEt, assuming $k_2 \ll k_3$, the following expressions for k_{cat} and K_m can be derived, using the steady-state assumption.

$$k_{cat} = k_{2,app} \frac{1 + \alpha[A]/K_A'}{1 + [A]/K_{A,app}'} \quad (4)$$

with

$$k_{2,app} = k_2/(1 + [H]/K_H') \quad (5)$$

and

$$K_m = K_S \frac{1 + [A]/K_{A,app}}{1 + [A]/K_{A,app}'} \quad (6)$$

From the comparison of eq 4 and 1, the following relationships can be obtained.

$$K_A' = K_A^*, r_{max} = \alpha \frac{K_{A,app}'}{K_A'} \text{ and } r_{max}/K_A^* = \alpha/K_A' \quad (7)$$

$K_{A,app}$ and $K_{A,app}'$ are functions of pH, which can be expressed as eq 8, where index, i, refers to the type of complexes.

$$K_{A,app}^i = K_A^i \frac{1 + [H]/K_H^i}{1 + [H]K_{AH}^i} \quad (8)$$

In the case of Ac-Gly-ONP, assuming $k_3 \ll k_2$

$$k_{cat} = k_{3,app} \frac{1 + \beta[A]/K_A''}{1 + [A]/K_{A,app}''} \quad (9)$$

with

$$k_{3,app} = k_3/(1 + [H]/K_H'') \quad (10)$$

and

$$K_m = K_S \frac{k_3(1 + \beta[A]/K_A'')(1 + [A]/K_{A,app})}{k_2(1 + \alpha[A]/K_A')(1 + [A]/K_{A,app}'')} \quad (11)$$

In these last equations, the $K_{A,app}^i$ parameters are expressed as in eq 8. Comparing eq 9 to eq 1, it can be deduced that in this case

$$K_A^* = K_A''$$

$$r_{max} = \frac{\beta K_{A,app}'}{K_A''} \text{ and } r_{max}/K_A^* = \beta/K_A'' \quad (12)$$

The ratio k_{cat}/K_m, which is measured under conditions where $[S] < K_m$, can be expressed in both cases by

$$k_{cat}/K_m = \frac{k_{2,app}}{K_S} \frac{1 + \alpha[A]/K_A'}{1 + [A]/K_{A,app}} \quad (13)$$

From this last equation, a precise interpretation can be given to the parameters of eq 2 which are given in Table I.

$$r_{max} = \frac{\alpha K_{A,app}}{K_A'}, K_A^* = K_{A,app}, \frac{r_{max}}{K_A^*} = \alpha/K_A' \quad (14)$$

Activation Phenomenon with Respect to the Rate-Determining Step. The parameters k_{cat} and K_m or k_{cat}/K_m as function of 2-heptylamine concentration for Ac-Gly-OEt and Ac-Gly-ONP could be analyzed according to the above equations. In the case of Ac-Gly-ONP, the increase in K_m as a function of 2-heptylamine concentration is reasonably described by eq 11 (see Figure 3). The fact that the K_m for this substrate is increased by all tested amines, in contrast to that is observed with Ac-Gly-OEt, could be explained by a larger activation of the deacylation step than the acylation ($\beta > \alpha$). Since the same acyl-enzyme is formed with Ac-Gly-OEt and Ac-Gly-ONP, data for the latter substrate can be used to evaluate the K_A'', k_3, and β parameters related to the acetyl-glycyl-enzyme. Values of K_A, K_A', K_A'', k_2, k_3, α, and β are given in Table V.

As shown in this table, the binding of 2-heptylamine and its activating properties appear to be strongly dependent on the type of enzyme intermediate. The order of decreasing stabilities for amine complexes in this case are Michaelis complex–2-heptylamine > free enzyme–2-heptylamine > acyl-enzyme–2-heptylamine. Similarly, the activation effect is inversely related to the affinity of 2-heptylamine for these two complexes: some compensation occurs between binding and activation since the β/K_A'' and α/K_A' ratios are very similar. These results indicate that the conformation of the active site (including substrate) is modified during the catalytic process, as reflected by the affinity of the different forms of enzyme toward amine-like 2-heptylamine.

Binding Site of Amines in Relation to the Activation Phenomenon. An interesting effect is the larger activation observed with 2-alkylamines than with 1-alkylamines, in agreement with the data of Erlanger and Castleman (1964) for the activation of Ac-Gly-OEt by 2- and 1-alkylamines. These last amines are more strongly bound to the enzyme but are relatively poor activators. For example, 1-butylamine partially inhibits Ac-Gly-ONP hydrolysis but 2-butylamine activates the reaction, under conditions where $[S] < K_m$. It was noted that although r_{max} ($=\alpha K_{A,app}/K_A'$) and K_A^* ($=K_{A,app}$) are very different for two homologous amines of series I and II, as shown in Table I, the r_{max}/K_A^* ($=\alpha/K_A'$) ratio does not differ greatly, except in the case of 1- and 2-butylamine. This shows that some compensating effect occurs between binding and activation, as already mentioned. Values of r_{max} and pK_A for 1- and 2-alkylamines with respect to the hydrocarbon chain length are given in Figure 6. It is clear that these profiles in the two series are sensibly parallel. In addition, Heiberg *et al.* (1967) have reported very similar profiles for the inhibition constants of a series of 1-alkylamines acting as competitive inhibitors of specific substrates (see Figure 6).

The apparent discrepancy between our pK_A values and their corresponding pK_I values can be easily rationalized by taking into account the differences in experimental conditions, especially ionic strength (Inagami and York, 1968)

TABLE V: Effect of 2-Heptylamine on the Tryptic Hydrolysis of Ac-Gly-OEt.[a]

k_2[b] (sec^{-1})	k_3[c] (sec^{-1})	α[b]	β[c]	K_A[b] (M)	K_A'[b] (M)	K_A''[c] (M)
3.2×10^{-2}	0.604	10 ± 1.5	104 ± 28	$6.9 \pm 0.4 \times 10^{-2}$	$2.02 \pm 0.22 \times 10^{-2}$	0.26 ± 0.08

[a] Ionic strength 0.58, 25°, at optimum pH. Parameters are those of Figure 5. [b] Values calculated from the data for Ac-Gly-OEt at pH 7.0, with $pK_H = pK_H' = 6.83$ and $pK_{AH}' = 6.14$ in eq 5 and 8. [c] Values calculated from the data for Ac-Gly-ONP at pH 6.31, with $pK_H'' = 6.83$ and $pK_{AH}'' = 6.14$ in eq 8 and 10.

and pH. Similarly, for a given amine (ethylamine or 2-heptylamine), the different K_A values obtained in this work with several substrates (Ac-Gly-OEt, Ac-Gly-ONP, Bz-Gly-OEt) at various pH's (ranging from 4.9 to 7) are rather close when corrected for the pH effect using eq 8.

Furthermore, it is reasonable to assume that the 1- and 2-alkylamines involved in the present activation of neutral substrates are bound at the same site implicated in the competitive inhibition of specific substrates.

Heidberg *et al.* (1967) described the active site of trypsin as formed by two hydrophobic subsites separated by a polar region. The first hydrophobic subsite is the receptor of the cationic chain of specific substrates as postulated by Mares-Guia *et al.* (1967). In the case of a large amine, up to 4 methylene groups of the aliphatic chain adjacent to the amino group are bound to the first site. Additional CH_2 groups in the chain extend into the polar region. If the chain is larger than seven carbon atoms (1- and 2-octylamines), the additional methylene or methyl groups are bound to the second hydrophobic site. It is important to note that in this model, the first hydrophobic site is completely filled by the butylamine molecule and by the larger amines.

From our data, a minimum activating effect is observed when the first site is occupied by butylamine, but, with larger amines, the activating effect increases again and reaches a maximum with 1- and 2-heptylamines, when the three carbons of the terminal alkyl part of these amines are located in the aqueous region postulated by Heidberg *et al.* (1967). The decreasing activation ratio, r_{max}, observed with 1- and 2-octylamines can be correlated with the location of the terminal methyl group of these amines in the second hydrophobic site.

Binding Site of Neutral Substrates. Inagami and Murachi (1964) interpret the absence of activation in the case of 1-butylamine as resulting from an overlapping between binding sites of this amine and Ac-Gly-OEt. This explanation is ruled out by the fact that activation phenomena reappear with larger amines, assuming that activation by smaller and larger amines takes places by the same mechanism.

Inagami's interpretation of the inhibition by butylamine can be reconciled with our data only if the large amines are not bound to the first hydrophobic site. From the available model of the hydrophobic binding sites of trypsin (Heidberg *et al.*, 1967; Hartmann and Holler, 1970), this hypothesis appears to be very unlikely.

As discussed by Sanborn and Hein (1968) neutral substrates are probably not bound at the same site as the corresponding specific substrates; our data strongly support this hypothesis. The binding of 1- and 2-alkylamines (which are not structurally related to specific substrate), at a primary site, could modify the conformation of a neighboring secondary site, which binds neutral substrates. In this sense, conformational requirements for the hydrolysis of neutral and specific substrates may be different. The observed decrease in K_m for Ac Gly-OEt in the presence of 2-heptylamine can be interpreted as resulting from a conformational change of the binding site of neutral substrate induced by the binding of the 2-heptylamine molecule at the primary site. Evidently, this interpretation which is close to that of Sanborn and Hein (1968) cannot account for the decrease in the activation phenomenon observed by Inagami and Murachi (1964) at high 1-propylamine concentrations. This effect is probably related to an unspecific binding of amines by trypsin as previously observed by d'Albis and Bechet (1967) in the case of benzylamine.

Trypsin probably contains other binding sites for amines with low affinity. For example, amines could be bound only at the second hydrophobic site. In the present paper, these secondary effects are completely neglected.

It can be tentatively assumed that the neutral substrate is bound to the second hydrophobic site. The decreasing r_{max} for 1- and 2-octylamines may be thus explained by an overlapping between substrate and the terminal methyl group of octylamine molecule.

No large activating effect has been observed with NPAc, a completely unspecific substrate. On the other hand, Bz-Gly-OEt hydrolysis is partially inhibited by ethylamine and 2-heptylamine. These results show that the activating effect is very closely related to a particular structural feature of the neutral substrate: an α-peptidic linkage is required but the activation phenomenon disappears with a large α-acyl group, like benzoyl, which favors the binding of the substrate to

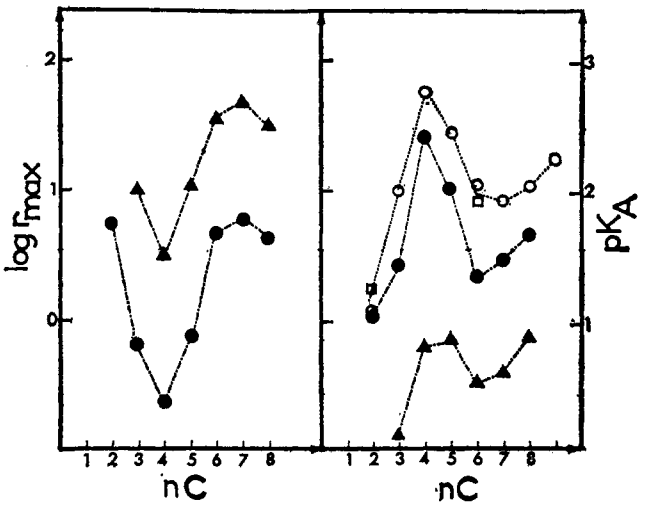

FIGURE 6: Amines as modifiers of the tryptic hydrolysis of Ac-Gly-ONP. Parameters r_{max} and K_A as function of hydrocarbon chain length (nC) of 1 (●) and 2 (▲) alkylamines. K_I values of Heidberg (○) and Inagami (□) are indicated on the K_A graph (see text).

enzyme (K_m of Bz-Gly-OEt is about 20-fold smaller than that of Ac-Gly-OEt).

These paradoxical features of the activation of trypsin-catalyzed hydrolysis are certainly related to the ability of the trypsin active-site geometry to be modified by effectors which are more or less related to specific substrates. Our data cannot be completely explained on the basis of a rigid enzymes active site.

Acknowledgment

We are indebted to Dr. D. Thusius for carefully reading the manuscript, and for many helpful criticisms. We also thank Professor E. Shaw for his generous gift of NPGB.

References

Baines, N. J., Baird, J. B., and Elmore, D. T. (1964), *Biochem. J. 90*, 470.
Bender, M. L., and Kézdy, F. J. (1965a), *J. Amer. Chem. Soc. 87*, 4954.
Bender, M. L., and Kézdy, F. J. (1965b), *Annu. Rev. Biochem. 34*, 49.
Chase, T., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29*, 508.
Cleland, W. W. (1967), *Advan. Enzymol. 29*, 1.
d'Albis, A., and Bechet, J. J. (1967), *Biochim. Biophys. Acta 140*, 435.
Erlanger, B. F., and Castleman, H. (1964), *Biochim. Biophys. Acta 85*, 507.
Hartmann, H., and Holler, E. (1970), *Eur. J. Biochem. 16*, 80.
Heidberg, J., Holler, E., and Hartmann, H. (1967), *Ber. Bunsenges. Phys. Chem. 71*, 19.
Inagami, T. (1964), *J. Biol. Chem. 239*, 787.
Inagami, T., and Mitsuda, J. (1964), *J. Biol. Chem. 239*, 1388.
Inagami, T., and Murachi, T. (1964), *J. Biol. Chem. 239*, 1395.
Inagami, T., and York, S. S. (1968), *Biochemistry 7*, 4045.
Ingles, D. W., and Knowles, J. R. (1967), *Biochem. J. 104*, 369.
Inward, P. W., and Jencks, W. P. (1965), *J. Biol. Chem. 240*, 1986.
Koshland, D. E., and Neet, K. E. (1968), *Annu. Rev. Biochem. 37*, 359.
Mares-Guia, M., Shaw, E., and Cohen, W. (1967), *J. Biol. Chem. 242*, 5777.
Sanborn, B. M., and Hein, G. E. (1968), *Biochemistry 7*, 3616.
Seydoux, F., and Yon, J. (1967), *Eur. J. Biochem. 3*, 42.
Yon, J. (1959), *Biochim. Biophys. Acta 31*, 75.

Studies on the Hydrogen-Transfer Reactions Catalyzed by Pyridine Nucleotide Linked Dehydrogenases*

William S. Allison,† Harold B. White, III,‡ and Michael J. Connors

ABSTRACT: Irreversible one-turnover reactions between GAP-*1-t* (generated from DHAP-*1-t*) and the lobster muscle glyceraldehyde 3-phosphate dehydrogenase–NAD complex have been carried out. Analyses of the products of these reactions have shown that NAD^{3}H is formed and tritium is not incorporated into the enzyme. These results show that the hydrogen-transfer reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase is direct and not mediated through the alternate oxidation and reduction of a tryptophan residue. Yeast alcohol dehydrogenase has been labeled with tritium by adding urea to a reaction mixture which contained yeast alcohol dehydrogenase, ethanol-*1-t*, and NAD at pH 11.0. Fractionation of tryptic digests of the labeled enzyme failed to reveal that a specific tryptophan residue at the active site of alcohol dehydrogenase was labeled. All attempts to label various lactate dehydrogenases with tritium derived from lactate-*2-t* failed.

It has been concluded from these experiments that the hydrogen transfers catalyzed by pyridine nucleotide linked dehydrogenases are direct and not mediated by the alternate oxidation and reduction of tryptophan residues at the active-sites of the enzymes.

It has been suggested that the hydride-transfer reactions catalyzed by pyridine nucleotide dependent dehydrogenases may be mediated through the alternate oxidation and reduction of the methylene group of tryptophanyl side chains in the enzymes (Schellenberg, 1965, 1970). The enzyme-mediated hydride-transfer mechanism is based on the following experimental observations. Tritium is incorporated into the alanine side chains of tryptophan residues of yeast alcohol dehydrogenase (Schellenberg, 1966), rabbit muscle lactate dehydrogenase (Schellenberg, 1967), and the mitochondrial pig heart malate dehydrogenase (Chan and Schellenberg, 1968) when the enzymes are incubated with their respective tritiated substrates under appropriate conditions. The results of studies with model systems have made the proposed mechanism of enzyme-mediated hydride transfer appear attractive indeed. For instance, it has been shown by Schellenberg and McLean (1966) that a model indolenine salt is reduced to the corresponding indole by reduction with 1-benzyl-1,4-dihydronico-

* Publication no. 775 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 01254, and from the Department of Chemistry, University of California, San Diego, California 92037. *Received January 18, 1971.* This investigation was supported by research grants from the Public Health Service, National Institute of General Medical Sciences (GM-14,481 and GM-16,974).

† To whom inquiries should be addressed at the University of California, San Diego, Calif.

‡ Present address: Department of Chemistry, Harvard University, Cambridge, Mass.