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Allosteric Activation of the Hydrolysis of Specific Substrates by Chymotrypsin

Bernard F. ERLANGER, Norbert H. WASSERMANN, Anne G. COOPER, and Raymond J. MONK

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York

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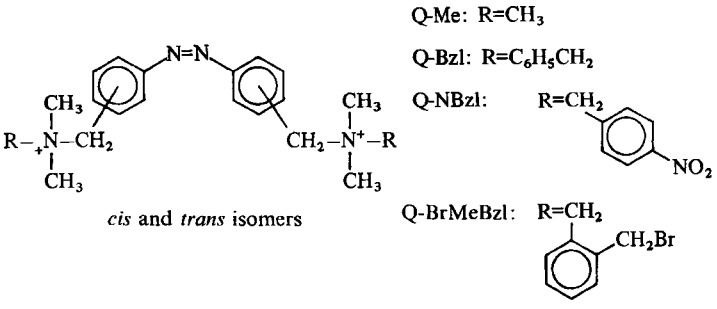
A variety of azobenzene compounds having bis-quaternary nitrogens have been shown to accelerate the hydrolyis by chymotrypsin of certain specific substrates by an allosteric mechanism. One of the most potent, 2,2′-bis[α-(benzyldimethylammonium)methyl]azobenzene dibromide (2,2′-Q-Bzl) accelerated the hydrolysis of glutaryl-L-phenylalanine *p*-nitroanilide 40-fold at saturating concentration. Acceleration was by increasing k_{cat} without altering K_m. The hydrolysis of acetyl-L-tyrosine *p*-nitroanilide and acetyl-L-tyrosine anilide was also accelerated by Q-Bzl (25-fold and 1.8-fold respectively) while the hydrolysis of hemoglobin, azocoll and a number of esters was not affected. The inactivation of chymotrypsin by diphenylcarbamyl chloride and diphenylcarbamyl fluoride was accelerated by 2,2′-Q-Bzl. Reactivation in the presence of NH_2OH was also accelerated, but in the absence of added nucleophile (*i.e.* of NH_2OH) no increase in rate was detectable. An allosteric effector was covalently attached to chymotrypsinogen A by reaction with 2,2′-bis[α-(*o*-bromomethylbenzyldimethylammonium)methyl]azobenezene dibromide. The product, when converted to active enzyme, was about 4 times more active than chymotrypsin as a result of an increase in k_{cat} of hydrolysis; K_m was unaffected. The mechanism of the allosteric acceleration process is not known but, because for all of the substrates affected acylation of the enzyme is rate-limiting, it is tentatively suggested that the effectors facilitate proton transfer to the leaving group by an inductive effect on the 'charge relay system'. Spectral studies indicate that the allosteric site is a portion of the enzyme with a polarity near that of water, possibly on the outside surface of the enzyme molecule.

In a preliminary communication [1] we reported acceleration of the chymotryptic hydrolysis of certain specific substrates by a number of compounds having the general structure:



Acceleration of substrate hydrolysis (for example, Glt-Phe-Nan) occurred as a result of the binding of the activator molecule to a site other than the normal

Abbreviations. Glt-Phe-Nan, glutaryl-L-phenylalanine *p*-nitroanilide; Ac-Tyr-Nan, α-*N*-acetyl-DL-tyrosine *p*-nitroanilide; Z-Tyr-NHOH, carbobenzoxy-L-tyrosine hydroxamide; Ac-Tyr-An$_2$, α-*N*-acetyl-L-tyrosine anilide; Q-Me, bis[α-(trimethylammonium)methyl]azobenzene dibromide; Q-Bzl, bis[α-(benzyldimethylammonium)methyl]azobenzene dibromide; Q-Nbzl, bis[α-(*p*-nitrobenzyldimethylammonium)methyl]azobenzene dibromide; Q-BrMeBzl, 2,2′-bis[α-(*o*-bromomethylbenzyldimethylammonium)methyl]azobenzene dibromide; K-Bzl, 2,2′-[α-(benzyldimethylammonium)-methyl]diketobenzene dibromide; Ac-Tyr-OEt, α-*N*-acetyl-L-tyrosine ethyl ester; Ac-Gly-OEt, α-*N*-acetyl-glycine ethyl ester; Bz-Gly-OEt, α-*N*-benzoyl-glycine ethyl ester; Ph_2NCF and Ph_2NC-chymotrypsin, diphenylcarbamyl chloride, fluoride and chymotrypsin, respectively; Ac-ONp, *p*-nitrophenylacetate; Bz-Arg-Nan, α-*N*-benzoyl-DL-arginine p-nitroanilide.

Enzyme. α-Chymotrypsin or chymotrypsinogen A (EC 3.4.21.1).

substrate binding site. No inhibition could be observed at any concentration tested, even by structurally related compounds that were poor activators. Kinetic experiments demonstrated that one of the most potent activators, Q-Bzl, caused an increase in k_{cat} of hydrolysis but did not change K_m. Because the substrates affected by the activation process were all amides, for which K_m approximates a true binding constant (*cf.* [2]), we can conclude that the activators did not change the binding affinity of chymotrypsin for substrate. None of the compounds increased the rate of hydrolysis of Bz-Arg-Nan by trypsin; nor did they inhibit.

We have referred to the activation as an allosteric process, since it resulted from the binding of an effector (or modifier) to a site that was topologically different from the active site at which substrate is hydrolyzed (*cf.* [3]). The process is different from most allosteric effects described in that the chymotrypsin molecule is not made up of multiple subunits. Moreover, in most allosteric phenomena, substrate binding is affected [4]. In this paper we present the full experimental details of the experiments and additional experiments designed to explore the phenomenon further with other activators, other substrates and with a specific inactivator of chymotrypsin. Moreover, we have been able to link an activator molecule covalently to chymotrypsinogen.

EXPERIMENTAL PROCEDURE

Enzymes

Worthington products were used: chymotrypsinogen A (CGC) was '5× crystallized, electrophoretically pure', α-chymotrypsin (CDI) was 3× crystallized.

Substrates

Glt-Phe-Nan was prepared as described previously [5]. Ac-Tyr-Nan was prepared from acetyl-L-tyrosine, *p*-nitroaniline and P_2O_5 by a procedure developed in this laboratory for the synthesis of peptides [6]. Its physical characteristics were as described by Inagami and Sturtevant [7]. Ac-Tyr-OEt, Ac-ONp and hemoglobin were products of Schwarz/Mann Laboratories. Azocoll was a gift from Dr I. Mandl. Ac-Gly-OEt was synthesized according to the procedure of Wolf and Niemann [8].

Z-Tyr-NHOH was prepared as follows. Carbobenzoxy-L-tyrosine (2 g; 6.3 mmol) [9] was dissolved in 25 ml of tetrahydrofuran. Tri-*n*-butylamine (1.16 g; 6.3 mmol) was added and the solution was cooled in an ice bath. Then 0.9 g (0.85 ml, 6.7 mmol) of isobutylchloroformate was added and the solution allowed to stand at 0 °C for 30 min. Meanwhile, 2.1 g (30 mmol) of hydroxylamine hydrochloride were dissolved in 5 ml of water and, just prior to its addition to the mixed anhydride solution, it was made alkaline with 10 ml of 3.5 N NaOH. The addition to the mixed anhydride solution was made with vigorous stirring, which was continued overnight at room temperature. The solvent was then removed *in vacuo*, 50 ml of water was added and the pH adjusted first to 3 with dilute hydrochloric acid and then to 6 with NH_4OH. The solution was taken to dryness *in vacuo* and the residue washed once with chloroform. The residue was now a white solid, which was extracted with ethanol, leaving behind a granular residue. Water was added to the ethanol solution until just before turbidity appeared. Upon standing, a crystalline product (needles) appeared. It was collected and recrystallized from ethanol/water to give 850 mg (41%) of product; m.p. 175–176. 5 °C with decomposition. Analysis: calculated for $C_{17}H_{18}O_5N_2$ (M_r 330.3): C 61.8; H 5.5; N 8.5. Found: C 62.0; H 5.3; N 8.7.

Activators

Of the activators listed in Table 1, the synthesis of 2,2′-Q-Bzl, 2,2′-Q-NBzl and 2,2′-Q-BrMe-Bzl will be given in detail here. The others will be described in another paper now in preparation, in which studies on acetylcholinesterase and the acetylcholine receptor will be described.

2,2′-Q-Bzl was prepared from 2,2′-bis(α-dimethylaminomethyl)azobenzene by benzylation.

Preparation of 2,2′-Bis(α-dimethylaminomethyl)azobenzene

9 g (0.05 mol) of *o*-(α-dimethylaminomethyl)-nitrobenzene [10] in 150 ml of dry ether were reduced with sodium bis[2-methoxyethoxy]aluminium hydride (45 ml of 70% solution; 0.05 mol) ('Vitride'-Eastman), diluted with 50 ml of dry ethyl ether. The reducing agent was added dropwise with stirring over a period of 1 h while the reactants were in an ice bath under an atmosphere of nitrogen. After addition was complete, the solution was stirred for an additional 0.5 h at room temperature. A solution of 20% sodium hydroxide (100 ml) was then added to decompose the reaction complex. The ether layer was recovered, washed twice with water, dried over anhydrous Na_2SO_4 and taken to dryness *in vacuo*. The red, oily residue (about 7 g) was purified by chromatography on Al_2O_3 (Woelm, neutral, activity 1) in a 45 × 2.5-cm column using ligroin (b.p. 100–115 °C)/1% methanol as the eluant. The product (red in color) appeared immediately after a small quantity of a yellow material. It absorbed in the ultraviolet with peaks at 235 nm and 332 nm in methanol. After distillation to dryness *in vacuo* the product crystalliz-

ed. It was recrystallized from isopropanol, m.p. 68–69 °C. (Yield, 3.5 g; 47%.) Analysis: calculated for $C_{18}H_4N_4$ (M_r 296.4): C 72.9; H 8.2; N 18.9. Found: C 72.8; H 8.2; N 18.7.

Preparation of 2,2'-Q-Bzl. Benzyl bromide (2.42 g, 17 mmol) was added to a solution of 1.2 g (4 mmol) of 2,2'-bis(α-dimethylaminomethyl)azobenzene in 20 ml methanol and the reaction solution was refluxed for 2 h. About 150 ml of ether was added to precipitate the quaternary salt, which was recrystallized from isopropanol/ether (4/1); m.p. 180–181 °C. (Yield, 2.1 g; 81%.) Analysis: calculated for $C_{32}H_{38}N_4Br_2$ (M_r 638.5): C 60.2; H 6.0; N 8.7; Br 25.0. Found: C 60.0; H 6.0; N 8.7; Br 25.2.

Preparation of 2,2'-Q-NBzl. 148 mg (0.5 mmol) of 2,2'-bis(α-dimethylaminomethyl)azobenzene and 432 mg (2 mmol) of *p*-nitrobenzylbromide (Aldrich) in 16 ml methanol were refluxed for 1 h. After cooling to room temperature and standing overnight, the crude quaternary salt precipitated. The orange crystals were filtered, washed with 20 ml of methanol and with 50 ml of ethyl ether. Yield, 310 mg; 79%, m.p. 217–218 °C. Recrystallization from methanol raised the melting point to 218–219 °C. Analysis: calculated for $C_{34}H_{36}N_6O_6Br_2$ (M_r 783.1): C 52.1; H 4.6; N 10.7; Br 20.4. Found: C 51.9; H 4.6; N 10.7; Br 20.2.

Preparation of 2,2'-Q-BrMeBzl. 66 g (0.25 mol) of α-α'-dibromo-*o*-xylene (Aldrich 96%, m.p. 92–94 °C) were dissolved in 150 ml of acetonitrile at reflux temperature. At 90–95 °C, a solution of 450 mg (1.5 mmol) of 2,2'-bis(α-dimethylaminomethyl)azobenzene in 20 ml acetonitrile was added and the mixture refluxed for 30 min, protected from external moisture with a $CaCl_2$ tube. After cooling to room temperature overnight, the precipitated quaternary salt was separated from the solution and was twice suspended in 150 ml of toluene to remove any excess dibromoxylene. The orange crystals of crude product were filtered, washed with ethyl ether and dried *in vacuo* (60 °C), weight 815 mg, m.p. 204–205 °C. Yield, 65% based on amount of azo derivative used. Recrystallization from 50 volumes of methanol raised the melting point to 207–208 °C. Analysis: calculated: for $C_{34}H_{40}N_4Br_4$ (M_r 824.4): C 49.5; H 4.9; N 6.8; Br 38.8. Found: C 49.5; H 4.9; N 6.8; Br 38.6. Nuclear magnetic resonance ($C^2H_3)_2SO$ + 80 °C): δ = 5.05 (singlet, 4H, $-CH_2Br$), 4.7–4.8 (doublet, 4H, $-\overset{+}{N}-CH_2-$), 2.97 (singlet, $-\overset{+}{N}-CH_3$).

Covalent Attachment of 2,2'-Q-BrMeBzl to Chymotrypsinogen

Chymotrypsinogen A (0.287 g, 11.2 μmol) was dissolved in 30 ml of 1 mM hydrochloric acid. To this solution was added 0.1 g (120 μmol) of Q-BrMeBzl in 25 ml of water. The total volume of the reaction mixture was brought to 125 ml by the addition of 50 mM Tris-chloride buffer, pH 8.5, containing 20 mM $CaCl_2$ and the solution was incubated at 25 °C for 48 h. The pH of the solution was then brought to 3 with 4 N HCl and dialyzed against deionized water in the cold room for 24 h. Approximately 300 mg of complex was recovered after lyophilization. It was dissolved in 5 ml of 1 mM HCl and introduced into a 2.5 × 80-cm column of Biogel P-6 which had been equilibrated with 1 mM HCl. The complex was eluted with 1 mM HCl and appeared as a brown band immediately after the void volume. A retarded band of free reagent was always observed. The product (250 mg) was recovered by lyophilization.

Kinetic Experiments

Glt-Phe-Nan. Kinetic experiments with Glt-Phe-Nan were carried out with a Gilford Model 2000 spectrophotometer using a procedure in which the rate of formation of *p*-nitroaniline was measured [5]. To a cuvette containing 2.8 ml of Glt-Phe-Nan (1.0 mM) in 0.05 M Tris-chloride buffer, pH 7.5, containing 0.03 M $CaCl_2$ and 2% methanol was added 0.1 ml of 1.5 mM activator in 0.05 M Tris-chloride buffer, pH 7.5, containing 0.03 M $CaCl_2$. At zero time 0.1 ml of a 66 μM solution of chymotrypsin in 1 mM HCl was added and the rate of *p*-nitroaniline produced was measured at 410 nm on the recorder. Controls without enzyme were routinely run at same time.

Tests for activity of various ligands were run by the simpler procedure of determining the amount of *p*-nitroaniline released in 10 min after quenching the reaction with acetic acid [5].

Ac-Tyr-Nan. The substrate was handled like Glt-Phe-Nan except that a stock solution of 10 mM in dimethylsulfoxide was prepared and appropriate quantities were used in assay procedures. If premixed with buffer, as was done with Glt-Phe-Nan, Ac-Phe-Nan precipitated from the stock solution.

Titrimetic Assays

Hydrolysis of Ac-Tyr-An_2, Ac-Tyr-OEt, Z-Tyr-NHOH, Ac-Gly-OEt and Bz-Gly-OEt was followed titrimetrically using a Radiometer pH-stat with a stirred, thermostated reaction vessel.

Ac-Tyr-An_2. Stock solutions: Ac-Tyr-An_2, 38.5 mM in dimethylsulfoxide; activator, 1 mM or 5 mM in a 'salt solution' (100 mM KCl and 25 mM $CaCl_2$); chymotrypsin, 112.5 mg in 5 ml 1 mM HCl. For the kinetic assays the total volume prior to enzyme addition was always 10 ml, of which 1 ml was dimethylsulfoxide and the rest 'salt solution'. The enzyme was always added in 0.2 ml of 1 mM HCl to start the reaction. The titrant was 25 mM or 50 mM NaOH, depending upon the rate of reaction. In a

typical series of experiments at pH 7.5 the final concentrations of reactants were as follows: chymotrypsin, 0.4 mg/ml; Ac-Tyr-An$_2$, 2.3 mM; Q-Bzl, 0.1 mM, 0.5 mM, 1 mM, 2 mM. Because the mixing of dimethylsulfoxide with water produces heat, a waiting period must be observed before addition of enzyme so that the temperature of the reaction mixture equilibrates with that of the water bath, *i.e.* 25 °C.

Ac-Gly-OEt and Bz-Gly-OEt. The conditions for these assays were adapted from Applewhite *et al.* [11] and Applewhite and Niemann [12]. The reaction medium contained 5 mM Tris-chloride buffer pH 7 and 20 mM $CaCl_2$. The titrant was 50 mM NaOH. Chymotrypsin was present at a concentration of 0.27 mg/ml. The stock solution of Ac-Gly-OET (50 mM) contained 6% methanol and was kept warm to prevent precipitation. Ac-Gly-OEt was water soluble. Substrate concentrations were in the range of 8 mM.

Z-Tyr-NHOH. Conditions of the assay were similar to those for Ac-Gly-OEt and Bz-Gly-OEt except that the final methanol concentration in the reaction solution was 15% in order to keep the substrate in solution. Concentrations of reactants were in the following range; chymotrypsin, 0.12 mg/ml; Z-Tyr-NHOH, 6 mM; 2,2'-Q-Bzl, 0.1 mM.

Proteolytic Assays

Hemoglobin. The substrate was a salt-free preparation from Schwarz/Mann. A 2% stock solution in 50 mM Tris-chloride and 30 mM $CaCl_2$, pH 7.5, was prepared and filtered clear. A chymotrypsin stock solution contained 40 mg in 10 ml of 1 mM HCl. The activator was dissolved in the same buffer used for the substrate. For an assay, 5 ml of substrate, 0.5 ml of activator (or buffer in the control) and 0.5 ml of enzyme were incubated for 10 min at 25 °C. Then unhydrolyzed hemoglobin was precipitated with 10 ml of 0.3 M trichloroacetic acid and, after standing 5 min, was filtered through Whatman No. 2 paper and measured at 280 nm. A blank containing 2,2'-Q-Bzl was also read.

Azocoll. A typical reaction mixture contained 25 mg of azocoll, 5 ml of 50 mM Tris-chloride buffer, pH 7,5, containing 30 mM $CaCl_2$ (with or without activator) and 0.4 mg of chymotrypsin. Incubation was for 1 h in a shaker bath at 37 °C. The reaction mixture was then centrifuged and the clear supernatant measured at 520 nm.

Experiments with Ac-ONp. Continuous release of *p*-nitrophenol was measured in a Gilford model 2000 spectrophotometer at 400 nm, at 26 °C. Stock solutions: Ac-ONp, 1 mM in 10 mM Tris, 10 mM maleate, 10 mM $CaCl_2$, pH 7.0, containing 10% methanol; 2,2'-Q-Bzl, 20 mM in Tris-maleate buffer pH 7.0; chymotrypsin, 60 μM (M_r 23000) in 1 mM HCl. The cuvettes serving as controls contained 2.8 ml of the Ac-ONp stock solution; to this was added 0.2 ml of buffer, or 0.1 ml of the activator stock solution. Two other cuvettes contained (a) 2.8 ml Ac-ONp, 0.1 ml activator and 0.1 ml chymotrypsin or (b) 2.8 ml Ac-ONp, 0.1 ml of buffer and 0.1 ml of chymotrypsin. The enzyme was always added last, at zero time.

Reaction of Chymotrypsin with Ph_2NCF and Ph_2NCCl

For the reaction of Ph_2NCF with chymotrypsin at 0 °C the following stock solutions were prepared: activator 10 mM in 10 mM Tris, 10 mM maleate buffer, pH 7.0, containing 10 mM $CaCl_2$; chymotrypsin, 0.375 mg/ml (16.3 μM assuming M_r 23000) in 1.5 mM acetic acid, 10 mM $CaCl_2$, pH 3.5; Ph_2NCF, 1.2 mM in methanol, kept refrigerated and just before the experiment it was diluted 75-fold with the above Tris-maleate buffer. In the reaction the total volume was 20 ml, of which 0.5 ml was the enzyme solution, which was added last. The rest of the solution was Ph_2NCF, activator and buffer made up so that the final concentrations were: chymotrypsin, 0.4 μM; Ph_2NCF, 0.4 μM; activators, various concentrations in 0.1 mM range. The reaction solution was kept in an ice-water bath. At 5, 10, 15 and 25 min, 1-ml aliquots were removed using chilled pipettes and diluted to 15 ml with 15 mM acetic acid, 10 mM $CaCl_2$, pH 3.5, and kept on ice until the last aliquot was taken from the inactivation mixture. These samples were then assayed for their chymotrypsin activity using acetyl-DL-phenylalanine β-naphthyl ester [13] at 25 °C.

Inactivation by Ph_2NCCl was carried out similarly but at 25 °C. The concentrations of reactants were: Ph_2NCCl, 2 μM; chymotrypsin, 1.1 μM.

Reactivation of Ph_2NC-Chymotrypsin

In absence of Hydroxylamine. The following solutions were prepared: (a) 0.4 μM chymotrypsin (M_r 23 000) in Tris/maleate buffer, pH 7 (see above). (b) 0.4 μM chymotrypsin and 0.4 μM Ph_2NCF in Tris/maleate buffer. (c) 0.4 μM chymotrypsin, 0.4 μM Ph_2NCF and 0.5 mM 2,2'-Q-Bzl in Tris/maleate buffer. The total volume for each of the solutions was 20 ml. They were incubated at 25 °C and at various times (5, 15, 30, 60, 90, 120 min, 6 h, 23 h, 24 h, 25 h and 26 h) aliquots were assayed for chymotrypsin activity using acetyl-DL-phenylalanine β-naphthyl ester as substrate [13]. Complete inactivation occurred in less than 1 h; no reactivation was ever detected.

In presence of 2 M NH_2OH. To assay this reaction it was not possible to use acetyl-DL-phenylalanine β-naphthyl ester as substrate because it is decomposed by NH_2OH. Hence, Glt-Phe-Nan assay was used [5], which requires much more enzyme. The reaction tubes, therefore, contained higher concentrations of

Ph_2NCF and chymotrypsin as follows: (a) 43.5 μM chymotrypsin (M_r 23000) in 10 mM Tris, 10 mM maleate buffer, pH 7.0, containing 10 mM $CaCl_2$. (b) 43.5 μM chymotrypsin, 43.5 μM Ph_2NCF in Tris/maleate buffer, pH 7.0. (c) 43.5 μM chymotrypsin, 43.5 μM Ph_2NCF, 0.5 mM 2,2′-Q-Bzl in Tris/maleate buffer, pH 7.0. (d) 43.5 μM chymotrypsin, 0.5 mM 2,2′-Q-Bzl in Tris/maleate buffer, pH 7. 1-ml samples were assayed for enzymic activity using Glt-Phe-Nan as substrate. Inactivation occurred in less than 200 s. Then reactivation was measured at time intervals shown in Fig. 1.

Conversion to π-Chymotrypsin of Chymotrypsinogen and Chymotrypsinogen Covalently Linked to Q-BrMeBzl

Zymogen solutions were made up in 50 mM Tris-chloride buffer, pH 8.5, containing 20 mM $CaCl_2$ at a concentration of 1.0 mg/ml. Trypsin was dissolved in 1 mM HCl at a concentration of 0.5 mg/ml. To activate the zymogen (or 'azozymogen') 0.15 ml of the trypsin solution was added to 1.5 ml of the zymogen solution, which was cooled to 0 °C in an ice bath. The reaction mixture remained at 0 °C for 2 h. Aliquots were then diluted appropriately with buffer and assayed with specific substrate.

RESULTS

Activation of Glt-Phe-Nan by Various Effectors

Shown in Table 1 are the effects of various related-bis-quaternary azo derivatives on the hydrolysis of Glt-Phe-*p*Nan by α-chymotrypsin. All activators were present at a concentration of 0.1 mM. The most active compounds were all 2,2′-isomers; of these, Q-BrMeBzl was the most active with 2,2′-Q-Bzl following closely behind. One compound lacking the azo group, 2,2′-K-Bzl, was also active. All of the activities shown are well beyond experimental error.

Effects on Various Substrates

As described previously [1], acceleration of the hydrolysis of Glt-Phe-Nan by chymotrypsin in the presence of 2,2′-Q-Bzl occurred as a result of an increase of k_{cat} with no change occurring in K_m of the reaction. The same was found for acceleration of the hydrolysis of acetyltyrosine *p*-nitroanilide and acetyl-L-tyrosine anilide. Shown in Table 2 are the increases of V for the three substrates in the presence of saturating amounts of activator as determined by Lineweaver-Burk double-reciprocal plots (see [1] for data on Glt-Phe-Nan). Also listed are substrates for which no activation was observed at concentrations of 2,2′-Q-Bzl as high as 1 mM.

Table 1. *Effects of ligands on hydrolysis of Glt-Phe-Nan*
Concentration of Glt-Phe-Nan = 1 mM; enzyme concentration = 2.3 μM; buffer, 0.05 M Tris-HCl, pH 7.5, with 30 mM $CaCl_2$. All azo derivatives are in the *trans* configuration. v_a/v_0, ratio of mol *p*-nitroaniline released in 10 min in presence of ligand (v_a) to rate in absence of ligand (v_0)

Ligand (0.1 mM)	v_a/v_0
2,2′-Q-Me	1.2
3,3′-Q-Me	1.1
4,4′-Q-Me	1.1
2,2′-Q-Bzl	4.8
3,3′-Q-Bzl	1.4
4,4′-Q-Bzl	1.1
2,2′-K-Bzl[a]	1.4
2,2′-Q-NBzl	2.0
2,2′-Q-BrMeBzl	6.0

[a] $-\overset{\overset{O}{\|}}{C}-\overset{\overset{O}{\|}}{C}-$ in place of $-N=N-$.

Table 2. *Effect of 2,2′-Q-Bzl on chymotryptic hydrolysis of several substrates*
k'_{cat}: rate constant in presence of saturating concentrations activator; k^0_{cat}: rate constant in absence of activator. Data were obtained by measuring rates at four concentrations of substrate between 0.1 mM and 1 mM and six concentrations of Q-Bzl between 1.25 mM and 0.2 mM. The data were graphed as Lineweaver-Burk (double-reciprocal) plots, V was extracted for each concentration of activator. These data were then plotted on a graph with [E]/V as the ordinate and 1/[A] as abscissa. The intercept on the [E]/V axis was taken as k'_{cat} at saturation of activator (see [1]; Fig. 1 and 2). For Ac-Gly-OEt and below, activation was sought at 1.25 mM 2,2′-Q-Bzl and not observed

Substrate	k'_{cat}/k^0_{cat}
Glt-Phe-Nan	42
Ac-Tyr-Nan	25
Ac-Tyr-An_2	1.8
Ac-Gly-OEt	1
Bz-Gly-OEt	1
Ac-Tyr-OEt	1
Z-Tyr-NHOH	1
Ac-ONp	1
Hemoglobin	1
Azocoll	1

The binding of 2,2′-Q-Bzl to α-chymotrypsin was studied previously [1] and K was found to be $(6.0 \pm 0.6) \times 10^{-4}$ M. This figure agrees well with the concentration of activator at which half-maximal acceleration of Glt-Phe-Nan occurred [1].

Effects on Inactivation of α-Chymotrypsin by Ph_2NCF and Ph_2NCCl

We next studied the effect of Q-Bzl on the reactions of Ph_2NCCl and Ph_2NCF with α-chymotrypsin (13–16]. As shown in Table 3, inactivation by either reagent was accelerated by 2,2′-Q-Bzl. The reaction with Ph_2NCF was accelerated to a considerably greater

Table 3. *Effect of 2,2'-Q-Bzl on inactivation of chymotrypsin by Ph_2NCCl and Ph_2NCF*
The ratio v_a/v_0 was determined as follows: in the case of Ph_2NCF inactivation was carried out in 50 mM Tris-HCl buffer, pH 7.0, containing 20 mM $CaCl_2$ at a temperature of 1 ± 0.5 °C (in an ice bath). Concentrations of reactants were as follows: Ph_2NCF, 0.4 μM; E, 0.4 μM; Q-Bzl, 0, 0.1 mM, 0.4 mM, 0.8 mM and 1.2 mM. Rates of inactivation were calculated from a semilogarithmic plot of data obtained by assaying aliquots for chymotrypsin activity at various time intervals using acetyl-DL-phenylalanine β-naphthyl ester as substrate (details in Experimental Procedure). Double-reciprocal plots of rate data *versus* concentration of activator were plotted and the rate at saturation concentration of activator was obtained from the intercept of the ordinate. This is v_a; v_0 = rate in absence of activator. The same was done for Ph_2NCCl except for the following differences in conditions: temperature, 25 °C; concentration of Ph_2NCCl, 2 μM; enzyme 1.1 μM

Inactivator	v_a/v_0
Ph_2NCF	9.3
Ph_2NCCl	1.9

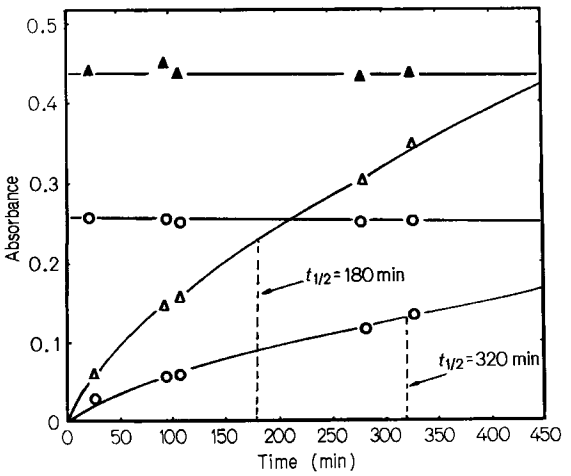


Fig. 1. *Reactivation of Ph_2NC-chymotrypsin by NH_2OH.* Reactivation in presence of 2,2'-Q-Bzl (Δ——Δ); reaction in absence of 2,2'-Q-Bzl (O——O). Controls: chymotrypsin (O——O); chymotrypsin + Q-Bzl (▲——▲)

extent than that of Ph_2NCCl. The reaction with Ph_2NCF was carried out at 0 °C instead of 25 °C because its rate of reaction was too fast to measure at the latter temperature; it is more reactive with chymotrypsin than Ph_2NCCl [16–17].

Effect on Reactivation of Ph_2NC-Chymotrypsin

We next were interested in studying the reactivation of Ph_2NC-chymotrypsin at 27 °C in 50 mM Tris-chloride buffer, pH 7.0, containing 30 mM $CaCl_2$. No measurable reactivation occurred either in the absence or the presence of 2,2'-Q-Bzl at concentrations as high as 0.5 mM, measured over a period of 26 h. On the other hand, reactivation by 2 M hydroxylamine was accelerated markedly by 0.17 mM 2,2'-Q-Bzl (Fig. 1).

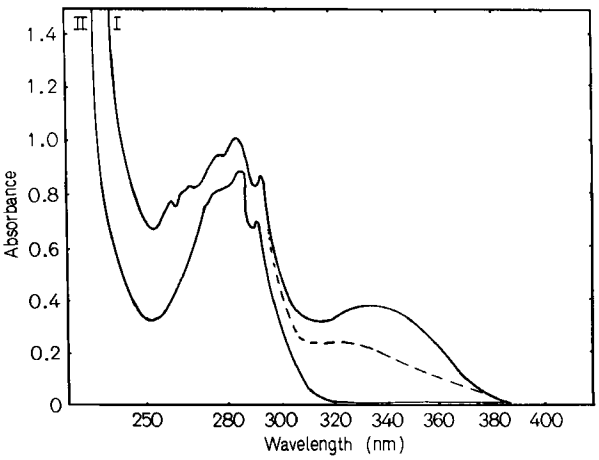


Fig. 2. *Ultraviolet spectra.* Curve I, chymotrypsinogen; curve II, zymogen covalently linked to effector molecule as result of reaction with 2,2'-Q-BrMeBzl, *i.e.* 'azozymogen'; (– – – –) 'azozymogen' after exposure to light of 330 nm

Covalent Linkage of 2,2'-Q-BrMeBzl to Chymotrypsin and to Chymotrypsinogen

Incubation of Q-BrMeBzl with chymotrypsin, followed by passage through BioGel P-6, yielded an activated product having about four times the reactivity of chymotrypsin with Glt-Phe-Nan as substrate. However, considerable denaturation of chymotrypsin took place during the reaction and separation procedures; hence, reaction was attempted with chymotrypsinogen A. A product ('azozymogen') was isolated which, by spectrophotometry, contained one molecule of azo compound per molecule of zymogen. Its spectrum is shown in Fig. 2. It has a maximum at 335 nm: 2,2'-Q-Bzl has a maximum at 331 nm. A similarly determined ultraviolet absorption spectrum of the modified enzyme in 0.01 M sodium dodecylsulfate showed a slight increase in absorption coefficient (approx. 2%) with no shift in the maximum. It should be recalled that azo compounds are photochromic [18] and can be converted to their *cis* isomer by exposure to light of appropriate wavelength (330 nm in this case) [19]. Hence, also in Fig. 2 is a spectrum of the zymogen derivative in which the covalently linked azo compound was converted to the *cis* isomer while attached to the zymogen.

The derivatized zymogen could be converted to the analogous active π-chymotrypsin derivative by exposure to trypsin at 0 °C for 2 h. When assayed against various concentrations of Glt-Phe-Nan along with identically activated native chymotrypsinogen, the kinetics could be described by the double-reciprocal plot in Fig. 3. Just as with the reversibly bound Q-Bzl, covalent linkage of the azo compound to chymotrypsin resulted in enzyme activation. Here too the effect was on V and not on K_m, the former being increased about four-fold. Exposure of the 'azo

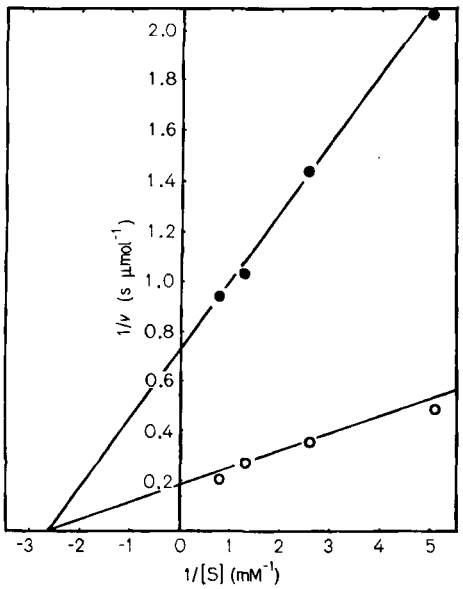


Fig. 3. *Kinetics of hydrolysis of Glt-Phe-Nan.* 'Azozymogen' (O——O); chymotrypsinogen (●——●). Both were activated by trypsin before assay (see text)

chymotrypsinogen' to light of 330 nm converted about 80% of the *trans* isomer to the *cis* isomer form; this product is about twice as active as π-chymotrypsin derived from native chymotrypsinogen A, *i.e.* one-half as active as that obtained from the *trans* isomer of 'azozymogen'.

DISCUSSION

The three-dimensional structure of chymotrypsin is known in detail to the extent that suggestions as to its catalytic mechanism extend down to the atomic level (*cf.* [20]). Its active site was shown to be a hydrophobic cleft or 'hole', in which specific and non-specific substrates were bound and hydrolyzed. Evidence for another hydrophobic binding site for specific substrates, expecially those with aromatic groups other than that of the amino acid residue, derives from experiments carried out in our laboratory [13, 21–24] and in a number of others [25–27]. Trypsin, an enzyme strikingly similar in structure to chymotrypsin, has a second substrate binding site which, when occupied, produces a considerable acceleration of the hydrolysis of certain specific substrates [28, 29].

Prior to our earlier report [1], activation of chymotrypsin by ligands had been reported only for non-specific substrates [30, 31] and the extent of activation, although significant, was not very great. The activation of chymotrypsin reported in this paper occurs with specific substrates and can be as high as 40-fold. Because of the bulk of the specific substrates, the activation process is not likely to be an isosteric one (in which the activator shares the catalytic site with the substrate); rather it is allosteric [3]. Despite the wealth of knowledge about the structure of chymotrypsin, it is not possible to predict where this allosteric site might be. If we could, it might be possible to propose a likely mechanism for the activation process.

On the other hand, some of the characteristics of the process can be listed and interpreted.

a) Substrates for which the rate-limiting step in hydrolysis is deacylation are not affected, *e.g.* Ac-Tyr-OEt and Ac-ONp.

b) The hydrolysis of amide substrates, for which the rate-limiting step is acylation, can be accelerated, but in the examples examined thus far the leaving group is limited to aromatic amines.

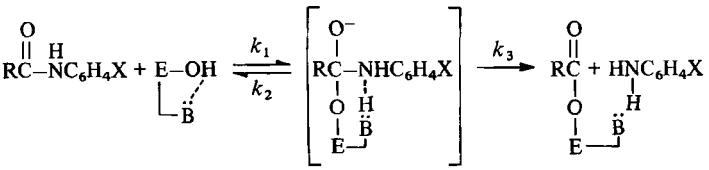
c) The extent of acceleration is determining by some aspect of the structure of the aromatic amine, *e.g.* hydrolysis of *p*-nitroanilides are accelerated more than that of anilides. Depending upon one's point of view (see below), this can be explained in terms of electronegativity or of hydrophobicity. Thus, aniline is less electronegative but also less hydrophobic than *p*-nitroaniline; anilides were accelerated to a lesser extent than nitroanilides.

d) Acceleration occurred as result of an increase in k_{cat}; K_m was unaffected.

e) Inactivation (=acylation) of the enzyme by diphenylcarbamyl derivatives was accelerated by 2,2'-Q-Bzl. Ph_2NCF, with the more electronegative group, was affected more than Ph_2NCCl. Unlike the leaving groups of affected substrates (above), the inactivator's leaving groups are not aromatic.

f) Deacylation (=reactivation) of Ph_2NC-chymotrypsin by water or Tris buffer is unaltered by the presence of effector. On the other hand, reactivation by hydroxylamine was accelerated.

We can conclude that the activators can accelerate some part of the acylation mechanism. Except for the case in which there was an added nucleophile, *i.e.* NH_2OH reactivation of Ph_2NC-chymotrypsin, deacylation was not affected. Thus, k_{cat} of hydrolysis of Ac-Tyr-OEt and Ac-ONp were not influenced by the presence of allosteric effector. The mechanism of this effect is not clear. According to some investigators [32, 33], the hydrolysis of anilides proceeds as follows:



with k_3 being rate-limiting. In this mechanism the electronegativity of the leaving group can influence k_{cat} of hydrolysis. Electronegativity would favour k_1

and, hence, the equilibrium concentration of the tetrahedral intermediate. It would work against k_3 because that step requires the transfer of a proton to the leaving group. However, k_{cat}/K_m, for the hydrolysis of Ac-Tyr-Nan is 72 M^{-1} s^{-1} [34] compared to 18 M^{-1} s^{-1} for the less electronegative acetyl-L-tyrosine *p*-methoxyanilide [35]. It appears then that the enzyme has a mechanism to facilitate proton transfer despite a barrier posed by a highly electronegative leaving group. Thus, with k_2 favored and k_3 not adversely affected, Ac-Tyr-Nan becomes a better substrate than Ac-Tyr-An_2. The proton transfer mechanism might be further facilitated by the positively charged quaternary compounds by means of an inductive effect on the 'charge relay' system [20]. Greater effects would be seen with highly electronegative leaving groups (nitroanilide > anilide; F > Cl) because the original energy barrier to proton transfer would be greater.

On the other hand, if one believes, as do Fersht and his colleagues [36, 37], that there is no accumulation of a tetrahydral intermediate and that variations in the hydrolysis of anilides result from differences in the extent of non-productive binding to a second hydrophobic site in chymotrypsin, then we might say that the activators somehow decrease the extent of non-productive binding. However, as Fastrez and Fersht [36] point out, non-productive binding lowers both K_m and k_{cat}. Therefore, we would have expected to see a change in both constants rather than just in k_{cat}. One might also question why the hydrolysis of Bz-Gly-OEt was not accelerated.

From an examination of the structures and relative activities of the activators (Table 1), we can conclude that hydrophobic as well as electrostatic forces are important in the binding interaction and in the subsequent activation process, *e.g.* Q-Bzl is better than Q-Me. The presence of the bridging azo group, or of some other group capable of donating electrons (as in K-Bzl), also appears to be important, and perhaps might explain why we found at concentrations as high as 1.33 mM that 1-nitro-2(benzyldimethylammoniummethyl)benzene bromide and the analogous 1-amino compound were inactive.

All of the activators of chymotrypsin are potent inhibitors of another serine esterase, acetylcholinesterase [38] (and unpublished results). Moreover, although they cause no noticeable effect on the hydrolysis of Bz-Arg-Nan by trypsin, we have preliminary evidence that they do bind to trypsin. Thus, in each of three serine esterases, there is a site capable of binding quaternary azo compounds which, perhaps, evolved from the same 'primitive' serine esterase [39].

A crucial question, of course, is the location of the allosteric site in chymotrypsin. The spectral data (Fig. 2) and the additional finding that no spectral shift occurs in 0.01 M sodium dodecylsulfate indicate that the chromophoric azobenzene moiety is located in a region with a polarity about the same as that of water. In earlier studies [40] with *p*-phenylazodiphenylcarbamyl-chymotrypsin (in which the serine at the active site was acylated by the reaction with *p*-phenylazodiphenylcarbamyl chloride), the ultraviolet spectrum of the azobenzene chromophore shifted from 334 nm in buffer to 345 nm in the presence of 0.01 M sodium dodecylsulfate. Thus, the active site of chymotrypsin is less polar than water and, in fact, hydrophobic in character (*cf.* [41]). On the other hand, the spectrum of *p*-phenylazodiphenylcarbamyl-trypsin is the same in buffer as in sodium dodecylsulfate, indicating that the microenvironment surrounding the inactivator in trypsin is similar in polarity to that of water. The allosteric site of chymotrypsin, therefore, is more polar in nature than the active site and may actually be on the outside surface of the chymotrypsin molecule. A more direct approach to the problem of locating the site will be pursued now that we have covalently linked an effector to chymotrypsinogen. We will proceed to digest the derivative and to isolate the peptide fragment to which the effector is attached.

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B. F. Erlanger, N. H. Wassermann, A. G. Cooper, and R. J. Monk, Department of Microbiology,
Columbia University College of Physicians and Surgeons, 630 West, 168th Street, New York City, New York, U.S.A. 10032