ALLOSTERIC ACTIVATION OF CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SPECIFIC SUBSTRATES

B.F. Erlanger, N.H. Wassermann and A.G. Cooper

Department of Microbiology, College of Physicians and Surgeons
Columbia University, New York, N.Y. 10032

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SUMMARY

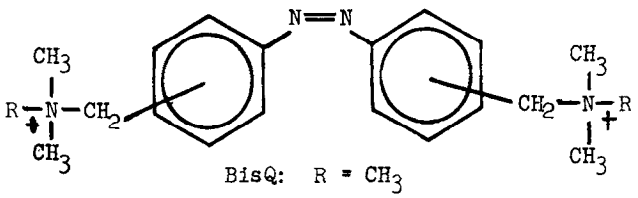
The rates of hydrolysis of three specific substrates of chymotrypsin, glutaryl-L-phenylalanine p-nitroanilide, acetyl-DL-tyrosine p-nitroanilide and acetyl-L-tyrosine anilide were enhanced by 2,2'-bis[α-(benzyldimethylammonium)methyl]azobenzene dibromide and less so by related compounds. Detailed studies with glutaryl-L-phenylalanine p-nitroanilide showed a 42-fold increase in k_{cat} with no change in Km. No acceleration (or inhibition) was noted with esters, hydroxamides or proteins as substrates. Tryptic hydrolysis of benzoyl-DL-arginine p-nitroanilide was unaffected. It was concluded that certain quaternary compounds can act as allosteric effectors of chymotrypsin.

Chymotrypsin is one of the few enzymes whose three-dimensional structure has been so well characterized that speculations about its catalytic mechanism extend down to the atomic level (cf. 1,2,3). Its active site was shown to be part of a hydrophobic pocket, called the "tosyl hole," in which specific and non-specific substrates were bound and hydrolyzed by a general base-general acid catalyzed reaction (4,5). That there may be at least one other site important for catalysis has been suggested from experiments in our laboratory (6) and a number of others (7,8,9), but, generally speaking, the x-ray crystallographic interpretations have given this suggestion little attention. The structurally related proteolytic enzyme, trypsin, does have a second, substrate-interacting site, which, when occupied, produces a very significant acceleration of the hydrolysis of certain specific substrates (10,11). On the other hand, activation of chymotrypsin by ligands has been reported only for non-specific substrates (12,13), and the extent of this activation has been far from dramatic.

Abbreviations: BisQ, bis[α-(trimethylammonium)methyl]azobenzene dibromide; BisQ Benzyl, bis[α-(benzyldimethylammonium)methyl]azobenzene dibromide; GPANA, glutaryl-L-phenylalanine p-nitroanilide; ATNA, acetyl-DL-tyrosine p-nitroanilide; ATA, acetyl-L-tyrosine anilide.

In the course of studies on bis-quaternary inhibitors of acetylcholinesterase and of the acetylcholine receptor (14,15), we were led to examine their action on chymotrypsin. Unexpectedly, some of them caused acceleration of the hydrolysis of the specific substrate, glutaryl-L-phenylalanine-p-nitroanilide (GPANA). One, in particular, 2,2'-bis[α-(benzyldimethylammonium)methyl]azobenzene dibromide (2,2'-BisQ Benzyl) caused an approximately 40-fold increase in k_{cat}.

BisQ Benzyl: R = $CH_2\,\phi$



BisQ: R = CH_3

In this report, we will describe our findings and speculate on the mechanism and significance of the activation process.

EXPERIMENTAL

Substrates - GPANA was prepared as previously described (16). Acetyl-DL-tyrosine p-nitroanilide (ATNA) was prepared from acetyl-L-tyrosine, p-nitroaniline and P_2O_5 (cf. 17). Its physical characteristics were the same as those described by Inagami and Sturtevant (18). 2,2'-BisQ Benzyl was prepared by the reaction of benzyl bromide with 2,2'-bis-dimethylaminomethylazobenzene (manuscript in preparation); m.p. 169-170. Acetyl-L-tyrosine anilide (ATA) was prepared by reaction of the ester with aniline; m.p. 241-242 (reported 236-7 (19)). Carbobenzoxy-L-tyrosine hydroxamide was prepared from the ester (20) by reaction with alkaline hydroxylamine; it melted at 175-176.5 and analyzed correctly. Acetyl-L-tyrosine ethyl ester and hemoglobin were products of Schwarz/Mann Research Labs. Azocoll was a gift from Dr. I. Mandl. Acetylglycine ethyl ester was synthesized (21). Alpha-chymotrypsin and Y-chymotrypsin were Worthington products.

Kinetic assays using GPANA and ATNA were run on a Gilford Model 2000 Spectrophotometer using a procedure in which the rate of formation of p-nitroaniline was measured (16). Titrimetric procedures were used for

TABLE I

Effect of ligands on the rate of hydrolysis of GPANA*

Ligand (10^{-4}M)	Va/Vo**
2,2'-BisQ	1.2
3,3'-BisQ (15)	1.1
4,4'-BisQ	1.1
2,2'-BisQ Benzyl	4.8
3,3'-BisQ Benzyl	1.4
4,4'-BisQ Benzyl	1.1
2,2'-diketo BisQ Benzyl***	1.4

*[GPANA] = 10^{-3}M, [E] = 2.3×10^{-6}M (assayed for operational normality according to ref. 22), 0.05 M Tris hydrochloride, pH 7.5, 0.03 M $CaCl_2$. Method according to ref. 16. Procedures for the synthesis of the ligands will appear in a paper now in preparation.

** Va/Vo = ratio of moles p-nitroaniline released in 10 minutes in presence of ligand (Va) to rate in absence of ligand Vo.

*** This compound differs from 2,2'-BisQ Benzyl by having

in place of $-N=N-$.

the kinetic assays in which ATA, acetyl-L-tyrosine ethyl ester and acetyl-glycine ethyl ester were the substrates. Some confirmatory experiments with GPANA were run titrimetrically.

RESULTS

Shown in Table I are the effects on the chymotryptic hydrolysis of GPANA of various structurally related bis-quaternary compounds at a concentration of 10^{-4}M. None were inhibitors. The most potent activator,

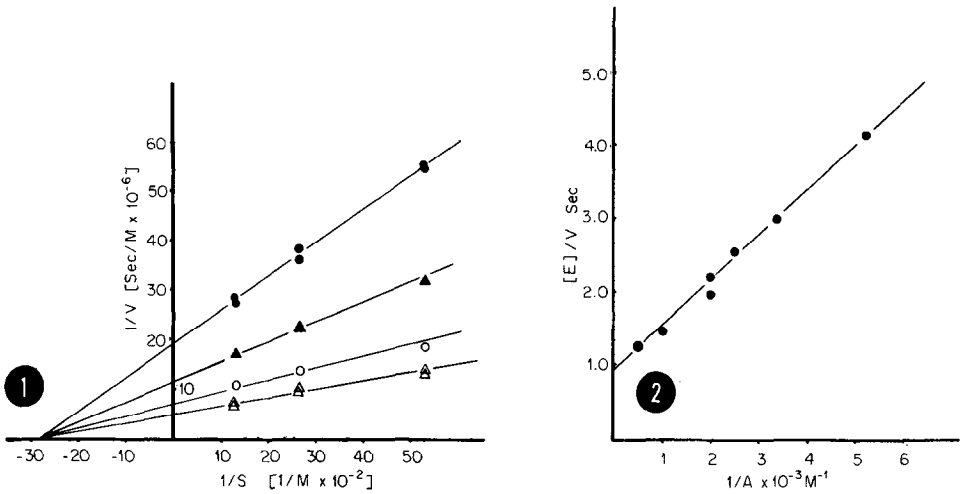


Fig. 1 - Reciprocal plot of kinetics of hydrolysis of GPANA in presence and absence of 2,2'-BisQ Benzyl. No 2,2'-BisQ Benzyl, ● ——●; 6.7 x 10^{-6}M BisQ Benzyl, ▲ ——▲; 2.5 x 10^{-5}M BisQ Benzyl, O —— O ; 5 x 10^{-5}M BisQ Benzyl Δ——Δ. [E], 2.2 x 10^{-6}M (MW = 23,500). Buffer, 0.05M tris-hydrochloride, 0.03M $CaCl_2$, pH 7.5 at 25°. Intercept of abscissa (-1/Km) yields Km of 3.6 x 10^{-4}M; literature (16) reported 2.8 x 10^{-4}M under similar conditions.

Fig. 2 - Reciprocal plot of kinetics of activation by 2,2'-BisQ Benzyl extrapolated to saturating concentration of activator (A). [GPANA], 8 x 10^{-4}M; [E], 0.46 x 10^{-6}M (MW 23,500). Buffer and temperature as in legend to figure 1. Intercept of ordinate ($1/k_{cat}$) is 0.95 sec.

2,2'-BisQ Benzyl, caused a four-fold acceleration. The 3,3' isomer was considerably less active, as was the 2,2' diketo analogue. Significant activity (20% enhancement) was also shown by 2,2'-BisQ. The activities of the other compounds might or might not be significant.

Lineweaver-Burk plots of data from an extensive kinetic study of the action of 2,2'-BisQ Benzyl, in which substrate and ligand concentrations were varied (Fig. 1), showed no change in K_m of the chymotrypsin-GPANA interaction. However, k_{cat} was increased 42-fold to 1.05 sec^{-1} (Fig. 2) compared with 0.025 sec^{-1} in absence of activator (16).

The rates of hydrolysis of acetylglycine ethyl ester, acetyl-L-tyrosine ethyl ester, carbobenzoxy-L-tyrosine hydroxamide, hemoglobin and azocoll

were unaffected by 2,2'-BisQ Benzyl at 10^{-4}M. Acceleration of hydrolysis was seen with ATNA and ATA, that of ATNA being about 25-fold at saturation. The effect on the hydrolysis of ATA was less marked, preliminary kinetic studies indicating a maximal 2-3-fold acceleration at saturation.

Preliminary experiments showed that the activity of γ-chymotrypsin vs. GPANA was accelerated to the same extent by 2,2'-BisQ Benzyl. Tryptic hydrolysis of benzoyl-DL-arginine p-nitroanilide (23) was unaffected by any of the compounds in Table I; all inhibited acetylcholinesterase (14, and paper in preparation).

Binding of 2,2'-BisQ Benzyl to α-chymotrypsin was measured (24). The binding constant was found to be $6.0 \pm 0.6 \times 10^{-4}$M. This agrees well with the concentration at which half maximal activation of GPANA hydrolysis occurs (Fig. 2).

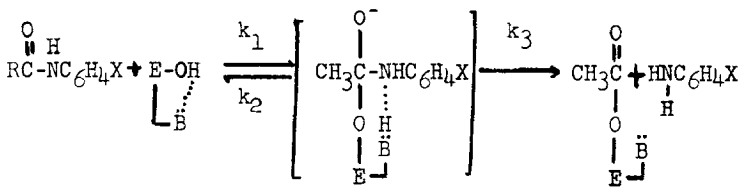
DISCUSSION

Our findings indicate that chymotrypsin has a functionally important binding site other than the "tosyl hole." The accelerator binding site may be near the "tosyl hole" but not near enough to produce steric problems when occupied by relatively large ligands. Hence, by definition, it is an allosteric site. It seems likely that the site is hydrophobic because 2,2'-BisQ Benzyl is a more effective activator than 2,2'-BisQ; it is also likely that it has an anionic component. We have no information as to whether the site binds both halves of the bis-quaternary compounds.

We can, at this time, only speculate about the mechanism of the acceleration process. Binding of the substrate is not affected; k_{cat} is. It is reasonable to assume that the cationic character of at least one of the quaternary groups is important in the acceleration mechanism and that their position in space is critical since the 2,2' isomer is most active. But what are the active ligands doing? There are two important clues: (a) Ester substrates are not affected. This is true whether the rate-limiting step is acylation (acetylglycine ethyl ester) or deacylation

(acetyl-L-tyrosine ethyl ester) (8). (b) Anilide substrates are affected but amides (i.e. peptides) are not. With respect to the anilides, the extent of acceleration is related to the electronegativity of the aniline moiety.

Chymotryptic hydrolysis of anilides is believed to include a tetrahedral intermediate and proton transfer:



with the rate-limiting step being governed by k_3 (25). The overall rate of hydrolysis by enzyme is influenced by the electronegativity of the leaving group, e.g. k_{cat}/K_m for ATNA is 261 (26) vs. 18 M^{-1} sec^{-1} for acetyl-L-tyrosine-p-methoxyanilide (5). We must assume, therefore, that in the enzymic hydrolysis there is a mechanism that facilitates proton-transfer to the nitrogen so that electronegativity does not have a marked negative effect on k_3. Overall rates would then increase with increasing electronegativity because of the favorable effect on k_1**. However, highly electro-

**We are aware of the finding of Parker and Wang (4) that k_{cat} increases with increasing basicity of some anilides. However, using their data, k_{cat}/K_m, the second order rate constant of acylation (27), does not follow basicity in any consistent fashion. Moreover, the data in the literature for amide substrates is more consistent with electron withdrawing groups favoring hydrolysis, e.g.

acetyl-L-tyrosinamide,	k_{cat}/K_m, 4.4 (28);	pKa, NH_4, 9.25 (29)	
acetyl-L-tyrosylglycinamide,	" " 19.1 (28);	"	glycinamide, 7.93 (30)
acetyl-L-tyrosinhydroxamide,	" " 45.1 (28);	"	hydroxylamine, 5.96 (29)
acetyl-L-tyrosine-p-nitroanilide,	" "261 (26);	"	p-nitroaniline, 1.11 (29)

negative leaving groups might pose a partial barrier to the proton-transfer facilitation mechanism. If BisQ Benzyl acts by aiding this mechanism (see below), it would have its greatest effect on nitroanilides, since for other substrates, near optimal conditions for proton-transfer might already prevail. It is interesting to note that BisQ Benzyl at saturation increased k_{cat}/K_m of ATNA to about 6,500 M^{-1} sec^{-1} so that it became 370X higher than k_{cat}/K_m of

acetyl-L-tyrosine p-methoxyanilide. This is similar to the ratio of the equilibrium constants (i.e. about 500) for transfer of the acetyl groups of these anilides to aminoazobenzenesulfonic acid by acetyl CoA-arylamine-N-transacetylase (27). (For GPANA, k_{cat}/K_m is about 3,000 M^{-1} sec^{-1} in the presence of 2,2'-BisQ Benzyl at saturation.)

We suggest that activation follows binding to a site on the surface of the enzyme via a hydrophobic linkage with the N-benzyl group, rather than the azobenzene portion of the compound. The cationic quaternary nitrogens (or one of them) must also play a part in binding, but their major function is probably to provide an environment that either screens unfavorable electronegative effects or, by induction, facilitates proton-transfer. Since the charge relay system is implicated in proton-transfer (1), the ligand might indirectly affect it. It would have to be indirect because Asp 102 is buried in the enzyme's interior. Alternatively, binding of the ligand might in some way induce favorable conformational changes at the active site of the enzyme.

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REFERENCES

1. Birktoft, J.J. and Blow, D.M., J. Mol. Biol. 68, 187 (1972).
2. Segal, D.M., Powers, J.G., Cohen, G.H., Davies, D.R. and Wilcox, P.E., Biochemistry 10, 3728 (1971).
3. Freer, S.T., Kraut, J., Robertus, J.D., Wright, H.T. and Xuong, N.H., Biochemistry 9, 1997 (1970).
4. Parker, L. and Wang, J.H., J. Biol. Chem. 243, 3729 (1968).
5. Inagami, T., York, S.S. and Patchornik, A., J. Amer. Chem. Soc. 87, 126 (1965).
6. Erlanger, B.F., Proc. Nat. Acad. Sci. USA 58, 703 (1968).
7. Hardman, M.J., Valenzuela, P. and Bender, M.L., J. Biol. Chem. 246, 5907 (1971).
8. Kaplan, H. and Laidler, K.J., Can. J. Chem. 45, 559 (1967).
9. Neil, G.L., Niemann, C. and Hein, G.E., Nature 210, 903 (1966).
10. Trowbridge, C.G., Krehbiel, A. and Laskowski, Jr., M., Biochemistry 2, 843 (1963).
11. Kallen-Trummer, V., Hofmann, W. and Rottenberg, M., Biochemistry 9, 3580 (1970).

12. Wallace, R.A., Peterson, R.L., Niemann, C. and Hein, G.E., Biochem. Biophys. Res. Comm. 23, 246 (1966).
13. Ponzi, D.R. and Hein, G.E., Biochem. Biophys. Res. Comm. 25, 60 (1966).
14. Wassermann, N.H., Bartels, E., Cooper, A.G. and Erlanger, B.F., Abstracts, 164th Amer. Chem. Soc. Meeting, Biol. 170 (1972).
15. Bartels, E., Wassermann, N.H. and Erlanger, B.F., Proc. Nat. Acad. Sci. USA 68, 1820 (1971).
16. Erlanger, B.F., Edel, F. and Cooper, A.G., Arch. Biochem. Biophys. 115, 206 (1966).
17. Erlanger, B.F. and Kokowsky, N., J. Org. Chem. 26, 2534 (1961).
18. Inagami, T. and Sturtevant, J.M., Biochem. Biophys. Res. Comm. 14, 69 (1964).
19. Bovarnick, M. and Clarke, H.T., J. Amer. Chem. Soc. 60, 2429 (1938).
20. Bergmann, M. and Zervas, L., Ber. 65, 1192 (1932).
21. Wolf, J.P., III and Niemann, C., Biochemistry 2, 493 (1963).
22. Erlanger, B.F. and Edel, F., Biochemistry 3, 346 (1964).
23. Erlanger, B.F., Kokowsky, N. and Cohen, W., Arch. Biochem. Biophys. 95, 271 (1961).
24. Hirose, M. and Kano, Y., Biochim. Biophys. Acta 251, 376 (1971).
25. Caplow, M., J. Amer. Chem. Soc. 91, 3639 (1969).
26. Bundy, H.F., Arch. Biochem. Biophys. 102, 416 (1963).
27. Jencks, W.P., Schafthausen, B., Tornheim, K. and White, H., J. Amer. Chem. Soc. 93, 3917 (1971).
28. Foster, R.J. and Niemann, C., J. Amer. Chem. Soc. 77, 1886 (1955).
29. Jencks, W.P. and Regenstein, J. in Sober, H.A. (Ed.), Handbook of Biochemistry, Chemical Rubber Co., Cleveland, Ohio, pp. J-150 - J-189 (1968).
30. Edsall, J.T. and Wyman, J., Biophysical Chemistry 1, 208 (1958).