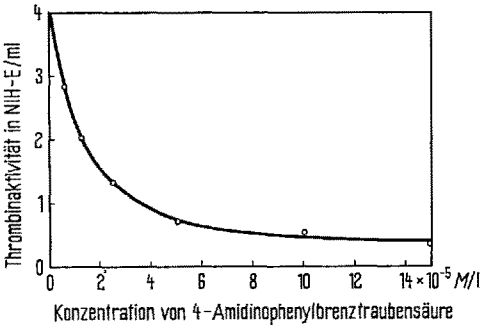
Antithrombinwirkung von Benzylamin-, Phenylguanidin- und Benzamidin-Derivaten

Hemmstoff	$Ki\,(M)$
Benzylamin	$1{,}2 \times 10^{-2}$
4-Aminomethylbenzoesäure	$> 2{,}0 \times 10^{-2}$
4-Methoxybenzylamin	$4{,}0 \times 10^{-3}$
4-Aminobenzylamin	$3{,}8 \times 10^{-3}$
4-Chlorbenzylamin	$2{,}2 \times 10^{-4}$
4-Aminomethylbenzoesäurebenzylester	$1{,}4 \times 10^{-3}$
Phenylguanidin	$9{,}0 \times 10^{-3}$
4-Guanidinobenzoesäure	$4{,}0 \times 10^{-2}$
Benzamidin	$2{,}0 \times 10^{-4}$
4-Aminobenzamidin	$8{,}0 \times 10^{-5}$
4-Amidinobenzoesäure	$> 2{,}0 \times 10^{-3}$
4-Amidinobenzoesäurebenzylester	$1{,}5 \times 10^{-4}$
4-Amidinophenylbrenztraubensäure	$3{,}0 \times 10^{-5}$

nenen kompetitiven Thrombinhemmstoffes Tosylagmatin[5,6] bei weitem.

Die Feststellung, dass die bisher gefundenen kompetitiven Thrombinhemmstoffe zugleich Hemmstoffe des Trypsins sind[7,8], weist auf eine enge Verwandtschaft der beiden Fermente in ihrem Wirkungsmechanismus und im Aufbau ihres aktiven Zentrums hin[9].

Summary. A series of aminomethyl, guanidino and amidino derivatives of benzene were investigated for their inhibitory effect on the activity of thrombin. Amidines were generally more potent than the other compounds. *p*-Amidinophenylpyruvic acid, a strong trypsin inhibitor, was found also to be the strongest small-molecular inhibitor on the activity of thrombin.



Hemmung der Thrombinwirkung auf Fibrinogen durch 4-Amidinophenylbrenztraubensäure (gemessen an der Gerinnungszeit von 1%igen Rinderfibrinogen-Lösungen in *Tris*-HCl-Puffer pH 7,4 bei 37 °C).

F. MARKWARDT und P. WALSMANN

Pharmakologisches Institut der Medizinischen Akademie Erfurt (DDR), 21. August 1967.

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Reaction of Lysozyme with Dithiothreitol and with Other Mercaptans

Most proteins contain disulfide bonds and these bonds have a unique structural role because they can link together otherwise separate polypeptide chains or tie noncontiguous parts of a single chain into loops. Scission of the disulfide bonds has frequently been used in studies of protein structure, of their function, and of the relationship between them. Disulfides may be cleaved by reaction with mercaptans, and various mercaptans have been used for this purpose with several proteins. However, little information is as yet available about the kinetics of the reaction and about the factors that determine reactivity[1].

In the present study, the rates have been measured for the reaction of 4 mercaptans with hen's egg-white lysozyme (E.C. 3.2.1.17)[2]. The rates vary widely and even the kinetic law may be different. The reactivity of lysozyme is of particular interest because its complete molecular structure is now known. The molecule consists of a single chain of 129 amino acid residues, folded to form a compact, rough ellipsoid, about $45 \times 30 \times 30$ Å. Four cystine residues are present, linking positions 6–127, 30–115, 64–80 and 76–94[3].

Two samples of lysozyme were examined, from Worthington Biochemicals (twice crystallized, salt-free) and from Calbiochem (grade A), with the same results. 2-Aminoethanethiol hydrochloride from Evans Chemetics was recrystallized from ethanol-ether to remove impurities that absorbed at 280 nm. Dithiothreitol (DTT) from Aldrich and all other chemicals were used without further purification.

Lysozyme was dissolved in 0.025 M borate buffer and mixed with a freshly prepared solution of thiol in the same medium. As the reaction proceeded, a precipitate formed. At appropriate intervals, a 0.2 ml aliquot of the reaction solution was withdrawn and was added to 5.00 ml of 0.1 M hydrochloric acid; the mixture was centrifuged at 2000 rpm for 3 min to remove any precipitate; and the absorbance of the supernatant was determined at 280 nm. The difference between the absorbance at any time and that at zero time gave the fraction of lysozyme that had reacted and precipitated.

A plot of log $[L]$ versus time ($[L]$ = lysozyme concentration) gave sensibly straight lines, i.e. first-order kinetics were followed. The Figure shows some representative results. Since the mercaptans were taken in large excess, the order does not reflect the molecularity of the reaction; this was investigated by varying the mercaptan concentration. The Table reports the half-reaction times obtained with the various mercaptans and in different conditions. The product obtained in the reaction with 2-mercaptoethanol was characterized in the

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[2] P. JOLLÉS, in *The Enzymes* (Eds. P. D. BOYER, H. LARDY and K. MYRBÄCK; Academic Press, New York 1960), vol. 4, p. 431.
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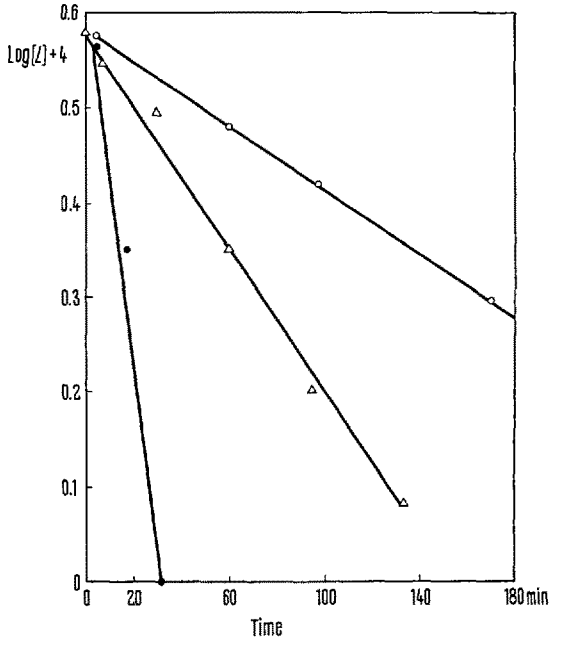
following way. After 50–75% of the reaction has occurred, the reaction mixture was centrifuged to separate the precipitate. This was washed twice with 0.001 *M* disodium ethylenedinitrilotetraacetate (EDTA) and then dissolved in 8 *M* urea-0.001 *M* EDTA. This solution was tested for -SH content with *N*-ethylmaleimide[4]. The result was 4.3 ± 0.2 SH groups/molecule; i.e. precipitation occurred after 2 of the 4 disulfide groups in the enzyme molecule has been reduced. A similar result has been obtained by electrolytic reduction[5].

It can be seen from the Table that the reactivity of the thiols at comparable concentrations and pH 10 is: DTT > 3-mercaptopropionate > 2-mercaptoethanol ⩾ 2-aminoethanethiol. This was not expected, because the mechanism of disulfide interchange involves the RS^- ion[6], and 2-aminoethanethiol is more extensively ionized than the other mercaptans[7]. The result indicates that other factors are of determining importance, e.g. the accessibility of the disulfide bonds – or of some particular disulfide bond – to the reagent.

Half-reaction periods

Thiol	Thiol concentration	pH	Half-reaction period, min
2-Mercaptoethanol	0.150	10.0	27
	0.100	10.0	37
	0.075	10.0	65
	0.050	10.0	110
	0.025	10.0	314
	0.050	9.0	860
DTT	0.050	10.0	20
	0.025	10.0	20
	0.012	10.0	20
	0.050	9.0	20
	0.050	7.8	200
	0.050	7.3	1100
3-Mercaptopropionate	0.050	10.0	56
2-Aminoethanethiol	0.050	10.0	ca. 4000



Rates of reaction of lysozyme with 0.05 *M* mercaptan at 25 °C. Empty circles: DTT at pH 7.8; triangles: 2-mercaptoethanol at pH 10; full circles: DTT at pH 10.

For 2-mercaptoethanol in the concentration range 0.15–0.05 *M*, the rate of reaction is inversely proportional to the thiol concentration, showing that the kinetic law is (L_i = insoluble product):

$$dL_i/dt = k[\mathrm{RSH}]\,[L] \qquad (1)$$

On the other hand, the reaction with DTT (at 0.05 to 0.012 *M*) is independent of the thiol concentration. This indicates that the thiol is not involved in the rate-determining step. A plausible explanation is that the disulfide group(s) is (are) not susceptible to reaction when the molecules of lysozyme are in the 'normal' (lowest-energy) configuration and that reaction becomes possible when appropriate deformations of the molecule expose or strain the group(s). Let L_r represent the reactive molecules:

$$L \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} L_r$$

$$L_r + \mathrm{RSH} \xrightarrow{k_2} L_i + {}^1/_2(\mathrm{RSSR})$$

Assume further that the steady-state approximation can be applied to L_r:

$$d[L_r]/dt = k_1[L] - k_{-1}[L_r] - k_2[L_r][\mathrm{RSH}] = 0$$

$$[L_r] = k_1[L]/(k_{-1} + k_2[\mathrm{RSH}])$$

It may then be seen that the reaction will reduce to the required form, independent of thiol concentration, if $k_{-1} < k_2(\mathrm{RSH})$:

$$dL_i/dt = k_2[L_r][\mathrm{RSH}] \sim k_1[L]$$

If, on the other hand, $k_{-1} \gg k_2[\mathrm{RSH}]$ – this implies that L_r would effectively be in equilibrium with L throughout the reaction – one obtains the rate expression (1).

Lowering the pH reduces the concentration of RS^- and therefore should decrease the rate of reaction with 2-mercaptoethanol; but not with DTT, since the rate-determining step does not involve the mercaptan. This is in agreement with the experimental observations as the pH is changed from 10 to 9. As the pH is lowered further, one might anticipate that the reaction with RS^- would become rate-determining in the case of DTT also, as is found in the range of pH 8–7[8].

Riassunto. Sono misurate le velocità di reazione della lisozima con 0.05 *M* ditiotreitolo, $HSCH_2CH_2OH$, ($HSCH_2CH_2COO^-$) e $H_2SCH_2CH_2NH_2$ in tampone borace di pH 10 e in alcune altre concentrazioni e pH. La cinetica della reazione col ditiotreitolo è differente da quella con $HSCH_2CH_2OH$ e viene data una interpretazione di questo fenomeno.

G. Gorin, R. Fulford and R. C. Deonier

Chemistry Department, Oklahoma State University, Stillwater (Oklahoma 74074, USA), 25 July 1967.

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[8] This work was aided by a grant and a Career Development Award (to G.G.) from the National Institutes of Health, U.S. Public Health Service.