Biochimica et Biophysica Acta, 527 (1978) 42—55
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BBA 68600

ENHANCEMENT OF α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SPECIFIC *p*-NITROANILIDE SUBSTRATES BY 4-PHENYLBUTYLAMINE DERIVATIVE OF HEN EGG-WHITE LYSOZYME *

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(Received May 19th, 1978)

Summary

Modification of hen egg-white lysozyme by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide in presence of 4-phenylbutylamine yielded derivatives, which contained 0.6—0.7 modified residues and retained about 60% of the original activity. Kinetic studies revealed that the modified-lysozyme increases approx. 20-fold the k_{cat} of hydrolysis of $SucGly_2Phe$-4-nitroanilide by α-chymotrypsin, without changing the K_m. The apparent dissociation constant of phenylbutylamine-modified lysozyme · chymotrypsin complex was found to be 0.03 mM and independent of substrate concentration. The accelerating effect of the modified lysozyme was also observed with other *p*-nitroanilide substrates of α-chymotrypsin. However, the hydrolysis of other substrates, acylation by active site titrant or inhibition by irreversible or competitive inhibitors were uneffected. The enhancing effect of the modified lysozyme seems to be very specific since other chymotrypsin-like enzymes, or serine proteinases except δ-chymotrypsin, were not influenced and phenylbutylamine derivatives of α-lactalbumin or ribonuclease were lacking any enhancing effect. Smaller, but significant enhancing effect was found also in lysozyme substituted by benzylamine, β-phenylethylamine and tryptamine and in inactive derivatives of lysozyme substituted by phenylbutylamine. Competitive inhibitors of lysozyme such as *N*-acetyl-D-glucose amine oligomers, $(GlcNAc)_2$ and $(GlcNAc)_3$ abolished partially the accelerating effect of phenylbutylamine-

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Abbreviations: TosPheCk, L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone; Ac, *N*-acetyl; Bz, *N*-benzoyl; Suc, *N*-succinyl; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; OMe, methyl ester; OEt, ethyl ester.

* Preliminary report of this work was presented at the 11th meeting of the Federation of the European Biochemical Societies in Copenhagen, Denmark in August 1977, Abstract No. A 6-1 857.

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modified lysozyme, indicating that the substituted group is located in the vicinity of the binding site.

Introduction

Chymotrypsin structure and chymotrypsin-catalyzed hydrolysis have been intensively studied in the last decade, resulting in a detailed elucidation of its mechanism on an atomic level (for review see ref. 1). It was also shown that the binding of the specific substrates occurs through hydrophobic interaction in the so-called 'specificity pocket' and that the stereospecificity of the substrate results mainly from the side-chain of the amino acid that contributes the carbonyl group of the hydrolyzed bonds [2,3]. However, careful kinetic analyses performed by Hansch and Coats [4] led them to conclude that the enzyme has one additional partially hydrophobic site that interacts with the leaving group. Although this site has not yet been identified, its existence and specificity was discussed on a basis of kinetic data [5].

Existence of an additional hydrophobic site was also proposed by Erlanger et al. [6,7] who found that interaction of certain azobenzene compounds having bis-quaternary nitrogens with chymotrypsin accelerates significantly the hydrolysis of some specific substrates. In the present communication we wish to describe another type of interaction between substituted lysozyme and α-chymotrypsin which results in a 20-fold enhancement of hydrolysis of certain *p*-nitroanilide substrates by the latter, and discuss its possible mechanism.

Materials and Methods

Materials. Hen egg-white lysozyme (2 X crystallized), bovine α-chymotrypsin (3 X crystallized) bovine δ-chymotrypsin and bovine trypsin (2 X crystallized) were purchased from Worthington, and subtilisin (Novo type) from British Drug House. Porcine elastase I [8] porcine elastase II [9] and *Streptomyces griseus* protease B [10] were prepared as described in the literature. 4-Phenylbutylamine, was purchased from Fluka AG and benzylamine, α-DL-phenylethylamine, β-phenylethylamine, *n*-butylamine, histamine, tryptamine, glycine ethyl ester, L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone, phenylmethanesulfonyl fluoride, *N*-acetyl-L-tyrosine ethyl ester, and 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide were obtained from Sigma. *N*-Acetyl-L-phenylalanine amide and *N*-acetyl-L-tryptophan amide were products of Miles-Yeda Ltd. *N*-Benzoyl-DL-arginine-4-nitroanilide hydrochloride and *N*-acetyl-L-tyrosine-4-nitroanilide were products of Merck. *N*-Succinylglycyl-glycyl-L-phenylalanine-4-nitroanilide was a gift of Dr. S. Blumberg and *N*-succinyl-L-alanyl-L-alanyl-L-alanine-4-nitroanilide was a gift of Dr. J. Bieth. *N*-Acetyl-D-glucosamine oligomers $(GlcNAc)_2$ and $(GlcNAc)_3$ prepared by the method of Rupley [11] were a gift of Dr. D.M. Chipman. *p*-Nitrophenyl-*p'*-(ω-dimethyl-sulfonioacetamido)-benzoate bromide, a specific active-site titrant of chymotrypsin [12] was a gift of Dr. E. Shaw, and 2',3'-epoxypropyl β-glycoside of di-(*N*-acetyl-D-glucosamine), an affinity labeling reagent of lysozyme was a gift of Dr. Y. Eshdat. *N*-Acetyl glycine ethyl ester was prepared

in our laboratory by acetylation of glycine ethyl ester with acetic anhydride. *N*-Chlorosuccinimide and 2-nitrophenylsulfenyl chloride were purchased from Pfaltz and Bauer, Inc. (New York, N.Y.).

Assay of enzymatic activities. The spectrophotometric assays of bovine α-chymotrypsin, δ-chymotrypsin, porcine elastase II, *S. griseus* protease B and subtilisin were carried out at 30°C in 0.05 M Tris-HCl buffer (pH 8.0)/0.02 M $CaCl_2$ with (0.5 mM) $SucGly_2Phe$-4-nitroanilide as a substrate. The activity of porcine elastase I and bovine trypsin was estimated similarly using 0.5 mM $SucAla_3$-4-nitroanilide [13] or 1 mM BzArg-4-nitroanilide [14], respectively. The esterolytic activity of chymotrypsin was determined titrimetrically, the assay being carried out at 30°C in a Radiometer Titrator in 0.1 M KCl/0.005 M Tris/0.05 M $CaCl_2$ buffer (pH 8.0) by using 10 mM Ac-Tyr-OEt or 0.3 M Ac-Gly-OMe. The proteolytic activity of chymotrypsin was determined by the casein digestion method [15]. Hydrolysis of 10 mM *N*-acetyl-L-tryptophan amide by chymotrypsin was performed in 0.05 M sodium phosphate buffer (pH 8.0) and the rate of the reaction was followed by measuring the released NH_3 by a ninhydrin method [16]. Hydrolysis of 10 mM *N*-acetyl-L-tryptophan amide and *N*-acetyl-L-phenylalanine amide was also followed titrimetrically at pH 8.8 and 40°C as described above. Lysozyme activity was estimated spectrophotometrically by following the rate of lysis of *Micrococcus lysodeikticus* cells [17].

Preparation of modified hen egg-white lysozyme. In a typical experiment 5 ml lysozyme (8 mg/ml) were mixed with 1 ml 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (6 mg/ml) and 3 ml 0.6 M 4-phenylbutylamine (or other nucleophile) previously adjusted to pH 5.2, giving an 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide/lysozyme molar ratio of 11.4. After 3 h incubation at 25°C, the sample was extensively dialyzed for 72 h and lyophilized. The weight yield of lysozyme ranged between 85 and 90%. Preparation of larger batches was carried out by scaling up the reaction mixtures without changing the concentrations of various reagents, although in some cases higher concentrations of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide were used.

Modification of lysozyme was also carried out in the presence of 4 M guanidinium chloride but the carbodiimide/lysozyme ratio was raised to 200.

Determination of kinetic constants for enhancement of chymotrypsin activity by modified lysozyme. Hydrolysis of $SucGly_2Phe$-4-nitroanilide by chymotrypsin was carried out in the presence of increasing amounts of modified lysozyme (usually 0—0.1 mM) as described above using very low concentrations of chymotrypsin (0.1—0.2 μM). Maximal velocity (V_a), in the presence of saturating concentration of the activator, was calculated from the intercept of $1/v$ axis; and maximal enhancement was calculated as a V_a/V_0 ratio, taking V_0 as a velocity in the absence of modified lysozyme. The 'activator constant' (K_A) was calculated from the intercept on $1/A$ axis as described by Dixon and Webb [18]. All data were calculated using least-squares analysis.

Inactivation of chymotrypsin by $TosPheCH_2Cl$ and $PhCH_2SO_2F$. The reaction was carried out in 0.1 M Tris-HCl buffer (pH 8.0)/0.4 M $CaCl_2$ at 0°C with or without modified lysozyme. The reaction was started by addition of an aliquot of $TosPheCH_2Cl$ or $PhCH_2SO_2F$ and the rate of inactivation was determined by measuring the residual esterolytic activity on Ac-Tyr-OEt. The time

required for 50% inactivation was calculated from semilogarithmic plots.

Determination of K_i for inhibition of chymotrypsin by Ac-D-Trp-OMe. The reaction was carried out using SucGly$_2$Phe-4-nitroanilide as a substrate (0.5 and 0.25 mM) in the presence of various concentrations of Ac-D-Trp-OMe with or without phenylbutylamine-modified lysozyme. The K_i was estimated by plotting the reciprocal of velocity as a function of Ac-D-Trp-OMe according to Dixon and Webb [18].

Preparation of inactive derivatives of lysozyme. 15 mg solid 2-nitrophenylsulfenyl chloride were added to 7 mg/ml lysozyme in 5 ml 0.06 M acetic acid and the reaction mixture stirred for 16 h at room temperature. Excess reagent was removed after centrifugation and the solution dialyzed against 0.06 M acetic acid and then water at 4°C and lyophilized.

Extent of sulfenylation was determined spectrophotometrically using the following extinction coefficients for the 2-nitrophenylsulfenyl moiety: ϵ_{365} = 4000 and ϵ_{280} = 9500 l · mol^{-1} · cm^{-1} [19]. Lysozyme concentration was determined from amino acid analysis with norleucine as an internal standard. Both values indicated modification of approx. 3.4 ± 0.5 tryptophan residue per mol protein.

N-Bromosuccinimide-treated lysozyme was prepared according to Spande and Witkop [20], resulting in over 99% loss of activity and oxidation of 1.3 residues tryptophan per mol protein.

Electrophoresis on cellulose acetate membranes. Electrophoresis was performed in a Beckman Microzone Electrophoresis System Model R-100 in 0.08 M collidine acetate buffer, pH 8.0 (the molarity is that of the cationic component). 0.25—0.75-µl samples of protein solution were applied to the membrane and electrophoresis was performed for 15 min at 400 V. The membranes were stained with 0.2% Amido Black in $CH_3OH/H_2O/CH_3COOH$ (4.5 : 4.5 : 1.0, v/v) for 5 min and quickly destained with the same solution prepared without the dye.

Inactivation of native and modified lysozyme by affinity labeling reagent. The inactivation experiment was carried out using both native and phenylbutylamine-modified lysozyme as described by Eshdat et al. [21].

Determination of the incorporated nucleophiles. 4 mg modified lysozyme derivatives were hydrolyzed in evacuated tubes in constant boiling HCl at 110°C for 22 h. The acid was then evaporated and the hydrolysates were dissolved in 0.2 M sodium citrate buffer (pH 2.2). The amines released by hydrolysis were quantitatively determined on LKB 3201 amino acid analyzer. The hydrolysates were applied to the short (0.9 X 4 cm) column filled with Durrum DC-6A amino acid analysis resin. The analysis was performed at 63°C with buffer and ninhydrin flow rates of 80 and 40 ml/h, respectively, but ninhydrin was applied 10 min after beginning of the analysis. The following buffers were used: 0.2 M sodium salicylate containing 0.5 M NaCl (pH 12.0) for 4-phenylbutylamine and pH 10.4 for α- and β-phenylethylamine, 0.2 M sodium borate (pH 9.5) containing 0.5 M NaCl for benzylamine and pH 8.5 for histamine and *n*-butylamine. The corresponding amines with and without hydrolysis served as standards; no destruction in hydrolysis was observed. The concentration of the modified enzyme was determined from the amino acid content of each hydrolysate. Extent of tryptamine incorporation was esti-

mated spectrophotometrically using the extinction coefficient for the tryptophan moiety at neutral pH ($\epsilon_{280} = 5560\ l \cdot mol^{-1} \cdot cm^{-1}$) and determination of lysozyme concentration by amino acid analysis. Glycine ethyl ester incorporation was also estimated from amino acid analysis of the modified enzyme.

Stopped flow experiments. The experiments were carried out in an Aminco stop-flow spectrophotometer at room temperature. In the first experiment, one syringe of the mixer contained 0.5 mM $SucGly_2Phe$-4-nitroanilide in 50 mM Tris-HCl buffer (ph 8.0)/20 mM $CaCl_2$ and the second syringe contained 40 μM chymotrypsin or 4 μM chymotrypsin with 200 μM phenylbutylamine-modified lysozyme. In the second experiment one syringe contained 0.2 mM *p*-nitrophenyl-*p'*-(ω-dimethyl-sulfonioacetamido)-benzoate bromide in 100 mM sodium diethylbarbiturate buffer (pH 8.3)/20 mM $CaCl_2$/4% dimethylformamide and the second syringe contained 8 μM α-chymotrypsin with or without 200 μM modified lysozyme. In both experiments the absorbance at 410 nm was monitored.

Results

Preparation of lysozyme modified with 4-phenylbutylamine

Modified lysozyme was prepared in the presence of increasing concentrations of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide. The properties of various preparations are summarized in Table I and Fig. 1. The maximal enhancing effect was obtained with 10—20 excess of carbodiimide resulting in incorporation of 0.6—0.7 mol 4-phenylbutylamine per mol lysozyme. However, electrophoretical analysis revealed that the modified enzyme was heterogeneous. Three bands could be distinguished. The least cationic protein was assumed to represent the unmodified enzyme. The more cationic protein, which was dominant in samples 2 and 3, is an enzyme with one modified

TABLE I

THE EFFECT OF PREPRATION OF 4-PHENYLBUTYLAMINE (PBA)-LYSOZYME IN PRESENCE OF INCREASING CONCENTRATIONS OF 1-ETHYL-3-(3-DIMETHYLAMINO-PROPYL)-CARBODIIMIDE (EDC) ON ITS RESIDUAL ENZYMATIC ACTIVITY AND ENHANCEMENT OF α-CHYMOTRYPSIN HYDROLYSIS OF $SucGly_2Phe$-4-NITROANILIDE

The maximal activity in presence of saturating concentration of 4-phenylbutylamine-lysozyme (V_a) and the activator constant (K_A) were calculated from double reciprocal plots at constant concentration (0.5 mM) of $SucGly_2Phe$-4-nitroanilide V_0 represents the activity without the activator.

EDC / lysozyme (mol/mol)	PBA incorporated / lysozyme (mol/mol)	V_a / V_0	K_A (μM)	Residual lysozyme activity (%)
70	0.85	15.3	19.6	36.4
40	0.75	18,3	26,5	39.4
20	0.68	21.3	32.9	48.8
10	0.58	19.6	34.7	58.5
5	0.29	15.3	40.5	70.1
2	0.19	10.3	45.2	79.8
0	0.00	1.0	—	100.0

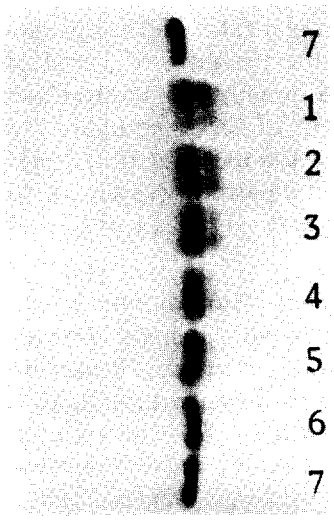

Fig. 1. Electrophoresis of phenylbutylamine-modified lysozyme derivatives prepared in presence of different concentrations of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC). The samples 1—7 were prepared accordingly using the following EDC/lysozyme molar ratios: 70, 40, 20, 10, 5, 2, 0. The arrow indicate the application point and the protein moved toward cathode (located on the right side).

carboxyl group (one extra positive net charge per molecule); the most cationic protein, whose relative quantity increases in samples 3 to 1, is most diffuse and probably represents the enzyme with two modified carboxyl groups. In view of these results it is obvious that the activator constant (K_A) is not a true dissociation constant of the modified lysozyme · chymotrypsin complex. If we assume that only two modified species of lysozyme exist, namely with one or with two modified carboxyl groups at least three different types of complexes are possible. The K_A represents therefore, an average of different constants some of which may result from unproductive binding. It should also be remembered that the K_A was calculated from the total concentration of lysozyme that also included the unmodified enzyme. Therefore, only the values of samples 1 and 2 in which the latter almost completely disappeared approach, approximately the value of the true dissociation constant of the complex. Increased modification was followed by gradual decrease of lysozyme activity.

It should also be noted that both native and modified enzyme could be completely inactivated by the affinity labelling reagent 2′,3′-epoxypropyl β-glycoside of di-(*N*-acetyl-D-glucosamine), although the inactivation of the latter was 2.7 times slower. Increasing concentration of 4-phenylbutylamine in the modification mixture beyond 0.2 M had no further effect on the properties of phenylbutylamine-modified lysozyme.

Phenylbutylamine itself at concentrations up to 0.1 mM, normally used for determination of the enhancing effect with modified lysozyme had no effect on the activity of chymotrypsin. At higher concentrations the activity was partially inhibited as it could be expected.

Modification of lysozyme by 4-phenylbutylamine in the presence of its competitive inhibitor

The modification was performed in the presence of 1 mM, 0.1 mM or

without $(GlcNAc)_3$ using an carbodiimide/lysozyme molar ratio of 18. Electrophoretic analyses revealed that the modification in the presence of 1 mM of competitive inhibitor yielded a product composed mainly of unmodified protein and having a maximal enhancing (V_a/V_0) effect of 2.9 as compared to the value 20.1 of the control. The sample prepared in the presence of 0.1 mM of $(GlcNAc)_3$ have V_a/V_0 of 14.7 and its electrophoretic pattern was similar to the control. The 4-phenylbutylamine content was respectively: 0.11, 0.48 and 0.65 residues per mol.

Modification of inactive derivatives of lysozyme by 4-phenylbutylamine

Two inactive or almost inactive derivatives of lysozyme, namely 2-nitrophenylsulfenyl-treated lysozyme and *N*-bromosuccinimide-treated lysozyme with respective residual activity of 9.0 and 1.1% were modified with 4-phenylbutylamine using an carbodiimide/lysozyme molar ratio of 14. The residual lysozyme activity was reduced to 6.0 and 0.6%, respectively, and the electrophoretic pattern showed three protein bands all more cationic than the native enzyme. The kinetic parameters of activation were, respectively: V_a/V_0 6.4 and 9.0 and K_A 28.1 and 37.0 μM, and the number of residues of 4-phenylbutylamine incorporated into one molecule of enzyme was 3.69 and 3.80.

Modification of lysozyme by 4-phenylbutylamine in the presence of 4 M guanidinium chloride

The modified protein product contained 4.58 residues of 4-phenylbutylamine per mol enzyme, was devoid of any lysozyme activity and had no enhancing effect on the hydrolysis of $SucGly_2$-Phe-4-nitroanilide by chymotrypsin. Moreover, the modified protein exhibited a slight inhibitory effect. Attempts to elucidate the type of inhibition and to calculate the K_i were unsuccessful since the linearity of the Dixon plots ($1/v$ versus [I]) was limited only to the low concentrations of modified lysozyme and they gradually flattened off above 15 μM.

The effect of substrate concentration on the enhancing activity of phenylbutylamine-modified lysozyme

In order to estimate whether the maximal enhancement of the modified lysozyme dependent on substrate concentration, the kinetic parameters of enhancement were calculated at four different concentrations of $SucGly_2$Phe-4-nitroanilide.

The basic data are presented at Fig. 2. Replotting of these results on a double reciprocal plot ($1/v$ versus 1/modified lysozyme) at four different $SucGly_2$Phe-4-nitroanilide concentrations allowed us to calculate the respective V_a/V_0 and K_A values. As shown in Table II these parameters are definitely not affected by substrate concentration.

Since the experiments were done using subsaturating concentrations of the substrate ($K_{m_{app}}$ = 0.58 mM) the existence of any effect of substrate concentration on the kinetic parameters of the enhancement should be easily detected.

The effect of phenylbutylamine-modified lysozyme on hydrolysis of other substrates by chymotrypsin

Hydrolysis of specific and non-specific ester substrates of chymotrypsin such

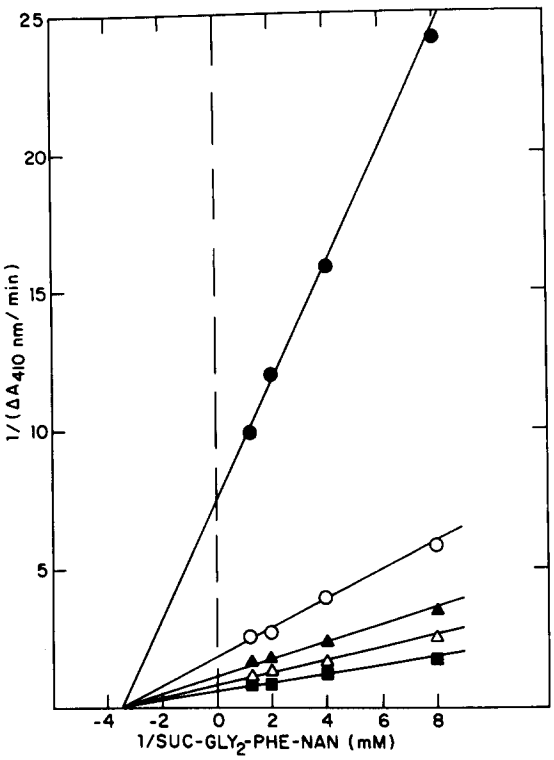

Fig. 2. The effect of phenylbutylamine-modified lysozyme on enhancement of chymotrypsin at different substrate concentrations. Chymotrypsin hydrolysis of SucGly$_2$Phe-4-nitroanilide was monitored at 410 nm at different concentrations of the modified lysozyme; ●, none; ○, 9 μM; ▲, 18 μM; △, 36 μM; ■, 72 μM.

as Ac-Tyr-OEt or Ac-Gly-OEt was uneffected by the presence of modified lysozyme. No effect on the hydrolysis of casein was observed.

It should be noted that the estimations of the hydrolysis of amide substrates such as *N*-acetyl-L-tryptophan amide and *N*-acetyl-L-phenylalanine amide in the presence of modified lysozyme were somewhat complicated because modified

TABLE II

THE EFFECT OF THE SUBSTRATE SucGly$_2$Phe-4-NITROANILIDE CONCENTRATION ON THE ENHANCEMENT OF α-CHYMOTRYPSIN BY 4-PHENYLBUTYLAMINE-MODIFIED LYSOZYME

The maximal activity in presence of saturating concentration of 4-phenylbutylamine-modified lysozyme (V_a) and the activator constant (K_A) were calculated from double reciprocal plot at four concentrations (0.125—0.750 mM) of SucGly$_2$Phe-4-nitroanilide. V_0 represents the activity in absence of the activator. The results of two experiments (±S.D.) are presented.

SucGly$_2$Phe-4-nitroanilide (mM)	$\frac{V_a}{V_0}$	K_A (μM)
0.750	20.1 ± 1.2	29.3 ± 3.0
0.500	18.5 ± 1.9	32.2 ± 2.8
0.250	19.1 ± 2.1	31.5 ± 1.5
0.125	19.5 ± 0.8	29.8 ± 2.1

as well as native lysozyme were both hydrolyzed by chymotrypsin at a rate exceeding that of the amides hydrolysis. However, since equivalent amounts of phenylbutylamine-modified lysozyme and lysozyme gave approximately the same results we concluded that the modified lysozyme does not enhance the hydrolysis of amides, although a small enhancement of 10—20% could have been overlooked.

On the other hand, hydrolysis of other *p*-nitroanilide substrates such as SucPhe-4-nitroanilide or AcTyr-4-nitroanilide was strongly enhanced.

The effect of phenylbutylamine-modified lysozyme on the inhibition of chymotrypsin by irreversible or competitive inhibitors

Phenylbutylamine-modified lysozyme at final concentrations of 70—400 μM and at molar excess over chymotrypsin of 40 : 1 and 20 : 1 did not change the rate of irreversible inhibition by phenylmethanesulfonyl fluoride and TosPheCH_2Cl. The K_i of chymotrypsin inhibition by Ac-D-Trp-OMe was also not affected.

Modification of lysozyme by different nucleophiles

The characterization of the modification products and their enhancing effect on the hydrolysis of SucGly_2Phe-4-nitroanilide by chymotrypsin is summarized in Table III. Like in phenylbutylamine-modified lysozyme, the modification products were heterogeneous and composed of one or two additional more cationic protein bands in addition ot unmodified lysozyme. It should be noted that the enhancing effect was found only when the nucleophile consisted of a phenyl or an indole ring bound to an unbranched aliphatic chain. It is interesting to note that α-phenylethylamine-lysozyme derivative, which differs from the benzylamine-lysozyme derivative by having an additional branched methyl group, was devoid of any enhancing activity.

The residual lysozyme activity was reduced in all derivatives in which modification occurred this indicating that the modified carboxyl group is somehow involved in the enzymatic activity of lysozyme.

TABLE III

THE EFFECT OF MODIFICATION OF LYSOZYME BY VARIOUS NUCLEOPHILES ON ITS RESIDUAL ACTIVITY AND ENHANCEMENT OF α-CHYMOTRYPSIN HYDROLYSIS OF SucGly_2Phe-4-NITROANILIDE

The modification was carried out in presence of 0.2 M of each nucleophile using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/lysozyme molar ratio of 11.4. Definition of kinetic parameters is given in Table I.

Nucleophile	Nucleophile incorporated / lysozyme (mol/mol)	$\frac{V_a}{V_0}$	K_A (μM)	Residual lysozyme activity (%)
Benzylamine	0.74	7.1	59.2	89.0
DL-α-Phenylethylamine	0.52	1.0		89.0
β-Phenylethylamine	0.47	5.4	33.4	76.0
Histamine	0.40	1.0		92.0
Tryptamine	0.58	6.2	20.5	70.0
n-Butylamine	0.70	1.0		78.5
Glycine ethyl ester	0.76	1.0		67.6

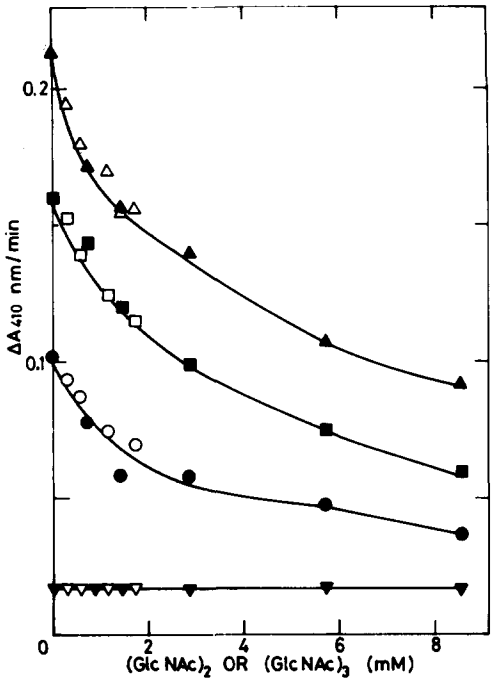

Fig. 3. The effect of $(GlcNAc)_2$ and $(GlcNAc)_3$ on the enhancement of chymotrypsin by phenylbutylamine-modified lysozyme. Chymotrypsin hydrolysis of $SucGly_2Phe$-4-nitroanilide was monitored at 410 nm in absence (▽▼) or at different concentrations: ○●, 10 μM; □■, 25 μM and △▲, 50 μM of the modified lysozyme. Solid symbols: with $(GlcNAc)_2$; open symbols, with $(GlcNAc)_3$.

The effect of competitive inhibitors of lysozyme on the enhancing activity of phenylbutylamine-modified lysozyme

The results presented in Fig. 3 clearly indicate that both competitive inhibitors partially abolished the enhancing effect of the modified lysozyme and that this is dependent on inhibitor concentration. Analysis of the data also reveals that the abolishing effect is slightly more pronounced at lower concentrations of the modified lysozyme in which the competitive inhibitors/modified lysozyme ratio was relatively higher.

In view of these results it seems that the modified lysozyme is not able to interact simultaneously with chymotrypsin and a competitive inhibitor and the interaction with the latter results in a loss of the enhancing effect.

The effect of phenylbutylamine-modified lysozyme on hydrolysis of p-nitroanilide substrates by other serine proteases

Hydrolysis of $SucGly_2Phe$-4-nitroanilide by various enzymes with chymotrypsin-like specificity such as porcine elastase II, subtilisin or *S. griseus* protease B was not affected by phenylbutylamine-modified lysozyme. Only δ-chymotrypsin activity was enhanced although the effect was lower than in α-chymotrypsin, resulting in $V_a/V_0 = 5.2$ and $K_A = 10.8\ \mu M$. Hydrolysis of $SucAla_3$-4-nitroanilide by porcine elastase I was not affected at all and the activity of bovine trypsin on Bz-DL-Arg-4-nitroanilide was even slightly inhibited.

Pre-steady-state kinetics of SucGly$_2$Phe-4-nitroanilide hydrolysis by α-chymotrypsin

Stopped-flow experiments using extended scale (full scale = 0.01 A) during the initial 50 ms after mixing (dead time = 4 ms), resulted in linear increase in absorbance, starting slightly above the origin. Similar linear curve was also obtained in the presence of phenylbutylamine-modified lysozyme. Those curves resemble the results obtained by Fastrez and Fersht [22] and can be interpreted accordingly as an evidence for lack of accumulation of tetrahedral intermediate both in the absence and in the presence of modified lysozyme.

The effect of phenylbutylamine-modified lysozyme on the acylation of the chymotrypsin by p-Nitrophenyl-p'-(ω-dimethyl-sulfonioacetamido)-benzoate bromide

The acylation of chymotrypsin in the presence or the absence of phenylbutylamine-modified lysozyme was monitored during the initial first second of the reaction, and the full extent of the acylation was monitored by an additional signal 1 min later. The rate of reaction was calculated by plotting the log of the ratio of unacylated/total enzyme as a function of time. The apparent acylation constants were, respectively, 1.54 and 2.24 s^{-1} in the presence or the absence of phenylbutylamine-modified lysozyme. Thus modified lysozyme does not enhance the acylation but causes a slight inhibition.

Discussion

Modification of hen egg-white lysozyme by a small excess of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide in the presence of relatively high concentrations of nucleophiles such as 4-phenylbutylamine or other similar aromatic compounds bound to an unbranched aliphatic chain (n = 1—4), yielded a derivative with strong enhancing effect on the hydrolysis of *p*-nitroanilide substrates by α- and δ-chymotrypsin. No other enzymes with chymotrypsin specificity or other serine proteases were affected. Moreover, phenylbutylamine itself, phenylbutylamine-modified lysozyme prepared in presence of guanidinium chloride and phenylbutylamine derivatives of other small proteins such as pancreatic ribonuclease or α-lactalbumin prepared under similar conditions are devoid any enhancing effect. This indicates that the described effect is very specific and restricted to lysozyme in which the native structure was preserved. The specificity of the reaction is further emphasized by the fact that only hydrolysis of *p*-nitroanilide substrates is accelerated. Other substrates such as Ac-Tyr-OEt for which the rate-limiting step in hydrolysis is deacylation, or Ac-Gly-OMe, *N*-acetyl-L-tryptophan amide, *N*-acetyl-L-phenylalanine amide for which acylation is the limiting step, or hydrolysis of casein are not affected. Acylation of chymotrypsin by the active site titrator as well as the rate of inhibition of chymotrypsin by irreversible inhibitors were also not enhanced by phenylbutylamine-modified lysozyme and its interaction with competitive inhibitors was not influenced. Kinetic analysis revealed that the enhancement results solely from the increase in k_{cat} while the apparent K_m (which equals to K_s in our case) was not changed. Since k_{cat} in the hydrolysis of *p*-nitroanilide substrates equals to the acylation constant (k_2) we shall try to elucidate the

mechanism of the enhancement by a more detailed analysis of this step. It is now generally accepted [1,5,23,24] that the acylation step is composed of two main substeps: (a) formation of the tetrahedral intermediate (TI) from the Michaelis complex (E · S) and (b) protonation of TI that results in the release of the leaving group (P_1) and formation of the acyl-enzyme (ES′). These may be represented according to Hirohara et al. [25] as:

$$E \cdot S \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} TI \overset{k_b}{\rightarrow} ES' + P_1 \qquad (1)$$

and

$$k_2 = k_a k_b / (k_{-a} + k_b) \qquad (2)$$

The increase in the apparent k_2 may therefore be the result of three main reasons: (1) increase in k_b that reflexes the facilitated proton transfer; (2) accelerated formation of TI resulting from increase of k_a or k_a/k_{-a} ratio; and (3) accelerated formation of TI resulting from increase of productive (E · S) to non-productive (E · S_{NP}) enzyme-substrate complex.

The first possibility seems rather improbable because no accumulation of tetrahedral intermediate was observed with *p*-nitroanilide substrates (see ref. 22 and our results) and therefore, in our case like in the hydrolysis of oxygen and sulfur esters of specific substrates [25], $k_b >> k_a$. Therefore if k_b is not the rate-limiting step of the acylation its increase would not increase the overall catalysis. The second possibility seems more acceptable although it has to be based on an unproven assumption that interaction of chymotrypsin with phenylbutylamine-modified lysozyme increases the nucleophilicity of Ser-195 or makes the carbonyl group of the substrate more favorably oriented. However, in this case we could expect, contrary to the experimental results, that hydrolysis of casein and amide substrates, sulfonylation by phenylmethanesulfonyl fluoride or acylation by *p*-nitrophenyl-*p*′-(ω-dimethyl-sulfonioacetamido)-benzoate bromide, should be also enhanced. This brings us therefore to the third possibility that interaction of chymotrypsin with phenylbutylamine-modified lysozyme accelerates the formation of TI due to an increase in the ratio of productive to non-productive binding. It has been shown [26] that this increase effects of both k_{cat} and K_m without changing their ratio. Fastrez and Fersht [22] suggested that non-productive binding predominates in hydrophobic anilides such as *p*-nitroanilide. If we assume that the modified lysozyme binds to chymotrypsin in a way that precludes or diminishes the non-productive binding, an increase in both k_{cat} and K_m should be expected. Since the latter is inconsistent with the experimental data we have to speculate that the change induced by the modified lysozyme not only increases the dissociation constant for non-productive binding but parallely decreases significantly the dissociation constant for productive binding. This can be easily shown theoretically by using the equations for calculation of apparent k_{cat} and K_m in the case of productive and non-productive binding [22]. For instance, 20-fold increase in the apparent k_{cat} without change in K_m (0.58 mM), could be achieved by a decrease in K_s from 16 to 0.81 mM with a parallel increase in K_{NP} (dissociation constant for non-productive binding) from 0.6 to 2.08 mM.

It should be remembered that although the primary binding site of chymotrypsin that effects its specificity accomodates the hydrophobic side chain of the amino acid that contributes the carbonyl group of the hydrolyzed bond [2,3] other additional binding sites may exist too. Existence of such a secondary partially hydrophobic binding site that is occupied by the leaving group was implicated by Hansch and Coats [4]. Later it was also shown that the leaving group specificity effects the rate of peptide hydrolysis [5].

It is therefore quite attractive to assume that the modified lysozyme interacts with this site, thus effecting the location of the leaving group. Whether this change would enhance the hydrolysis of *p*-nitroanilide substrates due to lowering the energy difference between the tetrahedral intermediate and the Michaelis complex by increasing the k_a, or due to a change in productive to non-productive binding is not clear. Therefore no choice between the second and the third possibility can be made at present. Although the three-dimentional structure of α-chymotrypsin is well known, we feel that the present data are not sufficient to suggest the position of the site that binds the phenylbutylamine-modified lysozyme. Only further experiments in which the interaction of the modified lysozyme with specifically modified chymotrypsin will be studied may enable us to clarify this point.

The initial idea that the catalytic apparatus of the modified lysozyme may be involved in the enhancing effect was abandoned in view of the fact that phenylbutylamine derivatives of modified and inactive lysozyme had also enhancing activity. Partial abolition of the enhancing effect by competitive inhibitors of lysozyme that interact with the binding site (subsites A, B, C) of the latter [27] is most likely due to structural changes in its vicinity. The formation of a complex between chymotrypsin and the modified lysozyme was indicated from kinetic data. Attempts to show its existence by means of electrophoresis were unsuccessful and the two bands migrated separately probably because the K_i of the complex is relatively high.

It should be noted that the acceleration of chymotrypsin activity by azobenzene compounds with bis-quaternary nitrogen [6,7] is most likely caused by a similar mechanism, although the interpretation provided by the authors was different [7]. We feel therefore that though the mechanism of chymotrypsin catalysis has been so widely investigated, elucidation of the described acceleration process may add to its further understanding, particularly concerning the role of the substrate leaving group.

The identification of the modified carboxyl groups in lysozyme was beyond the scope of this publication. However since the results indicate that at least two groups are partially and simultaneously modified, further experiments attempted to clarify this point through isolation of a homogeneous product are in progress.

Acknowledgements

We are grateful to Dr. J. Bieth, Dr. D. Chipman, Dr. Y. Eshdat and Dr. E. Shaw for their generous gifts and particularly to Dr. S. Blumberg who prepared for us the main substrate used in this work. We wish also to thank Ms. Nava Cohen for her excellent technical assistance. This research was supported in

part by Grant No. AMO 5098 from the National Institutes of Health, United States Public Health Service.

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