BBA 66049

CATHEPSIN C: AN ALLOSTERIC ENZYME

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(Received October 23rd, 1969)

SUMMARY

1. Cathepsin C requires Cl^- for activity. Activation is also found with bromide, iodide, thiocyanate, nitrate, and chlorate.

2. At saturating activator concentrations, substrates are split according to Michaelis–Menten kinetics; at non-saturating concentrations cooperativity is observed. Accordingly, in the absence of activators, a low concentration of the substrate Ala–Phe–amide enhances the rate of hydrolysis of Gly–Phe–*p*-nitroanilide.

3. The enzyme activity as a function of the activator concentration shows Michaelis–Menten kinetics. The A_{max} and K_a values are different for each activator.

4. Both high substrate and high activator concentrations are inhibitory.

5. The effects of activations and inhibitions on the individual reaction constants are discussed. The activators probably affect the substrate affinity K_s.

6. A method for rapid activity assay using Gly–Phe–*p*-nitroanilide is described.

INTRODUCTION

Cathepsin C (EC 3.4.4.9) is a lysosomal SH-enzyme which splits off certain dipeptides from peptides and proteins[1–6]. It also splits certain dipeptide esters[3,7], amides[1–3], anilides[8], and β-naphthylamides[9], and has a high transpeptidase activity[5,10], which accounts for the name dipeptidyl transferase used by some authors[11]. The enzyme from beef spleen has been purified about 1200-fold[12].

The activity of cathepsin C is usually assayed using the substrates Gly–Phe–amide, Gly–Tyr–amide, or Pro–Tyr–amide[1], but more recently, we have developed a method using Gly–Phe–*p*-nitroanilide. This substrate is used in low concentrations, and this led to the discovery of the chloride requirement of cathepsin C (see DISCUSSION) and, consequently, of the allosteric character of the enzyme. This paper describes our findings and their consequences. The effect of chloride was found independently by MCDONALD *et al.*[9] with one of our enzyme preparations and rat-spleen homogenate.

MATERIALS AND METHODS

Cathepsin C. Most of the experiments were performed with enzyme preparations according to DE LA HABA *et al.*[13]. The first steps of the isolation, in which large volumes have to be handled, were performed by N.V. Organon (Oss, The Netherlands), to which we wish to express our thanks. The specific activity of these preparations was 5–10 units/mg protein. The determination of the sedimentation coefficient and a comparison of the activities with Gly–Phe–*p*-nitroanilide and with Gly–Phe–amide were performed with an enzyme preparation of a higher degree of purity; this preparation had been chromatographed on Sephadex G-200 (Pharmacia, Uppsala, Sweden), and subsequently on DEAE-Sephadex *(idem)*[12]. To all elution buffers used in the column chromatographies 10 mM NaCl was added. After elution the active fractions were pooled, concentrated by ultrafiltration, and lyophilized. The specific activity was 80 units/mg protein. After 2 months of storage at −20° this preparation had retained 80% of its original activity.

Gly-L-Phe-p-nitroanilide·HBr (ref. 12) was a gift from Dr. G. I. Tesser (University of Nijmegen, The Netherlands), to whom we are greatly indebted.

Gly–L-Phe–p-nitroanilide acetate was prepared from the hydrobromide. It was dissolved in a small volume of hot water, and Gly–Phe–*p*-nitroanilide was precipitated as the free base with concentrated ammonia, redissolved in dilute acetic acid, and precipitated again with ammonia. After filtering and washing with a few drops of ice-cold water the Gly–Phe–*p*–nitroanilide was dried in a vacuum desiccator to remove traces of ammonia. After weighing, the Gly–Phe–*p*-nitroanilide was dissolved in a few ml of methanol containing an equivalent amount of acetic acid and precipitated as the acetate with ethyl acetate and petroleum ether. Yield, 75%; melting range 161–164° (decomp.). The molar absorbance at 315 nm of both Gly–Phe–*p*-nitroanilide preparations was measured. For the hydrobromide (net mol. wt., 423) we found a value of 11 900, for the acetate (net mol. wt., 402), a value of 12 100 (ref. 14, 13 000). After complete enzymatic hydrolysis the molar absorbances at 410 nm were 8200 for Gly–Phe–*p*-nitroanilide·HBr and 8400 for the acetate (*p*-nitroaniline[14], 8800). The substrate concentrations in the assays were calculated on the basis of absorbances at 410 nm which were derived from the absorbances of the substrate solutions at 315 nm.

L-Pro–L-Tyr–amide acetate. Carbobenzoxy–L-Pro (Mann, New York, N.Y.) was coupled to L-Tyr–methyl ester[15] by means of dicyclohexylcarbodiimide (Fluka, Buchs, Switzerland)[16]. The carbobenzoxy–L-Pro–L-Tyr–methyl ester was converted to the amide by dissolving it in superdry methanol which had been saturated with NH_3 at 0° and by keeping the solution in a pressure bottle at room temperature for 2 days. The carbobenzoxy group was removed by reduction with hydrogen gas and palladium–carbon catalyst (Fluka) in methanol containing an equivalent amount of acetic acid. The product obtained after recrystallization from methanol–ether yielded only one isatine-positive spot on paper (Schleicher and Schüll 2043b) chromatograms with an R_F value of 0.58 in *n*-butanol–pyridine–water–acetic acid (30:20:6:24, by vol.); the melting point was 156°.

L-Ala–L-Phe–amide acetate was prepared in the same way from a carbobenzoxy–Ala–Phe–amide preparation of unknown origin. Upon paper chromatography it was found to be slightly contaminated by ninhydrin-positive material. Ala–Phe–amide

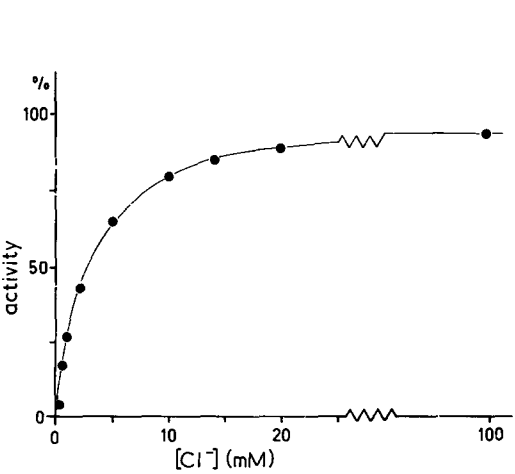
itself had an R_F value of 0.67 in *n*-butanol–pyridine–water (10:4:5, v/v/v).

Gly–L-Tyr–amide acetate was prepared in the same way from carbobenzoxy-Gly and L-Tyr–methyl ester. On a paper (Schleicher and Schüll 2043b) chromatogram the product showed a single spot with an R_F value of 0.37 in *n*-butanol–pyridine–water (10:4:5, v/v/v); the melting point was obscured by decomposition.

The other chemicals were of analytical grade from Merck (Darmstadt, Germany).

Standard activity assay with Gly–Phe–p-nitroanilide. 2.0 ml 0.1 M sodium acetate buffer (pH 5.0), containing 125 mM NaCl and enough Na_2SO_4 to yield a total I of 0.25 in the final volume of 2.5 ml, + 0.1 ml 150 mM cysteine hydrochloride in deionized water adjusted to pH 5.0 with concentrated NaOH, + 0.1 ml of enzyme solution were placed in a thermostat at 25° for 20 min to allow activation of the enzyme by cysteine. The reaction was started by the addition of 0.3 ml 3 mM Gly–Phe–*p*-nitroanilide·HBr in deionized water; after a suitable reaction time at 25° it was stopped by the addition of 0.5 ml of 60 mM monoiodoacetic acid. Absorbances at 410 nm were measured in a 1-cm glass cuvette in a Zeiss spectrophotometer PQM II. The activity in the assay mixture was calculated according to the equation $A = C \cdot t^{-1} \log [(E_{t \to \infty})/(E_{t \to \infty} - E_t)]$, which may be applied since with 0.36 mM Gly–Phe–*p*-nitroanilide the reaction is first-order ($K_m = 3$ mM). A is the enzyme activity in units present, t is the time in min, and C is a constant defining the unit. If one chooses C as 1500, A becomes equal to the concentration per ml of added enzyme in cathepsin units as defined by TALLAN *et al.*[1] for Gly–Phe–amide (the enzyme concentration effecting 1% hydrolysis per min with 50 mM Gly–Phe–amide in 0.15 M sodium citrate buffer (pH 5.0) with 6 mM cysteine hydrochloride at 37°).

All experiments were performed at 25°; unless otherwise specified, the ionic strength was always adjusted to 0.25 with Na_2SO_4.



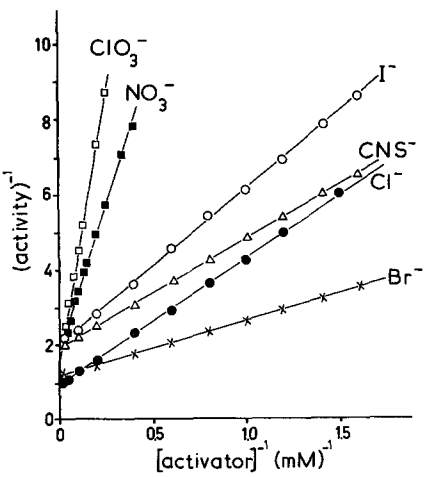


Fig. 1. Activity of cathepsin C as a function of the chloride concentration. Experimental conditions: standard assay procedure, but with varying NaCl concentration, HCl-free cysteine, Gly–Phe–*p*-nitroanilide acetate instead of the hydrobromide, and enzyme dialyzed against NaCl-free buffer.

Fig. 2. Activity of cathepsin C as a function of the concentration of various activating anions. Chloride and nitrate were the sodium salts; the other activators were potassium salts. Experimental conditions as in Fig. 1. The reciprocal activities are expressed in arbitrary units.

TABLE I

ACTIVATING PROPERTIES OF DIFFERENT ANIONS

Kinetic constants were calculated with the method of least squares from the experiments shown in Figs. 1 and 2. The activities are expressed in arbitrary units.

	Cl^-	Br^-	I^-	CNS^-	NO_3^-	ClO_3^-
A_{max}	1	0.86	0.50	0.52	0.51	0.59
K_a (mM)	3.5	1.3	2.1	1.5	7.7	16

RESULTS

At low substrate concentrations, as is the case in the assay with Gly–Phe–*p*-nitroanilide, Cl^- is required for cathepsin C activity (Fig. 1). Some other anions, *viz.* bromide, iodide, thiocyanate, nitrate, and chlorate, also act as activators. No activation was observed with many other anions and cations tried. The activators differ in their affinity as well as in the maximum activity they induce. Reciprocal activities plotted against reciprocal activator concentrations yield straight lines (Fig. 2). Therefore, to each activator the two kinetic constants A_{max} and K_a may be attributed; their values are given in Table I. A_{max} is the maximal activity induced by an activator at very low concentration of Gly–Phe–*p*-nitroanilide; K_a is the activator concen-

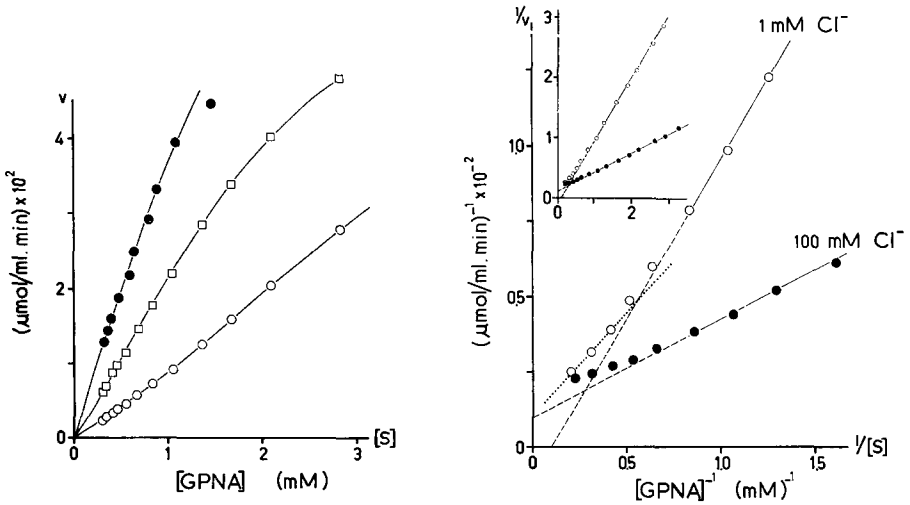


Fig. 3. Hydrolysis of Gly–Phe–*p*-nitroanilide by cathepsin C in the presence of different NaCl concentrations. Assay mixture: volume and concentrations as in Fig. 1, but with 100, 3, or 0.7 mM NaCl. In this experiment the reaction was started by adding enzyme preincubated at 25° in acetate buffer with 6 mM cysteine and the respective chloride concentrations; *I* 0.25. The enzyme concentrations and incubation times were varied for maximum accuracy. The substrate conversion never exceeded 10% and was taken into account. ●—●, 100 mM NaCl; □—□, 3 mM; ○—○, 0.7 mM.

Fig. 4. Lineweaver–Burk plots of Gly–Phe–*p*-nitroanilide hydrolysis by cathepsin C in the presence of 100 and 1 mM NaCl. Experimental conditions as in Fig. 3. The insert shows the plots over a larger substrate concentration range.

tration inducing half of the maximal activity obtainable with the same activator, also at very low substrate concentration.

The influence of the substrate concentration on the reaction rate is strongly dependent on the activator concentration. Thus, the hydrolysis of Gly–Phe–*p*-nitroanilide follows S-shaped curves at low chloride concentrations (Fig. 3); with 100 mM chloride, however, Michaelis–Menten kinetics are obeyed (Fig. 4). The kinetics at low activator concentrations indicate substrate activation, or cooperativity, so there must be more than one binding site for the substrate. Therefore, other substrates of cathepsin C, such as Ala–Phe–amide, should promote the hydrolysis of Gly–Phe–*p*-nitroanilide under the right conditions. Fig. 5 confirms this prediction: 5 mM Ala–Phe–amide enhances the reaction rate with Gly–Phe–*p*-nitroanilide 2.5-fold in the absence of chloride. At higher concentrations Ala–Phe–amide inhibits competitively. The occurrence of S-shaped curves and the enhanced reaction rate by a second substrate are typical features of allosteric enzymes.

The double-reciprocal plot with 1 mM chloride is also the type of curve found with cooperative enzymes: the linear part at low substrate concentration intersects the ordinate below the origin upon extrapolation. Because of substrate inhibition at higher concentrations and the poor solubility (10 mM) of Gly–Phe–*p*-nitroanilide, it is impossible to decide with certainty whether the v_{max} values at 100 and 1 mM chloride are identical. With another substrate, Pro–Tyr–amide, however, the v_{max} values are clearly independent of the activator concentration (Fig. 6).

On the other hand, v_{max} is affected by the type of activator. With 100 mM

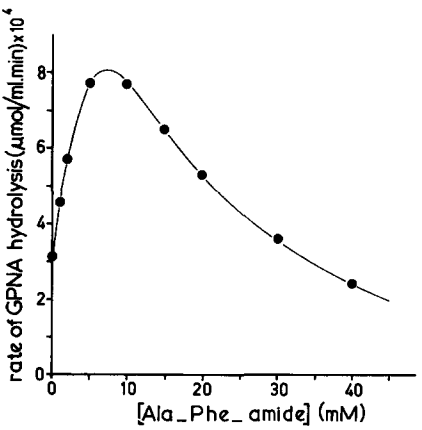


Fig. 5. Effect of Ala–Phe–amide on the rate of hydrolysis of Gly–Phe–*p*-nitroanilide by cathepsin C in the absence of activating anions. Assay mixture: volume and concentrations as in Fig. 1, but with no added activator. The enzyme concentration was 1 unit/ml.

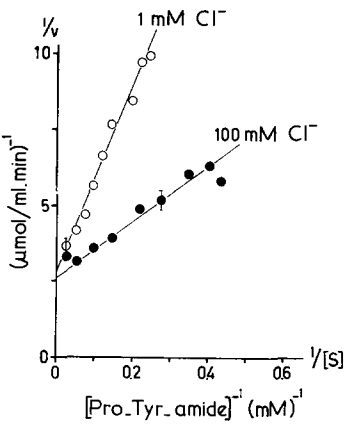


Fig. 6. Lineweaver–Burk plots of Pro–Tyr–amide hydrolysis by cathepsin C in the presence of 100 and 1 mM NaCl at pH 5.0. Assay mixture: 0.1 M sodium acetate buffer (molarity including the acetate ions of Pro–Tyr–amide acetate), either 100 or 1 mM NaCl, the appropriate concentration of Pro–Tyr–amide acetate, 6 mM cysteine, and Na_2SO_4 to total I 0.25. The reaction was started by the addition of preincubated enzyme as described in Fig. 3. The assay volumes, the enzyme concentrations, and incubation times were varied for maximum accuracy. All incubations were performed in duplicate. Four samples were taken at different times and pipetted into Conway dishes for standard ammonia assay. The initial rates were found graphically. The vertical bars indicate duplicate incubations.

Gly–Tyr–amide (K_m = approx. 3 mM) in 0.1 M acetate buffer (pH 5.0) at 25° and total I 0.25, the reaction rate with KBr (20 mM), KCNS (50 mM), and $NaNO_3$ (50 mM) is 100, 84, and 92%, respectively, of the rate with 50 mM NaCl. Identical results were obtained with Ala–Phe–amide.

At pH 5.0, Pro–Tyr–amide shows hardly any substrate inhibition, in contrast to the situation at pH 7.6 (Fig. 7). The substrate inhibition decreases with decreasing activator concentration. The Pro–Tyr–amide curves with 1 mM chloride at pH 5.0

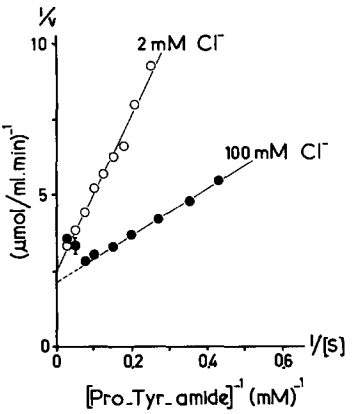


Fig. 7. Lineweaver–Burk plots of Pro–Tyr–amide hydrolysis by cathepsin C in the presence of 100 and 2 mM NaCl at pH 7.6. Buffer: 0.05 M sodium phosphate. Other experimental conditions as described in Fig. 6.

and with 2 mM chloride at pH 7.6 show apparent Michaelis–Menten kinetics; they presumably correspond to the high-substrate part of the curve with Gly–Phe–*p*-nitroanilide and 1 mM chloride. These Pro–Tyr–amide curves could not be determined at substrate concentrations below 2 mM because it is technically very difficult to determine initial rates at very low substrate concentrations from ammonia production. Until very recently[27] no substrate was available with which kinetics could be assayed in the entire concentration range; therefore, we had to combine the results obtained with two partially overlapping substrates.

Even in the absence of activators, there is always a certain residual activity towards Gly–Phe–*p*-nitroanilide, which amounts to 0.5–2.5% of the value at 100 mM chloride. This residual activity is, at least partially, due to traces of halides in the medium, traces which are extremely difficult to remove. In the absence of (added) activator, the cooperativity effect and the residual activity together result in a reaction rate with 50 mM Pro–Tyr–amide of about 30% of the rate in the presence of 100 mM chloride.

Very high concentrations of activators, with the exception of chloride, are inhibitory. The effects of several activators at 475 mM concentrations on the reaction rate with 0.36 mM Gly–Phe–*p*-nitroanilide were compared with their effects at 100 mM (bromide 20 mM) concentration with Na_2SO_4 added to equal ionic strength. The activity ratios were 1.00 with NaCl, 0.63 with KBr, 0.68 with KI, 0.43 with KCNS, and 0.79 with $NaNO_3$. With bromide, significant inhibition is already observed at a concentration of 40 mM.

Allosteric activators (and inhibitors as well) often have an effect on inactivation by heat, urea, proteolytic enzymes, *etc.* Therefore, we have studied the effect of 50 mM NaCl on heat denaturation of cathepsin C at 75° in 50 mM malonate buffer (pH 6.0). In the control, NaCl was replaced by Na_2SO_4 at the same I. After 60 min incubation with NaCl, 77% of the enzyme was still active, whereas with Na_2SO_4 only 6% of the enzyme activity was left.

The concentration of chloride has no effect on the sedimentation coefficient of cathepsin C at 20° and pH 6.9, which is 9.8 S at a protein concentration of 0.6 mg/ml, both with and without NaCl.

DISCUSSION

Why the activation phenomenon was not found before

Although cathepsin C has been investigated for many years there were no indications of a chloride requirement before the introduction of the assay with Gly–Phe-*p*-nitroanilide, in which much lower substrate concentrations are used than with other substrates, *e.g.* Gly–Phe–amide. The reason is clear: at high substrate concentrations, the reaction rate is largely independent of the activator concentration unless the latter is very low. This was never the case in the assay with Gly–Phe–amide, since this substrate is usually added as the hydrochloride or the hydrobromide, cysteine is always added as the hydrochloride, and the enzyme preparation often contains some NaCl. The chloride requirement became only apparent in a series of experiments in which hydrochloride had been replaced by mercaptoethanol. As in most if not all previous kinetic experiments[3,17], chloride (or other activators) had been present in unknown concentrations its effect on the reaction rate in those experiments is uncertain. The resulting K_m values are therefore variable and generally much higher than those found at high chloride concentration. On the other hand, it is clear from Fig. 5 that v_{max} values found by graphical extrapolation are independent of the chloride concentration.

Interpretation of the kinetic data

Cathepsin C is activated, and inhibited, by activators as well as substrates. The reaction sequence is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \text{acyl enzyme} \xrightarrow{k_3} E + P$$

so there are four velocity constants which may be affected by inhibition and activation. The constants k_1 and k_{-1} are considered jointly with the expression $K_S = k_{-1}/k_1$, since they cannot be determined separately. We tried to find out which constants were affected by the activation and inhibition and, if possible, to deduce an allosteric enzyme model which fits cathepsin C.

Little, if any, information can be gained from the substrate inhibition data. Proposed mechanisms should account for the differences in pH influence observed between Pro–Tyr–amide and Gly–Phe-*p*-nitroanilide. The dependence on halide activation is only apparent; therefore, inhibition need not be taken into consideration when interpreting the other phenomena. However, it makes a detailed investigation of them difficult.

Concerning inhibition by very high concentrations of activators we have the following data: compared to 100 mM, 475 mM of activator inhibits strongly the splitting of 0.36 mM Gly–Phe–*p*-nitroanilide. At saturating concentrations of the activator, the substrates are split according to Michaelis–Menten kinetics, and this is assumed to be still valid at inhibiting concentrations. Therefore, the hydrolysis rate of 0.36 mM Gly–Phe–*p*-nitroanilide (K_m = 3 mM, at 100 mM chloride) is proportional to

$$\frac{k_0}{K_m} = \frac{k_1 \cdot k_2}{k_{-1} + k_2} \simeq \frac{k_2}{K_S} \quad \left(k_0 = \frac{k_2 \cdot k_3}{k_2 + k_3},\ K_m = \frac{k_3 (k_{-1} + k_2)}{k_1 (k_2 + k_3)} \right),$$

so k_2/K_S is affected by the inhibition. The hydrolysis rate of 50 mM Ala–Phe–amide (v_{max}) is proportional to k_0. As 350 mM bromide, compared to 20 mM, causes a slight activation rather than inhibition of the hydrolysis rate of 50 mM Ala–Phe–amide, k_0 is not affected. $k_0 = k_3$, since it is known that, at least with chloride as the activator, the k_3 value of amide substrates is $\ll k_2$; in another part of the DISCUSSION it will be shown that the same holds true for bromide. In conclusion, inhibition by very high activator concentrations affects k_2 or K_S, or both, but not k_3.

There are more halide-activated enzymes for which high activator concentrations are inhibitory[18,19].

Any model of the mechanism of activation will have to account for the following data:

(i) At saturating activator concentrations, the substrates are split according to Michaelis–Menten kinetics; at non-saturating concentrations there is cooperativity, which is stronger when the activator concentration is lower.

(ii) v_{max} is independent of the activator concentration (at least with chloride).

(iii) The hydrolysis rate of 0.36 mM Gly–Phe–*p*-nitroanilide (at which concentration there is hardly any substrate activation), when plotted double-reciprocally against the activator concentration, yields a straight line over a large concentration range; in the absence of added activators there is only a very low residual activity.

(iv) The A_{max} and K_a values derived from the double-reciprocal plots are different for each activator.

(v) At non-saturating activator concentrations, not only the low-substrate concentration parts of the Lineweaver–Burk curves are linear, but the parts at high substrate concentration as well.

Regardless of any model to explain the enzyme's action, the following facts are clear from our experiments:

From the cooperativity observed with the substrate, it is concluded that there must be at least two substrate-binding sites per active unit; from the lack of cooperativity with the activator, it follows that there is either only one activator-binding site per active unit or that there are two or more independent sites.

The absence of substrate cooperativity at saturating activator concentrations and the independence of v_{max} of the activator concentration indicate that activation both by substrate and by activator affects the same velocity constants.

The occurrence of substrate cooperativity renders an activating effect on the reaction rate only (V-system) very unlikely. For that reason, activation of cathepsin C is expected to affect K_S; there may also be minor effects on k_2 and k_3.

To explain the different effects exerted by various activators, it is assumed

that at low activator concentrations the above-mentioned inhibition does not play a role. For the differences in A_{max} values between the activators, the same reasoning applies as for the inhibition by very high activator concentrations: the hydrolysis rate of 0.36 mM Gly–Phe–*p*-nitroanilide, *viz.* $k_0/K_m \simeq k_2/K_S$, is affected differently. Furthermore, there is a slight, but significant difference in the v_{max} values with amides as substrates. The differences in the A_{max} values are probably due to different activator effects on the K_S. The differences in the v_{max} values must be due to a separate small effect on the k_3 (or, but in our opinion less probably, to the k_2).

Cathepsin C obviously does not conform to the simplest allosteric enzyme model of MONOD *et al.*[20]. This model is based on an equilibrium between two, or a few, conformations of the enzyme preserving its symmetry under all circumstances and having a number of identical binding sites for the substrate(s) and for the activator(s). This model requires cooperativity for all ligands; cathepsin C does not meet this requirement in relation to the activators. Moreover, a situation of activators inducing different activities is not in accordance with a model having only a small number of possible conformations.

The model proposed by KOSHLAND *et al.*[21] is much more flexible because it does not require symmetry. A few mechanisms based on this model were devised which could account for the data (i), (ii), (iii), and (iv) but were found to be incapable of explaining (v) *viz.* the linearity of the high-substrate concentration part of the Lineweaver–Burk curves at low activator concentrations. Apparently, a more complex model is required, but the structure of cathepsin C is still completely unknown, and, without detailed data on the number of subunits and binding sites, it is useless to try and develop complex models. Anyway, the high molecular weight (200 000) renders a structure with subunits very likely, and the decrease of the sedimentation coefficient by detergents[11] supports this.

Halide-activated enzymes

A number of enzymes have been found whose activity is dependent on chloride, bromide, or iodide, or sometimes thiocyanate, nitrate, chlorate, and fluoride; all are hydrolytic enzymes. From beef liver an aryl sulfatase has been isolated[18]; in horse plasma[22] and in dog-lung tissue[23] an enzyme has been found which converts angiotensin I to angiotensin II by removing the C-terminal dipeptide His–Leu (ref. 22). More important for our investigations are a number of enzymes which split off N-terminal dipeptides. Like cathepsin C, these enzymes are very specific. From beef liver an enzyme has been isolated which splits off N-terminal His–Ser from glucagon[19]. Beef pituitary contains an enzyme which resembles cathepsin C in many respects[24]. Its ability to split off N-terminal Ser–Tyr from ACTH, which it has in common with cathepsin C (ref. 4), is especially noteworthy. The pituitary enzyme, however, does not utilize Ser–Tyr–amide and Gly–Phe–amide as substrates. In beef pituitary many more enzymes have been found which split off N-terminal dipeptides from peptide chains or dipeptide β-naphthylamides; a number of these enzymes are halide-dependent[24]. In hog kidney a chloride-activated enzyme has been found which splits Ala–Ala–β-naphthylamide[25]. In view of the recent results of MCDONALD *et al.*[27], it is possible that several of these enzymes are identical with or closely related to cathepsin C. Anyway, high chloride concentrations inhibit the above-mentioned glucagon-splitting enzyme[19]; in this respect it appears to differ from our enzyme.

Kinetics have not been investigated with any of these peptidases; in aryl sulfatase, chloride has been demonstrated to affect only v_{max}, not K_m; there is no cooperativity. Until now, cathepsin C has been the only hydrolytic enzyme in which cooperativity has been demonstrated. It is, however, not unreasonable to suppose that such cooperativity will be found in more, if not all, of the halide-dependent peptidases.

The biological importance of the chloride effect and the cooperativity cannot be assessed at this stage, at least until the biological function of cathepsin C is clearly defined.

ACKNOWLEDGMENTS

The authors are indebted to Dr. R. N. Campagne for his helpful suggestions in the preparation of this manuscript. We wish to express our thanks to Mrs. S. Degenhart-Kingma for skilful technical assistance. Furthermore Mr. W. Huisman is acknowledged for his contributions. The investigation was carried out under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.) and with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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