INACTIVATION OF CATHEPSIN B_1 BY DIAZOMETHYL KETONES

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SUMMARY. Benzyloxycarbonyl-phenylalanyl diazomethyl ketone and benzyloxycarbonyl-phenylalanyl-phenylalanyl diazomethyl ketone, which have been shown to inactivate the thiol protease papain by a mechanism different from that of substrate chloromethyl ketone derivatives, have now been examined as inhibitors of cathepsin B_1 of beef spleen. The dipeptide derivative irreversibly inactivates this protease rapidly, apparently by affinity labeling.

Cathepsin B_1 (E.C. 3.4.4.-), a lysosomal sulfhydryl protease present in many animal tissues (1), has been implicated in a number of physiological processes. For example, it has been shown capable of inactivating glucokinase, aldolase, and liver pyruvate kinase, while leaving unaffected the activities of other carbohydrate and amino acid metabolizing enzymes (2), indicating a regulatory role in protein turnover. More subtle effects of lysosomal proteases on enzymic activity are suggested by observations on 1,6 fructose biphosphatase (3). It has also been proposed that cathepsin B_1 may play some role in cellular detachment, invasive growth, and the primary step in metastasis formation (4). Cathepsin B_1 has recently shown to possess collagenolytic activity (5), which may be responsible for initial stages of extracellular breakdown of connective tissue (6). To explore these and many other possible roles of this protease, selective inhibitors would be valuable. Protease directed chloromethyl ketones do not permit a distinction between serine proteases and thiol proteases (7), therefore a chemically different class of reagents was sought. Diazomethyl ketone derivatives appeared promising as potentially selective for thiol proteases since initial observations indicate a lack of action on serine

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proteases (unpublished results). Studies with the thiol protease, papain, have established that Z-Phe[1] and Z-Phe-Phe[1] diazomethyl ketones are effective inactivators of that protease (8), therefore cathepsin B_1 was purified from bovine spleen and examined for its susceptibility to these inhibitors.

METHODS

Reagents: The synthesis of the inhibitors used in this investigation have been previously described (8,9,10). The organomercurial-Sepharose column was prepared by the procedure of Sluyterman and Wijdenes (11). The following chemicals were obtained from commercial sources: Aquasol from New England Nuclear; N^{α}-Z-Lysine' nitrophenyl ester from Cyclo chemical company; mercaptoethanol from Eastman Kodak; Sephadex gels from Pharmacia; Bio-Rex 70 from Bio Rad laboratories. Frozen bovine spleens were obtained from Pel-Freeze biochemicals and stored at -20° until needed.

Assay: Cathepsin B_1 was assayed spectrophotometrically, with N^{α}-Z-Lys-ONp[1] (12), by the addition of enzyme to 2.0 ml of 0.10 M acetate, 0.0025 M mercaptoethanol, 0.001 M EDTA (pH 5.4) followed by the addition of 20 µl of 10^{-2}M substrate in 95% acetonitrile. The increase in absorbance at 340 nm was measured. Enzyme activity was expressed as µmoles of p-nitrophenol produced min^{-1} ml^{-1} of enzyme solution.

The kinetic inactivation experiments were performed at room temperature. The inhibitors, at the appropriate concentration in methanol, were added at zero time to the enzyme solution. The final concentration of methanol was 10%. The extent of enzyme inactivation was determined by assaying an aliquot of the reaction and of a control mixture without inhibitor at various times.

Enzyme: Cathepsin B_1 was purified from bovine spleen by modification of the procedure of Otto and Risenkoning (13). Supernatent from the spleen extract (Fraction I) from one kilogram of tissue was fractionated with ammonium sulfate and the precipitate collected between 40 and 70% saturation was dissolved in 0.001 M EDTA and dialyzed overnight versus 8 liters of this solution. Dialysates from three batches were combined (Fraction II) and applied to a Bio-Rex 70 column (3.5 x 60 cm) previously equilibrated with 0.09 M citrate, 0.001 M EDTA (pH 5.4) buffer, and the column was developed with the same buffer. The fractions with high cathepsin B_1 activity were pooled and concentrated with an Amicon ultrafiltration apparatus, equipped with a UM-10 membrane. The concentrated pool (Fraction III) from the Bio-Rex 70 column was applied to a Sephadex G-75 column (2.0 x 100 cm) developed with 0.09 M citrate, 0.001 M EDTA (pH 5.4) buffer and fractions with high specific activity were pooled (Fraction IV).

Aliquots of this material were dialyzed overnight versus 0.10 M acetate, 0.10 M NaCl, 0.001 M EDTA (pH 5.4) buffer and applied to an organomercurial - Sepharose column (10 ml). Unadsorbed proteins were washed from the column with 0.10 M acetate, 0.10 M NaCl, 0.001 M EDTA (pH 5.4). Absorbed proteins were eluted from the column with the same buffer containing 0.01 M cysteine, and fractions containing significant amounts of protein were pooled for use (Fraction V) in the inactivation experiments described.

All the steps outlined in the purification procedure, with the exception of the initial acid extraction and the organomercurial Sepharose chromato-

[1]Abbreviations: Z- = benzyloxycarbonyl, -ONp = p-nitrophenylester.

graphy were carried out at 4°. The final specific activity of the cathepsin B_1 obtained as outlined above, ranged from 14.13 to 26.95 μmoles of nitrophenol released per minute per A_{280} per ml of enzyme solution in four different preparations of the enzyme. When this material was subjected to disc gel electrophoresis at pH 4.5 (13) followed by staining for amidase activity (14) a single band of color was observed although Coumassie blue revealed the presence of other proteins in addition. A summary of one preparation is shown below:

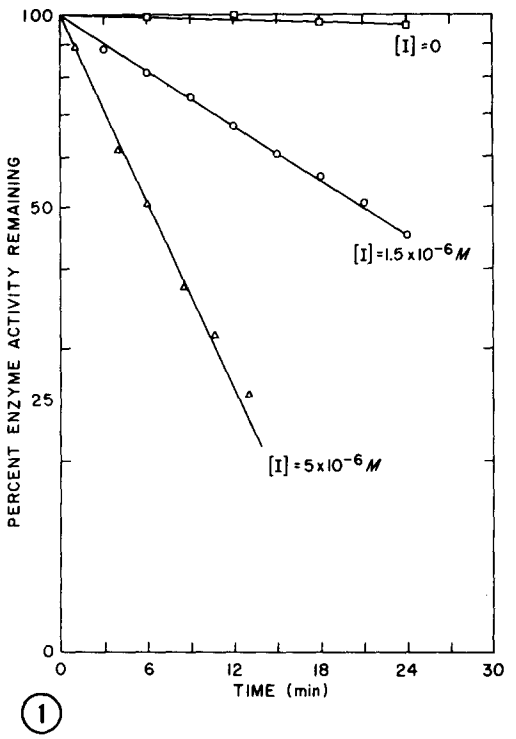
Fraction	Vol.	Total Units	Spec. Act.	Purification Factor	Recovery
I	5030 ml	5229	0.027	1	100%
II	260 ml	3489	1.00	37	67%
III	5.5 ml	1284	4.52	167	25%
IV	32 ml	894	10.62	392	17%
V	39 ml	713	14.13	523	14%

RESULTS AND DISCUSSION

The incubation of cathepsin B_1 with Z-Phe-CHN_2 (2.5×10^{-4}M) resulted in a gradual loss of activity reaching 50% in 173 minutes. This slow action permitted the demonstration that Z-Phe-CHN_2 was a competitive inhibitor for the enzyme with the substrate used with $K_i = 8.0 \times 10^{-4}$M.

The action of the dipeptide derivative, Z-Phe-PheCHN_2, was strikingly different, rapid inactivation following pseudo first order kinetics was observed with micromolar concentrations of the inhibitor (Figure 1). This marked increase in reactivity was also observed in the case of papain (8) and reflects the participation of an extended binding site.

Diazomethyl ketones are chemically unreactive in comparison with chloromethyl ketones and do not combine with the excess mercaptoethanol present in the buffer during inactivation experiments. This fact and the observation that Nα-Z-L-Phe diazomethyl ketone is a competitive inhibitor of the hydrolysis of Nα-Z-L-Lys nitrophenyl ester by cathepsin B_1 suggest that the inactivation of cathepsin B_1 by the diazomethyl ketones employed in this investigation results from prior complex formation of these reagents with the enzyme. This initial binding is thought to provide an orientation of the inhibitors at the active site of cathepsin B_1 such that the chemical potential of the essentially inert diazomethyl ketone function



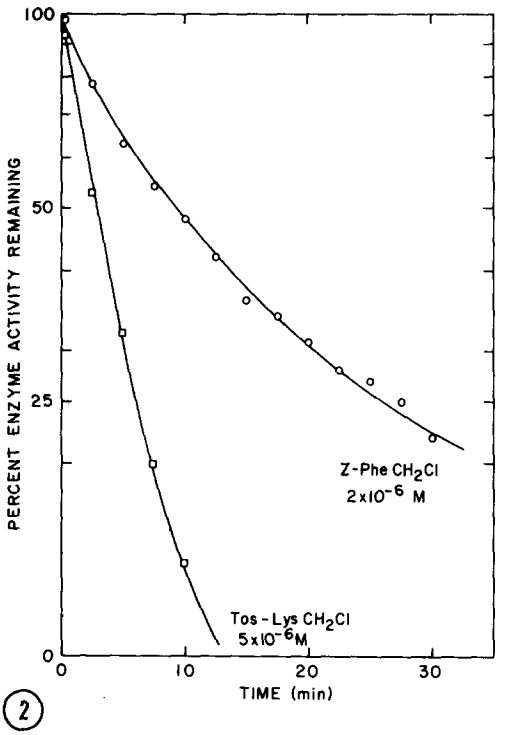


Fig. 1. Inactivation of cathepsin B_1 by Z-Phe-PheCHN$_2$ at pH 5.4 in 0.09 M acetate, 0.0020 M mercaptoethanol, 0.0009 M EDTA containing 10% acetonitrile at room temperature. Reaction mixture (2 ml) contained 40 µl of enzyme, specific activity 19.5. Aliquots (200 µl) removed at indicated times for assay.

Fig. 2. Inactivation of cathepsin B_1 by chloromethyl ketones. Conditions as in legend to Fig. 1.

is expressed and alkylation of the enzyme results. Continuing work on the kinetic characteristics of the reaction of diazomethyl ketones with cathepsin B_1 is expected to substantiate the affinity-labeling character of the inactivation. Structural variations in the peptide portion of the reagent are under way and can be expected to shed some light on the character of the binding sites in the cathepsin B_1 active center as well as indicate the biological potency attainable with this class of protease inhibitor. As pointed out, the reaction follows a different mechanism than chloromethyl ketone inactivation in the case of papain (8) shown, for example, by contrasting pH dependencies. It was not possible to examine this with

TABLE I

INACTIVATION OF CATHEPSIN B_1 BY CHLORO- and DIAZO-METHYL KETONES

REAGENT	CONCENTRATION (M)	$t_{\frac{1}{2}}$(min)	k_1(min^{-1})	$k_{1/I}$(min^{-1} mol^{-1})
N^{α}-Tos-LysCH_2Cl	5×10^{-6}	3	0.23	46,200
N^{α}-Tos-PheCH_2Cl	5×10^{-5}	5.3	0.131	2,610
N^{α}-Z-PheCH_2Cl	2×10^{-6}	7.25	0.096	48,000
N^{α}-Z-PheCHN_2	2.5×10^{-4}	173	0.004	16.1
N^{α}-Z-Phe-PheCHN_2	1.5×10^{-6}	21	0.033	22,000
	5.0×10^{-6}	6.4	0.11	22,000

cathepsin B_1 due to instability of the enzyme above neutrality. Contamination of diazomethyl ketone preparations by chloromethyl ketones is a source of error to be guarded against in this type of study (8).

To provide some basis for comparison of the two types of reagents, the rates of inactivation of cathepsin B_1 by Tos-Lys-CH_2Cl, Tos-PheCH_2Cl and Z-PheCH_2Cl were measured. The inactivation of human liver cathepsin B_1 by this type of reagent had been observed by Barrett without an attempt to follow rates (15). As shown in Figure 2, the reaction did not follow pseudo first order kinetics at the reagent concentrations used; mercaptoethanol, present in excess, most likely was reacting with the reagent. The initial linear part of the inactivation curve was utilized in calculating a rate of inactivation. Data are presented in Table I with $k_{1/[I]}$ (last column) presented as a basis for comparison of reactivity (8).

The reaction of cathepsin B_1 with the various chloromethyl and diazomethyl ketones reported in this study has not been investigated at the level of protein chemistry and cannot be definitely assigned to the reaction with a particular amino acid residue. However, previous studies of the reaction of chloromethyl ketones with other sulfhydryl proteases (16)

have definitely established that inactivation results from alkylation of the active center cysteine residue and the inactivation of papain by Nα-Cbz-L-Phe-diazomethyl ketone was shown to result from the alkylation of the active center cysteine residue (8), thus it seems reasonable to assume that the inactivation of cathepsin B_1 follows a similar course. The inactivation of pepsin, an acid protease, by active site directed diazomethyl ketones has been shown to result from the esterification of active site carboxyl groups (17), a reaction apparently requiring cupric ion activation.

Peptide diazomethyl ketone derivatives appear to be well suited for in vivo and in vitro investigations of the role of cathepsin B_1 in the physiological and pathological processes in which the enzyme has been implicated.

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