Inhibitory Spectrum of Talopeptin (MKI), a Specific Inhibitor of Thermolysin

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Talopeptin (*N*-(6-deoxy- α -L-talopyranosyloxyphospho)-L-leucyl-L-tryptophan), a specific inhibitor for thermolysin and some other endo-type metalloproteases, does not inhibit superoxide dismutase, alkaline phosphatase, or carbonic anhydrase, which are Zn(II)-enzymes, in the inhibitor concentration of the order of 10^{-4} M. Although the enzyme activity of carboxypeptidase A, a Zn(II)-containing exo-type metalloprotease, is slightly reduced by the addition of talopeptin, the inhibition is not significant at the 10% level judged by Student's *t* test. On the other hand, talopeptin seemed to act as an inhibitor for all dehydrogenases examined, most of which do not contain Zn(II), at the inhibitor concentration studied (around 10^{-4} M). It is possible that the phosphite group of talopeptin binds to the same site on the dehydrogenase as the one for the phosphate group of the coenzyme.

Talopeptin (MKI) specifically inhibits metalloproteases, including thermolysin.¹⁾ The X-ray crystallographic study of the three-dimensional structure of the complex between thermolysin and phosphoramidon (*N*-(α -L-rhamnopyranosyloxyphospho)-L-leucyl-L-tryptophan) has suggested that the phosphite oxygen of phosphoramidon and talopeptin binds to the zinc ion in the catalytic site of the enzyme.²⁾

The question naturally arises whether or not talopeptin can bind and inhibit other metalloenzymes, especially those containing Zn(II). In this paper, was investigated the inhibitory activity of talopeptin against Zn(II)-containing enzymes (carboxypeptidase A, carbonic anhydrase, alkaline phosphatase, superoxide dismutase, and alcohol dehydrogenase)³⁾ and also against other dehydrogenases that do not contain Zn(II).

MATERIALS AND METHODS

(a) Materials.

Enzymes. Commercial products from Boehringer Mannheim GmbH: alcohol dehydrogenase (ADH) (EC 1.1.1.1) from yeast and ADH from horse liver, lactate dehydrogenase (LDH) (EC 1.1.1.27) from rabbit muscle, carbonic anhydrase (CA) (EC 4.2.1.1) from beef erythrocytes, and carboxypeptidase A (CPA) (EC 3.4.17.1) from beef pancreas. From the Oriental Yeast Co., Ltd.: α -glycerophosphate dehydrogenase (α -GDH) (EC 1.1.1.8) from rabbit muscle, LDH from *Leuconostoc mesenteroides*, malate dehydrogenase (MDH) (EC 1.1.1.37) from yeast, MDH from pig heart, glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) from yeast (Grade I and II), and glutamate dehydrogenase (GLDH) (EC 1.4.1.3) from beef liver. From the Sigma Chemical Co.: MDH from beef heart and alkaline phosphatase (AP) (EC 3.1.3.1) from *E. coli*. From Miles Laboratories Limited: superoxide dismutase (SOD) (EC 1.15.1.1) from beef erythrocytes. From P.-L. Biochemicals, Inc.: xanthine oxidase (EC 1.2.3.2) from buttermilk.

Coenzymes, substrates. NAD, NADH, and NADP were purchased from P.-L. Biochemicals, Inc. Dihydroxy-

Abbreviations: talopeptin (MKI), *N*-(6-deoxy- α -L-talopyranosyloxyphospho)-L-leucyl-L-tryptophan; DH, dehydrogenase; ADH, alcohol dehydrogenase (EC 1.1.1.1); α -GDH, α -glycerophosphate dehydrogenase (glycerol-3-phosphate dehydrogenase) (EC 1.1.1.8); LDH, lactate dehydrogenase (EC 1.1.1.27); MDH, malate dehydrogenase (EC 1.1.1.37); G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GLDH, glutamate dehydrogenase (EC 1.4.1.3); AP, alkaline phosphatase (EC 3.1.3.1); CA, carbonic anhydrase (EC 4.2.1.1); SOD, superoxide dismutase (EC 1.15.1.1); CPA, carboxypeptidase A (EC 3.4.17.1); DL-HPLA, hippuryl-DL-phenyllactic acid; DAP, dihydroxyacetone phosphate.

TABLE I. THE COMPOSITION OF THE REACTION MIXTURES FOR THE DEHYDROGENASE ASSAYS

DH	Source	[E] ₀	[Coenzyme] ₀	[S] ₀ , etc.
ADH	Yeast	1 nM	1.5 mM [NAD]	0.015~0.12 M [ethanol]
	Horse liver	0.22 µg/ml	2.0 mM [NAD]	67 mM [ethanol]
α-GDH	Rabbit muscle	0.039 U/ml	0.14 mM [NADH]	0.19 mM [DAP]
LDH	Rabbit muscle	32 ng/ml	0.18 mM [NADH]	2.8 mM [pyruvate]
	<i>Leuconostoc mesenteroides</i>	0.033 U/ml	0.17 mM [NADH]	2.8 mM [pyruvate]
MDH	Yeast	0.10 U/ml	0.13 mM [NADH]	1.7 mM [oxalic acetate]
	Pig heart	0.18 U/ml	0.09 mM [NADH]	1.3 mM [oxalic acetate]
	Beef heart	0.25 U/ml	0.16 mM [NADH]	1.3 mM [oxalic acetate]
G6PDH	Yeast (Grade I)	0.028 U/ml	0.50 mM [NADP]	0.50 mM [G6P], 20 mM [MgCl ₂]
	(Grade II)	0.028 U/ml	0.50 mM [NADP]	0.50 mM [G6P], 20 mM [MgCl ₂]
GLDH	Beef liver	4.8 nM	0.16 mM [NADH]	3.3 mM [2-ketoglutarate], 0.13 mM [NH ₄ Cl], 83 µM [EDTA]

acetone phosphate (DAP) was obtained by hydrolyzing dihydroxyacetone phosphate dimethylketal (Boehringer Mannheim GmbH).

(b) *Methods.* Rates of the enzyme reactions were determined as follows: The initial rate was obtained from the course recorded by spectrophotometer or pH-stat, except for carbonic anhydrase (see below). Enzyme concentrations employed, except for superoxide dismutase, were confirmed to be within the range where the initial rate was proportional to the enzyme concentration.

Dehydrogenases. The rate of the enzymatic reaction was measured at 340 nm through the rate of formation or disappearance of NADH or NADPH at 25°C with a spectrophotometer Union-Giken SM-401. The composition of the reaction mixtures (1.5 ml, pH 7.5 Tris buffer) for the various enzymatic assays is listed in Table I (optical path: 1 cm).

Alkaline phosphatase. Enzymatic activity was estimated essentially according to the method of Neil and Horner,⁴⁾ measuring the increase in absorbance at 420 nm after the addition of the enzyme with a Shimadzu UV-200 spectrophotometer at 25°C. The reaction mixture contains *p*-nitrophenyl phosphate and 2 µg of the enzyme in 3 ml Tris buffer pH 8.0, and the concentration of *p*-nitrophenyl phosphate employed is 25 µM or 1 mM.

Carbonic anhydrase. Carbon dioxide solutions were prepared by bubbling CO₂ from a gas cylinder (Gasukuro Kogyo Co., Ltd.) into H₂O. The concentration of CO₂ in solutions was determined by the addition of an aliquot to a measured excess of 0.04 M Ba(OH)₂ and back titration with 0.01 N HCl, with phenolphthalein as the indicator.⁵⁾ The course of the reaction was followed spectrophotometrically (at 400 nm) through the rate of pH change by using *p*-nitrophenol as the indicator, after mixing the CO₂ solution, *p*-nitrophenol, and the enzyme, with a Union-Giken SM-401 spectrophotometer. The enzyme reaction was carried out with 4.3 mM CO₂, 91 µM *p*-nitrophenol,

4.1 ng/ml enzyme, and pH 6.4, 10 mM PIPES buffer at 14°C (total volume: 1.5 ml). The reaction obeyed first-order kinetics, and the apparent first-order rate constant was estimated from the course, which was used as the measure of the enzyme reaction rate.

Superoxide dismutase. Because of the instability of its substrate, the superoxide radical, an indirect assay had to be carried out for this enzyme. The inhibition of the rate of reduction of ferricytochrome *c* by superoxide anion formed in the aerobic xanthine oxidase-xanthine system was measured spectrophotometrically at 550 nm and 25°C.⁶⁾ The reaction mixture (2 ml, Tris buffer) contained cytochrome *c*, xanthine, and EDTA, whose concentrations were 10 µM, 50 µM, and 0.1 mM, respectively, and xanthine oxidase was added to produce a rate of reduction of cytochrome *c* of 0.023 absorbance change per min at 550 nm ([E]₀ ≈ 5 × 10⁻⁹ M), in the absence of superoxide dismutase. The addition of 1.38 U superoxide dismutase to the reaction mixture reduced the rate of the xanthine oxidase-catalyzing reaction to 53%.

Carboxypeptidase A. Esterase activity of carboxypeptidase A (CPA) was measured by using hippuryl-DL-phenyllactic acid (DL-HPLA) as a substrate with a pH stat apparatus (RTS 822 titration recording system, Radiometer, Copenhagen). The enzyme reaction was carried out in a thermostatted cell at 25°C under an N₂ gas stream with 10 mM HPLA, 16 nM enzyme, and 5 mM Tris buffer (50 mM NaCl) pH 7.5, in a volume of 3.0 ml.

RESULTS AND DISCUSSION

The results obtained are summarized in Table II. Student's *t* test was used to judge whether or not talopeptin inhibits the enzyme under the conditions employed. The number of stars indicates the confidence limit.

TABLE II. INHIBITION SPECTRUM OF TALOPEPTIN AGAINST Zn(II)-CONTAINING ENZYMES AND DEHYDROGENASES

Enzyme	Source	Essential Zn(II) involvement	[I] (mM)	Residual activity (%) ^a	Rough estimation of K_i (mM) ^b	Reaction conditions
AP	<i>E. coli</i>	○	0.46	99		pH 8.0, 25°C
CA	Beef erythrocytes	○	0.86	105		pH 6.4, 14°C
SOD	Beef erythrocytes	○	0.88	98		pH 8.0, 25°C
CPA	Beef pancreas	○	0.70	86		pH 7.5, 25°C
Dehydrogenase						
ADH	Yeast	○	0.2~0.4		0.4 ^c	
	Horse liver	○	0.66	92***	8	
α -GDH	Rabbit muscle	×	0.35	31***	0.2	
LDH	Rabbit muscle	×	0.86	62***	1.4	
	<i>Leuconostoc mesenteroides</i>	×	(?)	91**	9	pH 7.5, 25°C
MDH	Yeast	×	0.66	82**	3	
	Pig heart	×	0.66	58***	0.9	
	Beef heart	×	0.35	60***	0.5	
G6PDH	Yeast (Grade I)	×	0.66	72***	2	
	(Grade II)	×	0.66	88***	5	
GLDH	Beef liver	×	0.37	71***	0.9	

^a Student's *t* test was carried out to estimate the significance of the inhibition by talopeptin. The number of stars indicates the confidence limit. * Significant at 10% level. ** Significant at 5% level. *** Significant at 1% level.

^b The values are accompanied with a $\pm 50\%$ error, at maximum.

^c This value was estimated from an *s/v* vs. *s* (Hofstee plot).

Carboxypeptidase A, alkaline phosphatase, carbonic anhydrase, and superoxide dismutase

All of these four enzymes contain Zn(II). For carboxypeptidase A and carbonic anhydrase, the possibility is suggested that Zn(II) of the enzymes could act as a Lewis acid, withdrawing electrons from certain atomic groups of the substrate molecule,^{3,7)} as in the case of thermolysin.⁸⁾ Superoxide dismutase catalyzes an apparently diffusion-controlled oxidation of the superoxide radical at the Cu(II) ion of the enzyme catalytic center.⁹⁾ The function of the second metal, Zn(II), present in each subunit is not clear. It appears likely that Zn(II) is essential for stabilizing this enzyme.¹⁰⁾ Alkaline phosphatase from *E. coli* (MW 80,000) contains four atoms of tightly bound Zn(II) and two Mg(II) per mol.^{11,12)} Two zinc atoms of the four are essential to catalytic activity¹³⁾ and the additional two stabilize the protein structure.¹⁴⁾

Talopeptin does not inhibit superoxide dis-

mutase, alkaline phosphatase, or carbonic anhydrase at all in the inhibitor concentration range employed (around 10^{-4} M). The inhibition of carboxypeptidase A (Table II) cannot be deemed significant at the 10% level judged by Student's *t* test. It is noticeable that the interaction with talopeptin is entirely different for the two metalloproteases, carboxypeptidase A and thermolysin. One possible interpretation is that the dipeptide part of the inhibitor is too large to fit the substrate binding pocket (S_1') of the exopeptidase, carboxypeptidase A.¹⁵⁾ In fact, Kam *et al.* reported that *N*-phosphorylphenylalanine is a good inhibitor of carboxypeptidase A ($K_i = 5 \times 10^{-6}$ M), but a larger inhibitor, *N*-phosphorylleucyltryptophan, shows appreciably weaker inhibition ($K_i = 5.4 \times 10^{-5}$ M).¹⁶⁾

Dehydrogenases

Talopeptin inhibits all dehydrogenases examined in the inhibitor concentration range

around 10^{-4} M, as seen from Table II.

Horse liver alcohol dehydrogenase (a dimeric protein) contains two Zn(II) ions per monomer. One zinc ion at the bottom of the active site cleft is essential for the enzyme activity, while another lies in the periphery of the subunit and may be important in maintaining the protein structure.¹⁷⁾ The involvement of Zn(II) as a Lewis acid catalyst in liver alcohol dehydrogenase has been suggested.¹⁸⁾ Some investigations provide the evidence for zinc-bond water as the ionizing functional group involved in coenzyme binding, proton release, and conformational stability in the liver alcohol dehydrogenase reaction.^{19~22)} Alcohol dehydrogenases from other sources also contain catalytically essential Zn(II).¹⁸⁾ Some other dehydrogenases were originally thought to be zinc metalloproteins.^{23~26)} However, further studies demonstrated that the zinc ion in bovine glutamate dehydrogenase is not an essential constituent of the enzyme but may function as an allosteric effector,²⁷⁾ and that no significant amounts of zinc or other metals are contained in lactate dehydrogenases and malate dehydrogenases,^{28~30)} despite the structural similarities among dehydrogenases.³¹⁾

The inhibition for some of lactate dehydrogenases and malate dehydrogenases, which have been regarded as non-zinc enzymes, are stronger than that for horse liver alcohol dehydrogenase, a zinc-containing enzyme. Accordingly, it can be concluded that talopectin is able to bind also to dehydrogenases which do not contain Zn(II), and it does not necessarily bind to the Zn(II) of alcohol dehydrogenase. In fact, Colman and Foster found that 1,10-phenanthroline, a Zn(II)-chelating compound, inhibits zinc-free glutamate dehydrogenase.²⁷⁾

Probably the talopectin binding site is not far from the active sites of the dehydrogenases. One possibility is that talopectin binds near the coenzyme (NAD or NADP) binding site, and the phosphite group of talopectin binds to the binding site for the phosphate group of the coenzyme. This interpretation is not unreason-

able, since both talopectin and coenzyme have sugar(s) (6-deoxytalose and riboses, respectively) in addition to the similarity in the phosphite or phosphate group. Most dehydrogenases have the Michaelis constants, K_m 's, for coenzymes in the range of $10^{-5} \sim 10^{-3}$ M.³²⁾ The inhibitor constants of talopectin, K_i 's, for dehydrogenases are roughly $10^3 \sim 10^5$ times as large as its K_i for thermolysin (4×10^{-8} M at pH 7.5 and 25°C),³³⁾ but slightly larger than the K_m 's of the coenzymes for dehydrogenases.

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