Effect of Cobalt Ion on the Enzymatic Activity of *Pseudomonas aeruginosa* Alkaline Proteinase

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Received October 11, 1973

Pseudomonas aeruginosa alkaline proteinase was inactivated by *o*-phenanthroline in the presence of high concentration of Ca ion, which suggested that the enzymatic activity is related not to Ca ion but to another heavy metal ion. Among various metal ions tested, Co ion was effective in promoting hydrolysis, a several-fold increase in hydrolysis being observed in the presence of Co at a concentration higher than 5×10^{-3} M. A kinetic study indicated that the promotion related mainly with binding (*Km*) and little with catalysis (*k_{cat}*). The points of cleavage in oxidized insulin B chain were assumed to be identical in the presence or absence of Co ion.

Pseudomonas aeruginosa produces two proteinases which are separable by column-chromatography on DEAE-cellulose.¹⁾ Both the enzymes have been crystallized and their physico-chemical and enzymatic properties clarified.^{2~6)} One of the enzymes is most active in neutral pH range, and as it is active on elastin it can be called an "elastase".⁶⁾ The elastase is a typical metal-chelator sensitive neutral proteinase^{7,8)} which shows specificity against bulky or hydrophobic amino acid residues at the imino-side of the splitting point.

The other enzyme is most active at slightly alkaline pHs²⁾ but does not hydrolyze elastin,⁹⁾ and it might therefore be called an "alkaline proteinase." This enzyme is inactivated by various metal-chelators but not by DFP,* PCMB, etc.,^{2,4)} similarly as seen with the "elastase".⁶⁾ Nevertheless, the specificity of the alkaline proteinase against oxidized insulin B chain was completely different from that of the "elastase".⁷⁾ A study using various synthetic peptide substrates has shown¹⁰⁾ that

the specificity of the alkaline proteinase is determined rather by the amino acid residues distant from the splitting point (e.g., "secondary interaction") than by those on either side of the splitting point (e.g., "primary specificity") in an oligopeptide substrate.

The metal content of the "elastase" has not been determined, but like the other neutral proteinases from bacterial origin,^{11,12)} it may be a Zn-enzyme. The alkaline proteinase does not contain a significant amount of any metal ion except Ca (one to two gramatoms of Ca per mole of enzyme).⁴⁾ The question then arises whether the inactivation of the alkaline proteinase by metal-chelators occurs by a mechanism similar to that observed with usual neutral proteinases from bacterial origin. The present study was undertaken to investigate whether the constituent Ca ion is essential for activity of the alkaline proteinase, and whether any other metal ion is essential for its activity.

MATERIALS AND METHODS

Enzyme. The crystalline proteinase of *P. aeruginosa* IFO 3080 (twice recrystallized) was prepared according to the method described previously.²⁾ The preparation was found to be homogeneous in disc-electrophoresis at pH 8.

Substrates. Z-Phe-Ala-Ala, Z-Ala-Gly-Gly-Leu, and Z-Ala-Phe-Gly-Ala were synthesized according to

* Abbreviations used are: DFP, diisopropyl phosphorofluoridate; PCMB, *p*-chloromercuribenzoic acid; EDTA, ethylenediamine tetra-acetate. Abbreviated designations of peptides or their derivatives obey the tentative rules of the IUPAC-IUB Commission of Biochemical Nomenclature. Except where specified, the constituent amino acids were all of the L-configuration.

the methods given in the previous papers.^{10,13)} Zinc insulin (crystals) was kindly donated by Lilly Laboratories, Indianapolis, Indiana. Oxidized B-chain was prepared according to the method of Hayashi,¹⁴⁾ which is a modification of the method of Sanger.¹⁵⁾

Hydrolysis of synthetic peptides. A reaction mixture (1 ml) containing 0.05 M Tris-buffer of pH 7.0, an appropriate concentration of peptide, and a suitable amount of enzyme was incubated at 40°C. At three minute intervals 0.1 ml of the reaction mixture was transferred into a test tube containing 1 ml of a mixture of 0.5 M citrate buffer (pH 5) and 0.01 M EDTA solution to stop further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking.¹⁶⁾

The sites of action of enzyme upon substrates were determined by paper chromatography of the hydrolyzates in comparison with authentic compounds, or by paper-electrophoresis in a current of 1200 V at pH 2 (0.6 M formic acid and 2 M acetic acid, 1:1, by volume) for 50 min. The results indicated that Z-Phe-Ala-Ala,

Z-Ala-Gly-Gly-Leu, and Z-Ala-Phe-Gly-Ala were split
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by the enzyme at the peptide bonds shown by the arrows.

The color yields by the ninhydrin method of Ala-Ala, Gly-Leu, and Gly-Ala, based on L-leucine as 100%, were given in previous papers.^{13,17)} The initial velocity for each peptide was calculated using the corresponding standard curve.

Gelatin-hydrolysis. The activity was measured according to the method of Ottesen and Spector¹⁸⁾ using the pH-stat technique at pH 8.0 and 30°C with 10 ml of 0.5% gelatin solution as substrate and 0.05 N NaOH as titrant. Activities were estimated from initial rates and expressed as $\mu\text{eqv. H}^+/\text{min/mg enzyme/ml}$.

Kinetic study. In all cases, satisfactory Michaelis-Menten kinetics were observed, and plots of $1/v$ vs $1/S$ (Lineweaver-Burk plots) permitted the fitting of definite straight lines. For each determination of K_m and V_{max} derived from such plots, initial rates were measured from five (or more) values of the initial substrate concentration S . Depending upon the rate of enzymatic cleavage, the enzyme concentration was suitably adjusted; it was assumed that the molecular weight was 48,400.³⁾ This enzyme concentration was used to calculate k_{cat} from V_{max} values.

Determination of the points of cleavage in oxidized insulin B chain. The digestion of oxidized insulin B chain ($[S]=4$ mg/ml; $E:S=1:100$, w/w,) was carried out at 25°C in the presence of 0.1 M KCl and 0.01 M CoCl_2 . The pH of the reaction mixture was kept at 7.5 by the aid of a Radiometer pH-stat. After 20 hr reaction, the digest was frozen and lyophilized. Separation of peptides was performed by high voltage paper-

electrophoresis at pH 3.7 (pyridine, acetic acid, and water, 1:10:280, v/v) for 90 min at 2000 V. The peptides separated were detected by spraying ninhydrin solution onto guide strips, and eluted with 30% acetic acid. The eluates are designated by Roman numerals in order from right ("positive" charge) to left ("negative" charge) in the paper-electrophoresis.

After evaporation *in vacuo*, each peptide fraction was further separated by paper-chromatography (pyridine, *n*-butanol, acetic acid, and water, 20:30:6:24, v/v). For peptide nomenclature, an alphabetic suffix is added in order from lower to upper positions on the chromatogram. The peptides were eluted with 30% acetic acid, then dried and hydrolysed with 0.5 ml of twice distilled 6 N HCl for 24 hr at $105 \pm 1^\circ\text{C}$ in sealed and evacuated tubes. The hydrolyzates were dried in a rotary evaporator and the amino acid compositions were determined by an amino acid analyzer (Hitachi KLA-Type 2) for determination of the points of cleavage in oxidized insulin B chain.

RESULTS

Effect of o-phenanthroline in the presence of Ca ion

It is well-known¹⁹⁾ that *o*-phenanthroline binds Ca very weakly ($\log K_1=0.7$) while binding strongly heavy metal ions such as Zn ($\log K_1=6.36$; $\log K_2=5.64$; $\log K_3=5.20$). An *o*-phenanthroline-Ca solution should therefore be useful for preferential removal of such heavy metal ions. At a concentration of 2×10^{-3} M *o*-phenanthroline and 10^{-2} M CaCl_2 , less than one percent of the Ca ions are complexed. To investigate the role of the Ca ion constituent of the enzyme molecule, the hydrolysis of Z-Ala-Phe-Gly-Ala was determined in the presence of various concentrations of *o*-phenanthroline and 10^{-2} M CaCl_2 . The results are summarized in Table I, which indicates that the hydrolysis is inhibited almost completely in presence of *o*-phenanthroline at a concentration higher than 5×10^{-4} M, even in the presence of 10^{-2} M of CaCl_2 . It is therefore clear that the activity of the enzyme is related not to Ca ion but to other heavy metal ions.

The inhibition was instantaneous under the experimental conditions employed; no greater inhibition was obtained after preincubation with inhibitor than without preincubation.

TABLE I. EFFECT OF *o*-PHENANTHROLINE ON THE ENZYMATIC ACTIVITY OF *P. aeruginosa* ALKALINE PROTEINASE

The reaction mixture (1 ml) contained 10 mM peptide (Z-Ala-Phe-Gly-Ala), 0.02 mg enzyme, 10^{-2} M CaCl_2 , and 0.05 M Tris-buffer (pH 7.0). Initial velocities were determined at 40°C in the presence of various concentrations of *o*-phenanthroline shown in the table.

<i>o</i> -Phenanthroline (M)	Relative activity (%)
0	100
10^{-5}	74
2×10^{-5}	70
5×10^{-5}	44
10^{-4}	27
2×10^{-4}	10
5×10^{-4}	2
10^{-3}	0
2×10^{-3}	0

The enzyme inhibition was reversed upon dilution of chelator-enzyme solution or on addition of excess metal ions such as Zn, Co, Mn, etc. In a previous paper⁴⁾ it was shown that an EDTA concentration of more than 10^{-2} M was required for inactivation at pH 7, where the remaining activity was determined using casein as substrate. This inconsistency can be explained by considering that the EDTA-inactivated enzyme was reactivated by metal ions contaminating the casein used as substrate, thus necessitating a higher concentration of EDTA for the inactivation.

Effect of Co ion on hydrolysis

The above suggests that some heavy metal ion is essential for the activity of the enzyme, even though it has been shown⁴⁾ that the enzyme does not contain a significant amount of any metal ion except Ca. The effects of various metal ions on the enzymatic activity were therefore determined using Z-Ala-Phe-Gly-Ala as substrate. The results are summarized in Table II; of the metal ions tested, Co ion alone promoted the rate of hydrolysis. The effect of the concentration of Co ion on hydrolysis was further studied as shown in Table III. A considerable concentration of

TABLE II. EFFECT OF VARIOUS METAL IONS ON THE ENZYMATIC ACTIVITY OF *P. aeruginosa* ALKALINE PROTEINASE

The reaction mixture (1 ml) contained 10 mM peptide (Z-Ala-Phe-Gly-Ala), 0.02 mg enzyme, 0.05 M Tris-buffer (pH 7.0), and various metal ions (2×10^{-3} M) as seen in the table. Initial velocities were determined at 40°C .

Metal	Relative activity (%)
Control	100
CaCl_2	105
ZnCl_2	82
CoCl_2	220
NiCl_2	100
MnCl_2	90

TABLE III. EFFECT OF CONCENTRATION OF CO ION ON THE ENZYMATIC ACTIVITY OF *P. aeruginosa* ALKALINE PROTEINASE

Both preincubated and non-preincubated enzyme preparations were used; preincubated enzyme was treated with CoCl_2 solution (10^{-2} M) for one night at about 4°C . The concentrations of CoCl_2 in the reaction mixtures are as seen in the table. Other conditions are described in Table II.

CoCl_2 (M)	Without pre-incubation (Relative activity, %)	With pre-incubation (Relative activity, %)
0	100	—
10^{-5}	—	120
10^{-4}	—	160
10^{-3}	—	470
2×10^{-3}	220	630
5×10^{-3}	—	880
10^{-2}	430	840
2×10^{-2}	520	950
5×10^{-2}	710	—

Co ion ($> 5 \times 10^{-3}$ M) was required for maximum promotion of hydrolysis, though the requisite concentration differed considerably depending upon whether preincubation with Co ion had been done or not. At the optimum concentration, the rate of hydrolysis was increased several fold. The enzyme activation with Co ion was reversed upon dilution of the activated enzyme. This, together with the above results would indicate that Co ion binds loosely to the enzyme molecule. Similar promotion of hydrolysis by Co ion was observed when other synthetic

FIG. 1. Lineweaver-Burk Plots of Hydrolysis of Various Peptides and Gelatin by *P. aeruginosa* Alkaline Proteinase in the Presence or Absence of Co Ion.

The kinetic study was made in the presence (○) or absence (●) of 10^{-2} M CoCl_2 . The other experimental conditions are described in MATERIALS AND METHODS.

peptides or gelatin were used as substrates.

In order to investigate the role of Co ion in the promotion of hydrolysis, a kinetic study was made using Z-Phe-Ala-Ala, Z-Ala-Gly-Gly-Leu, Z-Ala-Phe-Gly-Ala, and gelatin as substrates. From the Lineweaver-Burk plots (Fig. 1) their kinetic parameters were determined. The results are summarized in Table IV; they indicate that in each case the promotion relates mainly with binding (K_m) and only little with catalysis (k_{cat}).

Specificity against oxidized insulin B chain in the presence of Co ion

We determined in a previous paper⁷⁾ the points of cleavage of oxidized insulin B chain in the absence of Co ion. In the present work

we have investigated whether these points of cleavage differ with the presence of Co ion in the reaction mixture. Digestion of B chain in the presence of Co ion was carried out according to the method described in MATERIALS AND METHODS. The peptides were separated by high voltage paper-electrophoresis, followed by paper-chromatography as shown in MATERIALS AND METHODS. The amino acid compositions of the respective peptides are shown in Table V. The amino acid contents of II, IVA, and VIA were too small to be determined. From the known structure of the insulin molecule, it was possible to relate the composition of each peptide to a sequence of amino acids, as seen in the table. The points of cleavage in the B chain

TABLE IV. KINETIC PARAMETERS OF *P. aeruginosa* ALKALINE PROTEINASE FOR HYDROLYSIS OF VARIOUS PEPTIDES AND GELATIN IN THE PRESENCE OR ABSENCE OF CO ION

The arrows show the bonds split.

Substrates	[Co] (M)	<i>K_m</i> (mM)	<i>k_{cat}</i> (sec ⁻¹)	<i>k_{cat}/K_m</i> (mM ⁻¹ ·sec ⁻¹)
Z-Phe-Ala-Ala ↑	10 ⁻² 0	0.4 1.7	0.62 0.30	1.55 0.18
Z-Ala-Gly-Gly-Leu ↑	10 ⁻² 0	5.4 25.0	6.8 5.0	1.26 0.2
Z-Ala-Phe-Gly-Ala ↑	10 ⁻² 0	2.5 13.0	11.4 7.7	4.6 0.6
Gelatin	10 ⁻² 0	0.94% 4.4%	182 89	194 20

TABLE V. AMINO ACID COMPOSITIONS OF PEPTIDES OBTAINED FROM THE OXIDIZED INSULIN B CHAIN

Amino acid	I	III	Peptides (residues/molecule)		V	VIB
			IVB	IVC		
CySO ₃ H	1.0 (1)	0.7 (1)	0.1 (0)		0.9 (1)	
Asp	0.1 (0)	0.2 (0)	1.0 (1)			
Glu	0.3 (0)	0.8 (1)	0.2 (0)	0.6 (1)	1.1 (1)	
Ser	0.1 (0)	0.2 (0)	0.1 (0)	0.4 (1)	0.5 (0)	
Thr		0.1 (0)				0.4 (1)
Gly	1.3 (1)	1.0 (1)		1.2 (1)	2.4 (2)	
Ala	0.1 (0)	0.4 (0)	0.1 (0)	0.5 (1)	0.3 (0)	1.0 (1)
Val	1.0 (1)	0.4 (0)	1.1 (1)	0.6 (1)	1.0 (1)	
Leu	0.7 (1)	0.9 (1)	0.2 (0)	1.9 (2)	1.0 (1)	
Pro		0.4 (0)				1.2 (1)
Phe		0.2 (0)	0.5 (1)			
Tyr		0.1 (0)		1.0 (1)		
His	0.2 (0)	0.7 (1)		0.4 (1)		
Lys		0.2 (0)				0.4 (1)
Arg					0.8 (1)	
Suggested segment	17~20	4~8	1~3	8~16	17~23	27~30

were thus deduced to be at Asn-Gln (3-4), CySO₃H-Gly (7-8), Gly-Ser (8-9), Tyr-Leu (16-17), Gly-Glu (20-21), Gly-Phe (23-24), and Tyr-Thr (26-27). Hydrolysis of B chain by the alkaline proteinase in the absence of Co ion yielded the same points of cleavage, as shown in the previous paper.⁷⁾ So it is shown that the specificity against the B chain by the alkaline proteinase is identical notwithstanding the presence or absence of Co ion in the reaction mixture.

DISCUSSION

The present study indicated that the *P.*

aeruginosa alkaline proteinase was inactivated by the addition of *o*-phenanthroline to the reaction mixture even in the presence of high concentration of Ca ion. This would indicate that the enzymatic activity is not related to Ca ion but to other heavy metal ions. Among various heavy metal ions tested, only Co ion was effective in promoting hydrolysis, and this was regardless of the kind of substrate, whether protein or synthetic peptide. For the maximum promotion of hydrolysis, a considerable concentration ($>5 \times 10^{-3}$ M) was required, indicating that the Co ion binds loosely to the enzyme molecule. The kinetic study indicated that the promotion with Co ion related mainly

with binding (K_m) and little with catalysis (k_{cat}).

An alkaline proteinase produced by a strain belonging to *Serratia* has been found to be a Zn-enzyme,²⁰⁾ similarly as seen with metal neutral proteinases from bacterial origin.^{11,12)} The specificity against oxidized insulin B chain is, however, considerably different²¹⁾ from that of usual metal neutral proteinases but similar to that of the *P. aeruginosa* alkaline proteinase presented here. The apo-enzyme can easily be prepared by treatment with metal chelators,²⁰⁾ and it is easily reversed upon addition of approximately 1 mg atom of Zn, Co, or Mn per mole of enzyme. It is of great interest to note that the Co-substituted enzyme is about ten times as active against Hippuryl-L-leucine-amide as the native or Zn-enzyme, although the activities against casein are comparable with each other. It has also been found²²⁾ that the hydrolysis of synthetic peptides by clostridial collagenase is activated by Co ion to some extent.

It still remains to be solved whether the *P. aeruginosa* proteinase is a Co-enzyme in a real sense, although it is shown that the specificity against oxidized insulin B chain is identical in the presence or absence of Co ion in the reaction mixture.

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