Phytochemistry, Vol. 33, No. 6, pp. 1317–1321, 1993
Printed in Great Britain.

0031-9422/93 $6.00+0.00

SPECIFICITY AND MOLECULAR PROPERTIES OF PENICILLOLYSIN, A METALLOPROTEINASE FROM *PENICILLIUM CITRINUM*

MEGUMI YAMAGUCHI, SATOSHI HANZAWA,* KEN-ICHI HIRANO,† YOUHEI YAMAGATA and EIJI ICHISHIMA‡

Laboratory of Molecular Enzymology, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1–1 Tsutsumidori-Amamiyamachi, Aobaku, Sendai 981, Japan; *Laboratory of Marine Biotechnology, Sodeshi-machi, Shimizu 424-91, Japan; †Research Laboratories of Amano Pharmaceutical Co., Ltd, Nishiharumachi, Nishikasugagun, Aichi-prefecture 481, Japan

(*Received* 8 *February* 1993)

Key Word Index—*Penicillium citrinum*; fungus; protease; metalloproteinase; specificity.

Abstract—The specificity and mode of action of penicillolysin, a metalloproteinase from *Penicillium citrinum*, were investigated with several bioactive-oligopeptides. The enzyme showed a high affinity toward the Pro-X (X = Gln, Lys, Leu or Arg) bonds of substance P, dynorphin A (1-13), neurotensin and chicken brain pentapeptide, and the R–R bonds in dynorphin A and neurotensin. Preferential cleavages of bonds by the enzyme with hydrophobic amino acid residues at the P_1 position were observed on the peptides used. The specificity of penicillolysin differs from that of other metalloproteinases. The M_r and *p*I were determined as 18 000 and 9.6, respectively. The first 50 amino acids in the N-terminal region were TKETĊSNASṘKSALĖKALSṄTVKLȦNAAATȦARSĠSASKḞSEYEḲTTSSṠ. CD spectra on the hollo- and apo-enzymes of penicillolysin were studied.

INTRODUCTION

Previous work in this laboratory led to the identification and purification of a metalloproteinase from *Penicillium citrinum* [1]. The purified enzyme migrated as a single band on SDS-PAGE at pH 9.4. The enzyme was found to contain 1 g-atom of zinc per mol of enzyme (M_r 17000). The enzyme showed a distinct mode of action and unique specificity towards the oxidized insulin B-chain at pH 7.0 [1]. The most susceptible bond at the initial site of cleavage was between Tyr16 and Leu17, and additional cleavage of the bonds Glu13-Ala14 and Ala14-Leu15 was noted. The activity of the enzyme was inhibited by 2 mM EDTA and *o*-phenanthroline. A remarkable pH-dependency on inactivation of the enzyme by EDTA was observed. The enzyme was completely inactivated by EDTA at acidic pH values below 5.0, but the inactivation was slight at alkaline pH values such as 8.0.

The important feature of a protease is its specificity at various levels. Since neprilysin (EC 3. 4. 24. 11) [2], thimet oligopeptidase (EC 3. 4. 24. 15) [3], envelysin (EC 3. 4. 24. 12) [4], neurolysin (EC 3. 4. 24. 16) [5] and thermolysin (EC 3. 4. 24. 27) can each hydrolyse the peptide bonds of bioactive-peptides and share some common mechanistic features, we compared the specificity of metalloproteinase from *P. citrinum*.

The inactive Zn^{2+}-free apoenzyme was reactivated by Co^{2+}, Zn^{2+} and Mn^{2+}, whereas Mg^{2+}, Fe^{2+} and Ca^{2+} were ineffective. CD spectral studies were performed with native, apo-, and Co^{2+}-reconstructed enzymes. The amino terminal sequence for the first 50 amino acids of the metalloproteinase was analysed.

The present work confirmed that the metalloproteinase from *P. citrinum* is a new type of metalloproteinase with a distinct mode of action and unique specificity. The trivial name penicillolysin is suggested for this new type of metalloproteinase.

RESULTS

Specificity of penicillolysin towards bioactive oligopeptides

A summary of the action of penicillolysin on oligopeptides is shown in Fig. 1. The enzyme degrades a variety of peptides which possess various amino acids at the P_1 position, including proline, basic arginine and hydrophobic amino acids such as tryptophan, phenylalanine, tyrosine and leucine. Penicillolysin most rapidly degraded substance P and dynorphin A (1–13). A characteristic feature of penicillolysin was the hydrolysis of Pro-X (X = Gln, Lys, Leu, or Arg) bonds in substance P, dynorphin A, neurotensin and chicken brain pentapeptide. The Pro-X cleaving activity is the specific action of prolyl oligopeptidase (post-proline cleaving enzyme, EC 3. 4. 21. 26), a serine proteinase from vertebrates, plants and *Flavobacterium*.

Penicillolysin recognizes a structural feature in dynorphin A and neurotensin. The enzyme showed a high affinity towards the Arg^6–Arg^7 bond in dynorphin A (1–13) and the Arg^8–Arg^9 bond in neurotensin.

The preferential cleavage by penicillolysin of bonds with hydrophobic amino acid residues at the P_1 position was observed for Phe^8-Gly^9 in substance P, Leu^2-Tyr^3 in

‡Author to whom correspondence should be addressed.

neutrotensin, Phe^5–Ser^6 in bradykinin, Trp^3–Ser^4 and Tyr^5–Gly^6 in luteinizing hormone releasing hormone (LHRH), Trp^{10}-Gly^{11}, Tyr^2-Ser^3 and Phe^7-Arg^8 in α-melanotropin (α-MSH), and Leu^5-Arg^6 and Phe^4-Leu^5 bonds in α-neoendorphin. Penicillolysin shows no preferential cleavage specificity for bonds with hydrophobic amino acid residues at position P_1, and its specificity is different from that of microbial metalloproteinases such as thermolysin (EC 3. 4. 24. 27) [6] and *Aspergillus oryzae* neutral protease I [6]. Penicillolysin was unable to hydrolyse angiotensin I, angiotensin II, or cholecystokinin (CCK)-octapeptide (26–33).

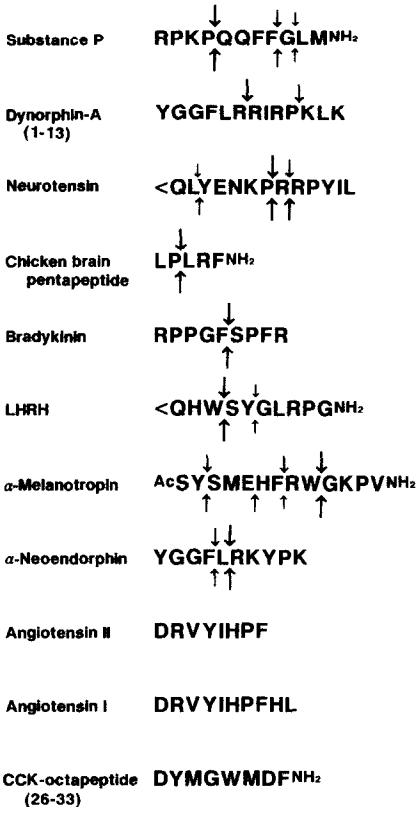

Fig. 1. Summary of cleavage specificity of penicillolysin towards various oligopeptides. The arrows above and below the sequences indicate cleavages for 1 hr hydrolysis and 24 hr hydrolysis by the enzyme, respectively. The degree of hydrolysis being: ↑ > ↑ > ↑.

Molecular properties

A M_r value of 18 000 was obtained by laser small angle scattering method [7]. A value of 17000 had been determined by SDS-PAGE previously [1]. The *p*I value was found to be 9.6. The N-terminal sequence of penicillolysin was determined as shown in Fig. 2. Homology of the enzyme was compared with other microbial metalloproteinases such as *Aspergillus oryzae* neutral protease II [8] and thermolysin [9]. The homology of penicillolysin with that of *A. oryzae* neutral protease II was 52 %, and only a low degree of sequence homology with thermolysin was found.

The specific activities of penicillolysin for clupeine and casein hydrolysates were 3.04×10^{-1} and 5.23×10^{-3} kat kg^{-1} protein at pH 7.0, respectively. The rate of clupeine hydrolysis was 60-fold greater than that for casein hydrolysis. When zinc is removed, the enzyme is completely inactive, and readdition of zinc restores the dual activities towards clupeine and casein (Table 1). Depending on the casein substrate, the cobalt-penicillolysin could be up to *ca* 1.6 times more active than the native zinc enzyme. On the other hand, in clupeine-hydrolysis, the cobalt-enzyme is about 70 % as active as the native enzyme. Thus, replacement of the zinc-penicillolysin with cobalt markedly decreases the activity towards clupeine while it increases it towards casein.

The curve in Fig. 3A shows the CD spectrum of penicillolysin. The α-helix and β-structure content of penicillolysin were *ca* 19 and 58 %, respectively. The CD curve of the apoenzyme is shown in Fig. 3B and is notably different from that of the native one. The α-helix and the β-structure content of the apoenzyme were *ca* 9 and 61 %, respectively. About 50 % of the α-helix was destroyed in the conformational changes from native to apoenzyme. The CD curve of Co^{2+}-reconstructed enzyme is shown in

Table 1. Specific activities of penicillolysin, and Zn^{2+}- and Co^{2+}-penicillolysin at pH 7.0

Enzyme	Specific activity (kat kg^{-1} protein)	
	Clupeine	Casein
Native	3.04×10^{-1} (100)	5.23×10^{-3} (100)
Zn^{2+}-penicillolysin	2.85×10^{-1} (94)	5.20×10^{-3} (99)
Co^{2+}-penicillolysin	2.10×10^{-1} (69)	8.26×10^{-3} (158)

The relative activity is shown in parentheses.

Fig. 2. N-Terminal sequence determination of penicillolysin and homology of the enzyme with those of other metalloproteinases. 1, Penicillolysin from *Penicillium citrinum*; 2, *Aspergillus oryzae* neutral protease II [8]; 3, thermolysin [9].

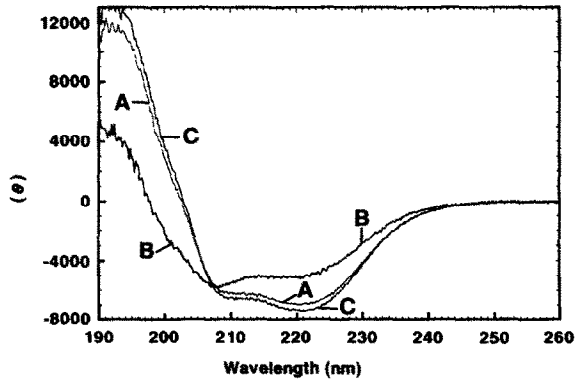

Fig. 3. CD spectra of penicillolysin, apoenzyme and Co^{2+}-penicillolysin in the region of 190–260 nm. (A) Penicillolysin; (B) apoenzyme; (C) Co^{2+}-penicillolysin.

Table 2. Secondary structure content (%) in penicillolysin, apoenzyme and Co^{2+}-penicillolysin

Enzyme	α-Helix	β-Structure	Turns	Random
Penicillolysin	19	58	3	21
Apoenzyme	9	61	2	28
Co^{2+}-penicillolysin	20	58	2	20

Fig. 3C. The contents of the α-helix and β-structure were *ca* 20 and 58 %, respectively. This CD curve was almost the same as that of the native enzyme. Parameters of the secondary structure of penicillolysin are shown in Table 2.

DISCUSSION

Metalloproteinases, which are most active at neutral pH and are sensitive to metal-chelating agents such as EDTA or *o*-phenanthroline, but not against DFP and sulphydryl reagents, are widely distributed in microorganisms [6], together with serine alkaline proteinases. The M_rs of these enzymes are 35000–40000, i.e. greater than those of serine alkaline proteinases. The *p*Is of the *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptomyces naraensis* enzymes are at pH 9.0, 5.9 and 4.2 [6], respectively. So far as determined, these are zinc enzymes.

Zinc is fairly universal in its choice of ligand atoms, coordinating with oxygen, nitrogen and sulphur atoms, and its rates of ligand substitution are fast. It is a strong Lewis acid, and yet does not hydrolyse readily at neutral pH to form hydroxy-derivatives [10].

The most common role for zinc in metalloproteinase (EC 3. 4. 24.—) is its direct participation in catalysis; other functions are structural or regulatory. The zinc atom is indispensable to the catalytic activity of penicillolysin, which contains 1 g-atom of the Zn^{2+} per M_r of 17000 [1]. To the native enzyme, the zinc atom is indispensable to both catalytic activity and structural function. Comparison of the CD spectral data of zinc penicillolysin and the apoenzyme reveals the features of their metal–protein interaction showing both similarities and specific characteristic differences. The ultraviolet CD spectrum of cobalt-penicillolysin above 190 nm is identical with that of zinc-penicillolysin. Minor differences among the spectra may reflect differences in the specific activity changes towards clupeine and casein hydrolysis.

Neutral protease II (Np II) from *Aspergillus oryzae* [8] is a zinc-containing metalloproteinase with smaller M_r, which consists of 177 amino acid residues. *Aspergillus sojae* neutral protease II contains 1 g-atom of zinc and 2 g-atoms of calcium per molecule with M_r of 19800 [11].

The specificity of penicillolysin differs from other metalloproteinases so far characterized [12] in respect to both substrate size and amino acid sequence. Highly purified penicillolysin has features which are similar to those of prolyl oligopeptidase (EC 3. 4. 21. 26) [13, 14], site specific endopeptidase [15] and chymotrypsin (EC 3. 4. 21. 1). Peptide bonds involving proline residues are often resistant to the action of endo- and exo-peptidases. The specificity of proline-specific endopeptidase activity of penicillolysin, i.e. preferential cleavage of the Pro-X bond, is similar to that of prolyl-oligopeptidase (EC 3. 4. 21. 26) as shown in Fig. 1.

Thermolysin has a strong preference for Phe, Tyr, Leu and Ile at the P_1' position of the substrate. According to the specificity of thermolysin, the rate of hydrolysis of the Xaa-Phe bond is 40-fold greater than of the Xaa-Leu bond. The Pro^7-Phe^8 in bradykinin and the Pro^7-Phe^8 in angiotensin I are cleaved by thermolysin [4]. Neprilysin (EC 3. 4. 24. 11) has a similar cleavage specificity on the Pro^7-Phe^8 bond in bradykinin [2]. Specific cleavage of the Pro^{10}-Tyr^{11} bond in neurotensin is reported by neurolysin (EC 3. 4. 24. 16) [5]. Procollagen N-endopeptidase (EC 3. 4. 24. 14) cleaves N-propeptide of procollagen chain α 1(I) at Pro-Gln [15]. Envelysin (EC 3. 4. 24. 12) cleaves the Pro^7-Phe^8 bond in both bradykinin and angiotensin I, and the Pro^4-Gln^5 bond in [Sar^9] - substance P [4]. Thermolysin is unable to hydrolyse the Pro-X bonds in substance P, dynorphin A (1–13) and neurotensin [4], and envelysin cannot hydrolyse the Pro-X bond in dynorphin A (1–13) [4]. Thimet oligopeptidase (EC 3. 4. 24. 15) is unable to hydrolyse any of the Pro-X bonds in several bioactive oligopeptides [3].

Davidson *et al.* [16] reported that Ca-dependent acidic endopeptidase one (type I) cleaves exclusively on the C-terminal side of Arg^{31}-Arg^{32} (B-chain/C-peptide junction); the other (type II) preferentially cleaves on the C-terminal side of Lys^{64}-Arg^{65} of proinsulin (C-peptide/A-chain junction). Penicillilysin, on the other hand, preferentially cleaves the peptide bond in a pair of basic amino acid residues, Arg-Arg, in dynorphin A and neurotensin. The specificity of penicillolysin is different from that of site specific endopeptidase type I in the strict sense. Thimet oligopeptidase (EC 3. 4. 24. 15) has a similar cleavage specificity of the Arg^8-Arg^9 bond in neurotensin [3], while neprilysin (EC 3. 4. 24. 11) is unable to hydrolyse the Arg^6-Arg^7 bond in dynorphin A (1–9) [2]. Envelysin cannot hydrolyse the Arg^8-Arg^9 in neurotensin or the Arg^6-Arg^7 bond in dynorphin [3].

The preference of chymotrypsin (EC 3. 4. 21. 1) for a bulky hydrophobic group at the P_1 position is supported. Similar preferential bond cleavages by penicillolysin with hydrophobic amino acid residues at the P_1 position were observed as shown in Fig. 1: Trp-Gly, Trp-Ser, Phe-Leu, Phe-Gly, Phe-Ser, Phe-Arg, Tyr-Gly, Tyr-Ser, Leu-Tyr and Leu-Arg bonds.

Penicillolysin shows a V-8 protease-like specificity towards the Glu^5-His^6 bond in α-MSH.

It can be concluded that penicillolysin, a metalloproteinase from *P. citrinum*, has a specificity unique from those of other metal proteinases.

EXPERIMENTAL

Materials. Penicillolysin was purified from a commercial 'protease B' [17] from *Penicillium citrinum* of Amano Pharmaceutical Co. Ltd, Aichi Prefecture, as described [1]. Bioactive-oligopeptides were obtained from the Peptide Institute Inc., Minoh-shi, Osaka, Japan.

Assay of the penicillolysin. Routine assay of penicillolysin employed 2% clupeine previously denatured at 100° for 30 min in 100 mM NaPi buffer, pH 7.0, as substrate. The heat denatured clupeine soln (150 μl) was dissolved in 0.4 ml 100 mM NaPi buffer, pH 7.0, and incubated for 20 min at 30° with 50 μl of penicillolysin. The reaction was stopped with 0.6 ml 12.5% TCA. An aliquot (0.1 ml) of the supernatant soln was dissolved in 2 ml 0.5 M citric acid buffer, pH 5.0, and 2 ml ninhydrin reagent. A ninhydrin assay [18] was then carried out on these filtrates. One katal of the penicillolysin is defined as the amount of enzyme which yields the colour equivalent of 1 mol of tyrosine sec^{-1} with the ninhydrin reagent using clupeine as a substrate at pH 7.0 and 30°. Specific activity is expressed in kat kg^{-1} of protein by the method of ref. [19].

Hydrolysis of peptides by penicillilysin. The peptides were dissolved to 1 mM in 100 mM NaPi buffer, pH 7.0. Substance P and α-MSH were dissolved in 100 mM Tris–HCl buffer, pH 7.5. CCK-octapeptide was dissolved in 10% DMSO soln. For hydrolysis of neurotensin, chicken brain peptapeptide, angiotensin I, angiotensin II, and CCK-octapeptide, the enzyme/substrate ratio was 1: 50 (mol: mol). For hydrolysis of substance P, dynorphin A, LHRH, α-neoendorphin and α-MSH, the enzyme/substrate ratio was 1: 400 (mol: mol). The reaction was started by adding 24 μl of enzyme soln in NaPi buffer to 72 μl of soln, and the mixt. was incubated at 30° for 1 or 24 hr. The reaction was terminated by freezing at −20° followed by lyophilization.

Isolation of hydrolysis products. The lyophilisate was dissolved in 200 μ H_2O, and 150 μl was loaded on a reverse-phase TSK gel ODS 120-T column and eluted with a linear gradient of MeCN in 0.1% TFA at room temp. The absorbance was monitored at 215 nm on a Shimadzu LC-3A liquid chromatograph. An adequate gradient was chosen for each peptide to achieve the best resolution of the product fragments. Each peak was collected with care so as not to lose those of low absorbance due to the absence of aromatic amino acid residues. The peak fractions were lyophilized and used for the following analysis.

Amino acid analysis. The purified peptides were hydrolysed by the method of ref. [20]. After dabsylation [21], amino acids were analysed with a Beckman System Gold Programmable Solvent Module 126 analyser. Tryptophan was determined by the NBS titration method of ref. [22].

N-Terminal sequence determination. The peptides purified by reverse phase chromatography were subjected to N-terminal sequence determination on an Applied Biosystem 473A protein sequencer with a 610A data analysis system.

Molecular weight. M_r was determined by low-angle laser light-scattering photometry and precision differential refractometry combined with gel-filtration HPLC (LALLS-HPLC). LALLS-HPLC was performed essentially according to ref. [7]. Samples were applied to a TSK-gel $G3000SW_{XL}$ column (0.78 cm × 30 cm, TOSOH) equilibrated with 50 mM NaPi buffer (pH 7) and containing 0.2 N NaCl according to ref. [17].

Isoelectric focusing (IEF). IEF of the enzyme was carried out on agarose gel. An IEF gel kit of pH 3.5–9.5 from ACI Japan was used. The following prepns were used as standard proteins: phycocyanin (4.65), β-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), horse myoglobin (7.0), whale myoglobin (8.05), α-chymotrypsin (8.8), and cytochrome c (9.6); the values in parentheses are the respective *p*Is.

Circular dichroism (CD). CD measurements were made on a JASCO automatic recording spectrophotometer, model J-720, at room temp. (22°) and a 1 mm path length quartz cell from 200 to 260 nm. A CD scale setting of 0.001 m° cm^{-1} was used in all experiments. Spectra were connected to the baseline shift by a running scale of solvent buffer. Results are expressed as mean residue ellipticities (θ) in deg cm^2 $dmol^{-1}$. The contents of α-helix and β-structure of the enzyme were calculated according to the SSE-338 program given in ref. [23].

Acknowledgements—This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Ichishima, E., Yamaguchi, M., Yano, H., Yamagata, Y. and Hirano, K. (1991) *Agric. Biol. Chem.* **55**, 2191.
2. Matsas, R., Kenny, A. J. and Turner, A. J. (1984) *Biochem. J.* **223**, 433.
3. Orlowski, M., Reznik, S., Ayala, J. and Pierotti, A. R. (1989) *Biochem. J.* **261**, 951.
4. Nomura, K., Tanaka, H., Kikkawa, Y., Yamaguchi, M. and Suzuki, N. (1991) *Biochemistry* **30**, 6115.
5. Barelli, H., Vincent, J.-P. and Checker, F. (1988) *Eur. J. Biochem.* **175**, 481.
6. Morihara, K. (1974) *Adv. Enzymol.* **41**, 179.
7. Maesawa, S. and Takagi, T. (1983) *J. Chromatogr.* **280**, 124.

8. Tatsumi, H., Murakami, S., Tsuji, R. F., Ishida, Y., Murakami, K., Masaki, A., Kawabe, H., Arimura, H., Nakano, E. and Motai, H. (1991) *Mol. Gen. Genet.* **228**, 97.
9. Titani, K., Hermondson, M. A., Ericsson, L. H., Walsh, K. A. and Neurath, H. (1972) *Nature* **238**, 35.
10. Auld, D. S. and Vallee, B. I. (1987) in *New Comprehensive Biochemistry, Hydrolytic Enzymes* (Neuberger, A. and Brocklehurst, K., eds), Vol. 16, p. 201. Elsevier, Amsterdam.
11. Sekine, H. (1972) *Agric. Biol. Chem.* **36**, 2143.
12. Nomenclature Committee, International Union of Biochemistry and Molecular Biology (1992) *Enzyme Nomenclature, Recommendations.* Academic Press, Orlando.
13. Kida, M. and Walter, R. (1976) *J. Biol. Chem.* **251**, 7593.
14. Walter, R. (1976) *Biochim. Biophys. Acta* **422**, 138.
15. Hojima, Y., McKenzie, J., van der Rest, M. and Prockop, D. J. (1989) *J. Biol. Chem.* **264**, 11 336.
16. Davidson, H. W., Rhodes, C. J. and Hutton, J. C. (1988) *Nature* **333**, 93.
17 Yamamoto, N., Matsumoto, K., Yamagata, Y., Hirano, K. and Ichishima, E. (1993) *Phytochemistry* **32**, 1395.
18. Ichishima, E. (1972) *Biochim. Biophys. Acta* **258**, 274.
19. Lowry, D. H., Roseburgh, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
20. Tsugita, A. and Scheffler, J. J. (1982) *Eur. J. Biochem.* **124**, 585.
21. Knecht, R. and Chang, J. Y. (1986) *Analyt. Chem.* **58**, 2375.
22. Spande, T. F. and Witkop, B. (1967) in *Methods in Enzymology* (Hirs, C. H. W., ed.), Vol. 11, p. 498. Academic Press, New York.
23. Yang, J. T. (1986) in *Methods in Enzymology* (Jakoby, W. B. and Griffith, O. W., eds), Vol. 130, p. 208. Academic Press, New York.