Use of Site-Directed Mutagenesis To Identify Valine-573 in the S'1 Binding Site of Rat Neutral Endopeptidase 24.11 (Enkephalinase)†

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Received March 9, 1990; Revised Manuscript Received May 9, 1990

ABSTRACT: On the basis of the identity of a segment of the amino acid sequence within the active site of the bacterial enzyme thermolysin and the mammalian enzyme neutral endopeptidase 24.11, the possible involvement of valine-573 of neutral endopeptidase 24.11 in substrate binding was investigated. Valine-573 was changed to leucine and to alanine by site-directed mutagenesis. The effect of these mutations on inhibitor binding and substrate catalysis was examined with a series of compounds containing variable P'1 residues. With a small P'1 residue such as alanine, both mutant enzymes exhibited kinetic properties essentially the same as the wild-type enzyme. However, with larger P'1 residues such as phenylalanine, tyrosine, and leucine, the Val573Leu mutant showed a 24–100-fold decrease in inhibitor affinity. Similarly substrates containing bulky P'1 residues showed a 10–40-fold decrease in V_{max} with little change in K_m. In contrast, the Val573Ala mutant showed only modest changes in terms of inhibitor binding or substrate turnover. These results support the proposed role of valine-573 as a part of the hydrophobic binding pocket, S'1 binding subsite, of neutral endopeptidase 24.11.

Neutral endopeptidase 24.11 ("enkephalinase", CALLA, NEP) is a zinc metalloendopeptidase that hydrolyzes a number of physiologically active peptides. The enzyme is believed to play a major role in the inactivation of endogenously released enkephalins at or near enkephalinergic synapses (Hersh, 1982). Other potential physiological roles for NEP include regulation of blood pressure through its hydrolysis of atrial natriuretic factor (ANF) (Olins et al., 1987) and endothelin (Vijayaragahavan et al., 1990), regulation of chemotaxis by hydrolysis of chemotactic peptide (Painter et al., 1988), modulation of smooth muscle contraction by inactivation of tachykinins (Dusser et al., 1989), and a possible role in the development and/or differentiation in the immune system (Shipp et al., 1989).

In 1980, Roques and co-workers (Roques et al., 1980) showed antinociceptive activity in mice by the NEP inhibitor thiorphan. This observation has been followed by a number of reports on the development and analgesic effects of NEP inhibitors (Carenzi et al., 1983; Schwartz et al., 1985; Ehrenpreis, 1985; Roques et al., 1986; Chipkin, 1986; Rupreht et al., 1983; Dickenson, 1986; Hersh, 1986; Bateman & Hersh, 1987). The mode of action of these compounds is presumably to inhibit inactivation of synaptically released enkephalins by NEP, thereby prolonging the analgesic effect of these opioid peptides. Thus, there is considerable interest in the synthesis of neutral endopeptidase inhibitors as pharmacological agents (Chipkin, 1986). An understanding of the structure of the active site of the enzyme should be of considerable use in the design and synthesis of such inhibitors.

NEP cleaves peptide substrates on the amino side of hydrophobic amino acids (Pozsgay et al., 1986; Hersh & Morihara, 1986) and in this respect is similar to a group of bacterial neutral endoproteases of which thermolysin is the best characterized. Both enzymes prefer bulky P'1 residues such as phenylalanine and leucine and are less reactive with a P'1 alanine or glycine. The S'1 site of NEP does, however, appear to accommodate larger P'1 residues. The similarities between NEP and the bacterial endoproteases extend beyond substrate and inhibitor specificity. Although there is little overall amino acid similarity between the mammalian and bacterial enzymes, there are two functional regions of the bacterial enzymes which can be found in the mammalian enzyme (Devault et al., 1987; Malfroy et al., 1987, 1988). One is an 11 amino acid sequence in the bacterial enzymes comprised of residues 228–238 (G-G-V-H-I-N-S-G-I-I-N) in which histidine-231 is believed to function in the stabilization of a tetrahedral reaction intermediate (Mathews, 1988). However, within the corresponding sequence in NEP which includes residues 627–635[1] (G-G-Q-H-L-N--G-I--N), histidine-630 is nonfunctional as determined by site-directed mutagenesis (Erdos & Skidgel, 1989). The other region is a 12 amino acid segment in the bacterial enzymes comprised of amino acids 135–146 (G-G-I--D-V-I-G-H-E-L-T-H for the *Bacillus cereus* enzyme) (Sidler et al., 1986). This helical segment contains the two histidines which serve as zinc ligands (histidines-142 and -146) and, as demonstrated with thermolysin, glutamate-143 which is postulated to act as an acid–base catalyst (Matthews, 1988). The corresponding sequence in NEP is residues 568–580 (G-G-I-G-M-V-I-G-H-E-I-T-H). It has been demonstrated that glutamate-577 is an essential residue, since its conversion to an aspartate or a valine residue causes a loss of catalytic, but not binding, activity (Devault et al, 1988a). This suggests that glutamate-577 in NEP is analogous to glutamate-143 in thermolysin. Similarly, changing histidine-576 or histidine-580 to phenylalanine generates inactive enzyme (Devault et al.,

† This work was supported in part by grants from the National Institute on Drug Abuse (DA 02243) and the Welch Foundation (I 391).

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[1] The numbering systems used for NEP from rat (Malfroy et al., 1987) and rabbit (Devault et al., 1987) differ due to presumed differences in the site of initiation of protein synthesis. The numbering system for the rat enzyme is used throughout this paper with residues identified in the rabbit enzyme renumbered accordingly.

0006-2960/90/0429-8052$02.50/0

1988b), suggesting that these histidines act as zinc ligands and are analogous to histidines-142 and -146 of thermolysin.

In addition to these residues, valine-139 on the bacterial enzymes forms a part of the S'1 binding site (Colman et al., 1972; Kester & Matthews, 1977). This binding subsite confers upon the enzyme its primary specificity which is directed at cleavage on the amino side of hydrophobic amino acids. In order to determine whether valine-573 in NEP is analogous to valine-139 of thermolysin, site-directed mutagenesis was used to change this residue either to the bulkier leucine residue or the smaller alanine residue. The mutant and wild-type enzymes were expressed in COS cells and their kinetic properties compared. The results of these experiments support a role for valine-573 in substrate binding.

Materials and Methods

cDNA Clone to Rat NEP. A cDNA clone to rat NEP was isolated as two overlapping fragments as previously decribed (Bateman et al., 1989). The 3' fragment, NE-1, was subcloned into M13 and used for mutagenesis.

Site-Directed Mutagenesis. The conversion of valine-573 in NEP to a leucine or to an alanine residue was accomplished by site-directed mutagenesis using a modification (Craig, 1985) of the method of Zoller and Smith (1982) as previously described (Bateman et al., 1989). Mutagenesis was conducted in M13 with the NE-1 subclone of rat kidney NEP (Bateman et al., 1989) using the oligonucleotides GTGATTTCATGTCCtATTtAgCATGCCGATGCCCC (Val → Leu) and GTGATTTCATGTCCtATTgCCATGCCG-ATGCCCC (Val → Ala), where the lower case letters represent the base changes introduced. These oligonucleotides, in addition to changing valine-573 to leucine or alanine, included silent third base mutations which facilitated identification of the mutant enzyme by differential hybridization. The mutant DNA was subcloned into the *Eco*RI site in pBluescript and the orientation of the clones determined by restriction mapping. The full-length cDNA was reconstructed by ligating in the 5' end of the cDNA, previously subcloned into pBluescript (Bateman et al., 1989), through an overlapping *Bgl*II site. The reconstructed cDNA was excised with *Xba*I and *Hin*dIII and cloned into the complementary sites of the expression vector pCMV (Andersson et al., 1989).

Wild-type and mutant cDNAs were transiently expressed in COS cells for 72 h. A membrane fraction was prepared, and the recombinant enzyme was solubilized from the membrane fraction with Triton X-100 as previously described (Bateman et al., 1989). The amount of NEP protein was determined by immunoblot analysis (Bateman et al., 1989). Immunoblot analyses were conducted in triplicate on separate gels. A variation in enzyme concentration of ±30% was observed.

Determination of Enzyme Activity. Enzyme activity was determined by using as substrates glutaryl-Ala-Ala-X-Y, where X = a variable amino acid in the P'1 position and Y = the fluorophore 2-naphthylamine or 4-methoxy-2-naphthylamine. In the case of the substrate containing a P'1 valine, the succinyl derivative containing 7-amino-4-methylcoumarin as the fluorophore was used. A two-step assay was employed in which the wild-type or mutant NEP was first incubated with the substrate to generate the aminoacyl fluorophore X-Y. Reaction mixtures contained 100 mM MES buffer, pH 6.5, 300 mM NaCl, variable substrate concentrations, and enzyme in a total volume of 50 μL. The reaction mixture was incubated at 37 °C for 15–30 min after which time the reaction was stopped by boiling. Aminopeptidase M (Sigma) (6 milliunits) pretreated with 1 μM phosphoramidon was added, leading to the formation of the free amino acid X and the free fluorophore Y. The amount of fluorophore produced was determined with an Aminco–Bowman spectrofluorometer using the following excitation and emission wavelengths (in nanometers): 2-naphthylamine, 335, 410; 4-methoxy-2-naphthylamine, 340, 425; 7-amino-4-methylcoumarin, 380, 460. In each case, the reaction of aminopeptidase M with the product amino acyl-fluorophore was shown to have gone to completion. Mock transfected COS cells were tested for endogenous activity with each substrate. Negligible activity was detected at the highest protein concentrations used in these studies.

For the determination of the K_i values of the various inhibitors, 0.1 mM glutaryl-Ala-Ala-Phe-2-naphthylamide was used at a fixed concentration, and rates were measured in the presence of variable concentrations of inhibitor. Since these compounds have previously been shown to act as competitive inhibitors (Pozsgay et al., 1986), the apparent K_i ($K_{i_{obs}}$) was thus determined as the horizontal intercept of plots of $1/v$ versus [I]. K_i was calculated from the relationship $K_i = K_{i_{obs}}(1 + [S]/K_m)$.

All kinetic data were computer fit to the kinetic programs of Cleland (1979).

The 2-naphthylamide substrates containing variable P'1 residues and the carboxyphenylethyl amino acid *p*-aminobenzoates were synthesized as previously described (Pozsgay et al., 1986). Glutaryl-Ala-Ala-Ala-4-methoxy-2-naphthylamide was purchased from Sigma Chemical Co. while succinyl-Ala-Ala-Val-7-amido-4-methylcoumarin was purchased from Chemical Dynamics Corp.

Determination of Cleavage Sites. The site of cleavage in the peptide substrates with both the wild type and mutant enzymes was determined by thin-layer chromatography in three solvent systems: solvent system 1, 2-propanol/EtOAc/5% HOAc (2:2:1 v/v); solvent system 2, 1-butanol/HOAc/water (60:15:25 v/v); solvent system 3, 1-butanol/pyridine/water (1:1:1 v/v). In each case, the product of the enzymic reaction was found to comigrate with the authentic amino acyl fluorophore.

Results

In order to determine whether valine-573 of neutral endopeptidase 24.11 comprises a part of the S'1 subsite of the enzyme, site-directed mutagenesis was used to construct mutant enzymes in which this residue was replaced either by a leucine to decrease the size of the putative binding pocket or by an alanine to increase the size of the putative binding pocket. The effect of these changes was assessed by measuring the K_i for the binding of competitive inhibitors with variable P'1 amino acids as well as by measuring the K_m and V_{max} for the reaction of substrates containing variable P'1 residues. The inhibitors used in these studies are [*N*-[(*R*,*S*)-1-carboxy-2-phenylethyl]amino]acyl *p*-aminobenzoates which have previously been used to examine the specificity of the P'1 site of the enzyme isolated from rabbit kidney (Pozsgay et al., 1986). As shown in Table I, there is no significant difference in the binding of the inhibitor containing a P'1 alanine between the wild-type enzyme and either the Val573Ala or the Val573Leu mutant. However, with compounds containing a bulky P'1 residue, the Val573Leu mutant shows a dramatic 24–100-fold decrease in its affinity for the inhibitors (Table I). In contrast, the Val573Ala mutant shows little difference in affinity for the inhibitors as compared to the wild-type enzyme.

We also compared the affinity of the mutant enzymes for several other known transition-state inhibitors. As shown in Table II, these inhibitors bind 20- to more than 500-fold less tightly to the Val573Leu mutant as compared to the wild-type

Table I: Inhibition of Wild-Type and the V573L and V573A Mutant Forms of Neutral Endopeptidase 24.11 by Carboxyphenylethyl Amino Acid *p*-Aminobenzoates[a]

P'1 residue	K_i (μM)			ratio	
	wild type	V573A	V573L	K_i(V573A)/ K_i(wild type)	K_i(V573L)/ K_i(wild type)
Phe	0.13 ± 0.02	0.16 ± 0.01	15.2 ± 2.49	1.2	115
Tyr	1.22 ± 0.12	0.98 ± 0.14	34.7 ± 4.29	0.8	28
Leu	3.88 ± 0.27	2.27 ± 0.39	93.4 ± 9.40	0.6	39
Trp	6.24 ± 0.29	3.57 ± 0.47	241.5 ± 14.8	0.6	24
Ala	24.72 ± 2.33	23.00 ± 2.68	34.3 ± 2.5	0.9	1.4

[a] All K_i values are reported as ± their standard error.

Table II: Inhibition of Wild-Type and the V573L and V573A Mutant Forms of Neutral Endopeptidase 24.11 by Transition-State Inhibitors[a]

inhibitor	K_i (nM)			ratio	
	wild type	V573A	V573L	K_i(V573A)/ K_i(wild type)	K_i(V573L)/ K_i(wild type)
phosphoramidon (*N*-[[(α-L-rhamnopyranosyl)oxy]-hydroxyphosphinyl]-Leu-Trp)	6.2 ± 0.2	3.7 ± 0.2	125 ± 12	0.6	20
thiorphan (*N*-[(*R*,*S*)-3-mercapto-2-benzylpropanoyl]glycine)	8.9 ± 0.7	7.1 ± 0.8	967 ± 32	0.8	109
(*S*)-homoPhe-[N]-L-Phe-β-Ala	44.3 ± 2.5	38.4 ± 5.6	>25000	0.9	>500

[a] All K_i values are reported as ± their standard error.

Table III: Comparison of the Reaction of Wild-Type NEP to the Val573Leu and Val573Ala Mutants with Substrates Containing Variable P'1 Residues[a]

(X) P'1 residue[b]	wild type			Val573Ala			Val573Leu		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Phe	0.25 ± 0.02	24.6 ± 1.5	97.6 ● 3.9	0.09 ± 0.01	25.0 ± 0.7	295.9 ± 15.7	0.18 ± 0.01	1.6 ± 0.07	9.1 ± 0.42
Leu	0.45 ± 0.05	27.2 ± 1.6	61.0 ± 3.5	0.31 ± 0.03	31.4 ± 1.4	100.6 ± 5.7	0.73 ± 0.16	0.7 ± 0.07	0.9 ± 0.12
Tyr	0.22 ± 0.02	6.4 ± 0.1	28.6 ± 1.2	0.20 ± 0.02	8.8 ± 0.3	44.4 ± 2.8	0.25 ± 0.04	1.2 ± 0.08	4.6 ± 0.40
Ala	0.31 ● 0.01	0.9 ± 0.02	3.0 ± 0.1	0.24 ± 0.02	2.2 ± 0.05	9.2 ± 0.4	0.18 ± 0.03	1.4 ± 0.08	7.4 ± 0.86
Val	0.76 ± 0.11	0.9 ± 0.07	1.1 ± 0.1	0.59 ± 0.09	1.8 ± 0.1	3.1 ± 0.2	0.90 ± 0.5	0.08 ± 0.02	0.09 ± 0.02

[a] K_m expressed in millimolar. V_{max} expressed as nanomoles of product formed per minute per microgram of immunoreactive enzyme. All kinetic constants are reported as ± their standard error. [b] The P'1, Phe-, Leu-, and Tyr-containing substrates were of the type glutaryl-Ala-Ala-X-2-naphthylamide. The substrate containing a P'1 Ala was the same except it contained the 4-methoxy-2-naphthylamide reporter group, and the P'1 Val-containing substrate was the succinyl derivative containing the 4-methyl-7-aminocoumarin reporter group. There is a small but significant effect on V_{max} of varying the fluorophore. Almenoff and Orlowski (1983) observed a 34% decrease in rate with the 2-naphthylamide reporter group relative to the 4-methoxy-2-naphthylamide reporter group. In preliminary experiments, we found a 25% change.

enzyme. In contrast, the Val573Ala mutant showed little difference in its affinity for these inhibitors as compared to the wild-type enzyme.

The effect on catalysis of changing valine-573 to either leucine or alanine was determined by using a series of synthetic substrates of the type glutaryl-Ala-Ala-X-Y in which X represents a variable P'1 amino acid and Y a fluorophore. As shown in Table III, when the P'1 residue is alanine the mutant enzymes exhibit little, if any, difference compared to the wild-type enzyme in terms of either K_m or V_{max}. When the P'1 position is occupied with bulky amino acids, there is little dependence of K_m on the size of the P'1 residue with either mutant. However, with these substrates, there is a rather large effect of changing valine-573 to leucine on V_{max} and thus V_{max}/K_m. In contrast to the results obtained with the Val573Leu mutant, only modest differences were observed between the wild-type and Val573Ala mutant (Table III).

Discussion

The X-ray crystallographic structure of thermolysin in the presence of inhibitors and pseudosubstrates shows that valine-139 is a part of the S'1 binding site and contributes to substrate binding by making a hydrophobic contact with the P'1 residue. An example is shown in Figure 1. Valine-573 of NEP is contained within a 12 amino acid sequence which is conserved between NEP and thermolysin (Devault et al., 1987; Malfroy et al., 1987, 1988) and appears to correspond to valine-139 of thermolysin. Thus, it seemed reasonable to suggest that valine-573 might be a part of the S'1 binding site in NEP. To test this model, site-directed mutagenesis has been used to modify this residue with the resultant mutant enzymes

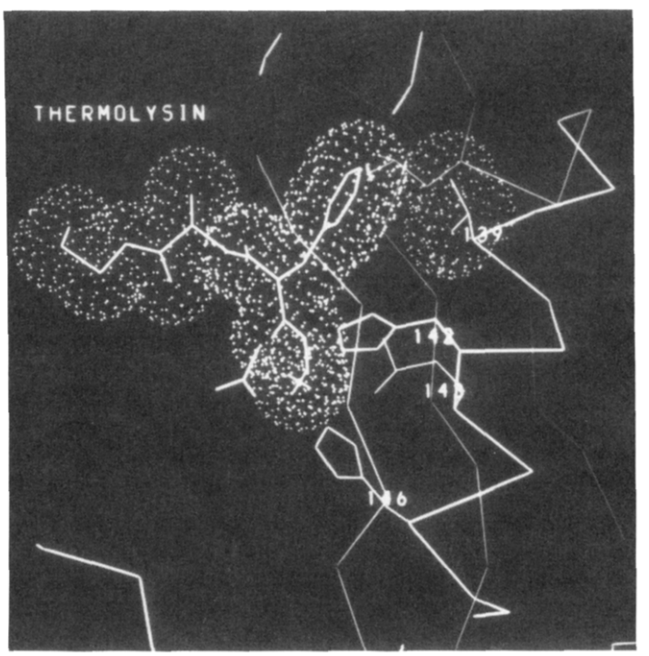

FIGURE 1: View of thermolysin showing the binding interaction of valine-139 with the benzyl ring of HONH-benzylmalonyl-L-Ala-Gly-*p*-nitroanilide in the S'1 binding pocket of thermolysin. The figure was taken from the protein data bank coordinates deposited by Holmes and Matthews (1981).

expressed in COS cells. The putative S'1 binding site was modified by substituting valine-573 with either a leucine residue or an alanine residue. When the resultant mutant enzymes were examined with either an inhibitor or a substrate containing a P'1 alanine, little, if any, difference between the wild-type and mutant enzymes were observed. This finding demonstrates that neither of these amino acid substitutions leads to a gross distortion of the enzyme such that it becomes catalytically incompetent.

On the other hand, changing the isopropyl side chain of valine to the isobutyl group of leucine results in dramatic changes in inhibitor binding and substrate catalysis with compounds containing bulky P'1 residues. In the case of the *p*-aminobenzoate inhibitors, there is a 24–100-fold decrease in binding affinity. The Val573Leu mutant also shows a decreased affinity for other known transition-state inhibitors which contain either a phosphoryl, a sulfhydryl, or a carboxyl metal-chelating group. In this case, the affinity of the mutant enzyme for a given transition-state inhibitor decreases with decreasing affinity of the inhibitor for the wild-type enzyme. For example, the Val573Leu mutant binds phosphoramidon 20 times less tightly than does the wild-type enzyme. This inhibitor, which contains a P'1 leucine, exhibits a K_i of 6 nM for the wild-type enzyme. On the other hand, (*S*)-homo-Phe-[N]-L-Phe-β-Ala (Mumford et al., 1982), which contains a P'1 Phe and has a K_i of 44 nM for the wild-type enzyme, binds more than 500-fold less tightly to the mutant enzyme.

With substrates containing bulky P'1 residues, the effect of converting valine-573 to a leucine is manifested not in K_m but instead in V_{max}. Depending on the P'1 amino acid, V_{max} is decreased 10–40-fold. Even with the wild-type enzyme, the predominant effect of variable P'1 residues is to affect V_{max} rather than K_m. The expression of enzyme specificity in terms of V_{max} is thought to reflect the utilization of binding energy for catalysis (Jencks, 1987). The NEP reaction seems to be an example of such a situation. The effects of the conversion of valine-573 to leucine can best be explained by a steric effect which results either from decreasing the size of the S'1 binding pocket or from a rearrangement of this binding site such that substrates and inhibitors bind in an unfavorable orientation. X-ray crystallography and computer graphic analysis of inhibitor binding to crystalline thermolysin (Kester et al., 1977; Hangauer et al., 1984) indicated that an increase in the size of residues binding to the S'1 subsite of the enzyme would create bad contacts with the bottom of the hydrophobic cleft. This would be predicted to result in a new alignment between the bond-breaking residues in the active center and the scissile bond that could lead to a decrease in the catalytic efficiency (k_{cat}) of the enzyme. A similar effect would be expected for a mutation that would increase the bulkiness of an amino acid residue at this site of the enzyme. Our mutant enzyme form in which the valine residue is replaced by the more bulky leucyl residue shows indeed this predicted change.

The conversion of leucine-573 to the smaller alanine residue has only a small effect on inhibitor binding and substrate reactivity. There appears to be a slight increase in V_{max} with a P'1 alanine or valine and a general increase in V_{max}/K_m. This latter effect is derived from small changes in both K_m and V_{max} and may reflect subtle changes in the conformation of the S'1 binding site. Thus, it would appear that in contrast to the rather major effects observed when the size of the binding pocket is reduced, increasing its size does not dramatically affect the enzyme. This could result from a rotation of the substrate relative to the S'1 alanine residue to maximize binding interactions. Alternatively, binding interactions with other residues in the S'1 site may still be available and may represent the major source of binding energy with both the wild-type and mutant enzymes.

In either case, these studies provide evidence that valine-573 is a part of the S'1 binding site of NEP and that this residue may function in a similar fashion to valine-139 of thermolysin.

Acknowledgments

We thank Dr. Betsy Goldsmith for assistance in the analysis of the X-ray structure of thermolysin and for consultation regarding modification of valine-573 in NEP. We also thank Dr. Paul Cook for providing us with the kinetic programs used in this study.

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Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus* Combines Intrinsic Phosphotransferase and Cyclic Phosphodiesterase Activities: A ^{31}P NMR Study†

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Received December 12, 1989; Revised Manuscript Received May 14, 1990

ABSTRACT: The inositol phosphate products formed during the cleavage of phosphatidylinositol by phosphatidylinositol-specific phospholipase C from *Bacillus cereus* were analyzed by ^{31}P NMR. ^{31}P NMR spectroscopy can distinguish between the inositol phosphate species and phosphatidylinositol. Chemical shift values (with reference to phosphoric acid) observed are −0.41, 3.62, 4.45, and 16.30 ppm for phosphatidylinositol, *myo*-inositol 1-monophosphate, *myo*-inositol 2-monophosphate, and *myo*-inositol 1,2-cyclic monophosphate, respectively. It is shown that under a variety of experimental conditions this phospholipase C cleaves phosphatidylinositol via an intramolecular phosphotransfer reaction producing diacylglycerol and D-*myo*-inositol 1,2-cyclic monophosphate. We also report the new and unexpected observation that the phosphatidylinositol-specific phospholipase C from *B. cereus* is able to hydrolyze the inositol cyclic phosphate to form D-*myo*-inositol 1-monophosphate. The enzyme, therefore, possesses phosphotransferase and cyclic phosphodiesterase activities. The second reaction requires thousandfold higher enzyme concentrations to be observed by ^{31}P NMR. This reaction was shown to be regiospecific in that only the 1-phosphate was produced and stereospecific in that only D-*myo*-inositol 1,2-cyclic monophosphate was hydrolyzed. Inhibition with a monoclonal antibody specific for the *B. cereus* phospholipase C showed that the cyclic phosphodiesterase activity is intrinsic to the bacterial enzyme. We propose a two-step mechanism for the phosphatidylinositol-specific phospholipase C from *B. cereus* involving sequential phosphotransferase and cyclic phosphodiesterase activities. This mechanism bears a resemblance to the well-known two-step mechanism of pancreatic ribonuclease, RNase A.

Phosphatidylinositol-specific phospholipase C (PI-PLC)[1] (EC 3.1.4.10) catalyzes cleavage of the membrane lipid phosphatidylinositol (PtdIns), producing a membrane-soluble product, diacylglycerol, and a water-soluble product, inositol phosphate. In mammalian cells, PI-PLC occurs as a family of proteins with limited structural homology (Rhee et al., 1989). These enzymes are believed to play a key role in the PtdIns-dependent pathway of transmembrane signal transduction of calcium-mobilizing hormones and growth factors (Berridge, 1986; Berridge & Irvine, 1989). Because of the importance of PtdIns-dependent signaling for understanding cellular processes such as metabolism, secretion, contraction, proliferation, neural activity, and associated diseases, interest in these enzymes has increased rapidly in recent years and a number of eukaryotic enzymes have now been cloned and sequenced [reviewed by Rhee et al. (1989)].

An enzyme with similar high specificity for inositol phospholipids also has been purified from the culture media of several microorganisms including *Staphylococcus aureus*

†This work was supported by U.S. Public Health Service Grants GM 25698 and GM 27137.

[1] Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; PtdIns, phosphatidylinositol; Ins(1)P, *myo*-inositol 1-monophosphate; Ins(2)P, *myo*-inositol 2-monophosphate; Ins(1:2cyc)P, *myo*-inositol 1,2-cyclic monophosphate (as used here, the abbreviation Ins refers to either the D- or L-enantiomer of the *myo*-inositol phosphates; for clarity and because of the nature of the work described, enantiomers or racemic mixtures of the inositol phosphates are specified by the prefixed D-, L-, or D,L-, respectively); BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid (disodium salt); Tris, tris(hydroxymethyl)aminomethane; d, doublet (NMR).

0006-2960/90/0429-8056$02.50/0