Substrate Specificities of Growth Factor Associated Kallikreins of the Mouse Submandibular Gland†

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ABSTRACT: The kinetic constants for the hydrolysis of a series of tripeptide *p*-nitroanilide substrates by mouse epidermal growth factor binding protein (EGF-BP), the γ-subunit of mouse nerve growth factor (γ-NGF), bovine pancreatic trypsin (BPT), and porcine pancreatic kallikrein (PPK) have been evaluated. These substrates correspond to the carboxyl-terminal three amino acids of the mature forms of epidermal growth factor (EGF) and β-nerve growth factor (β-NGF), as well as various substitutions in the penultimate and antepenultimate positions, and, as such, represent potential recognition sites for precursor processing. The mouse kallikreins (EGF-BP and γ-NGF) preferentially hydrolyze the substrates with the sequences of their specifically associated growth factors; however, the constants derived from these reactions do not account for the association constants observed with the mature growth factors, and additional significant binding interactions between EGF-BP and EGF and between γ-NGF and β-NGF are predicted to exist outside of the catalytic binding site, i.e., the P_3 to P_1 positions. A comparison of the kinetic constants of BPT, PPK, and the mouse kallikreins indicates that EGF-BP and γ-NGF display a hybrid catalytic character. A favorable substrate P_1 arginine guanidinium group interaction exists for the mouse kallikreins, similar to that of BPT, but a preference for a hydrophobic side chain in the substrate P_2 position makes the mouse kallikreins, especially EGF-BP, more closely resemble PPK than BPT. These findings have significant implications with regard to molecular modeling of the mouse kallikreins.

Epidermal growth factor (EGF)[1] and nerve growth factor (NGF) may be isolated from the mouse submandibular gland as stable high molecular weight complexes (Varon et al., 1967; Taylor et al., 1970) involving particular arginine-specific esterases (glandular kallikreins) (Greene et al., 1968; Taylor et al., 1974a). EGF is found complexed with the EGF binding protein (EGF-BP) (Taylor et al., 1974b), and the β-subunit of NGF is associated with two glandular kallikreins, α-NGF and γ-NGF (Varon et al., 1968). The α-NGF subunit is sequentially similar to γ-NGF and EGF-BP but is catalytically inactive (Isackson & Bradshaw, 1984) while γ-NGF and EGF-BP are both active arginine-specific esterases. The observation that the carboxyl-terminal residue of the mature forms of the growth factors is an arginine in both cases (Savage et al., 1972; Angeletti & Bradshaw, 1971) led to the prediction that precursor forms of the growth factors would exist and that they were processed by these specific kallikreins (Angeletti & Bradshaw, 1971; Taylor et al., 1974b). Support for these hypotheses [that suggested that the carboxyl-terminal arginines of the growth factors occupied the substrate binding sites of the kallikreins (Figure 1)] was provided by the observations that the kallikreins are inhibited by complexation with the growth factors (Greene et al., 1969; Server et al., 1976), small substrates compete with the growth factors for complex formation (Bothwell & Shooter, 1978), and removal of these arginines prevented complexation with the kallikreins (Moore et al., 1974; Server et al., 1976).

EGF-BP and γ-NGF share a high degree (85%) of amino acid sequence identity (Blaber et al., 1987a; Thomas et al., 1981; Ullrich et al., 1984), yet they are highly selective in the complex formation with their appropriate growth factor (Server & Shooter, 1976, 1977). This high degree of specificity, afforded by a relatively small number of amino acid substitutions, underscores the value of these kallikreins in analyzing binding pocket geometries (Blaber et al., 1987b).

In this report, the kinetic constants for γ-NGF, EGF-BP, and the related proteases bovine pancreatic trypsin (BPT) and porcine pancreatic kallikrein (PPK) have been determined for a series of N^{α}-acetyl tripeptide *p*-nitroanilide substrates. The carboxyl-terminal, penultimate, and antepenultimate positions of these tripeptides occupy the P_1, P_2, and P_3 positions, respectively, on each enzyme and are designated as such. The substrates include the carboxyl-terminal sequences of the mature forms of EGF and β-NGF as well as various substitutions at the P_2 and P_3 positions. The effect of different side chains in these positions on both the equilibrium dissociation constant and acylation rate constant has been evaluated as have changes in pH on the kinetic parameters of EGF-BP and γ-NGF for the range covering optimum growth factor/kallikrein interactions. Finally, the ability of these kallikreins to process the precursor of EGF was directly evaluated by utilizing a synthetic heptapeptide representing the P_4 to P_{-3} positions of the EGF precursor molecule.

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[1] Abbreviations: EGF, epidermal growth factor; NGF, nerve growth factor; γ-NGF, γ-subunit of mouse NGF; EGF-BP, EGF binding protein of mouse; BPT, bovine pancreatic trypsin; PPK, porcine pancreatic kallikrein; P_3, P_2, P_1, P_{-1}, etc., substrate binding pockets for amino acid side chains where the scissile bond occurs between the P_1 and P_{-1} residues; mGK, mouse glandular kallikrein; *N*-Ac-X-*p*Na, *N*-acetyl-*p*-nitroanilide derivative of peptide X; L-BAPNA, N^{α}-benzoyl-L-arginine-*p*-nitroanilide.

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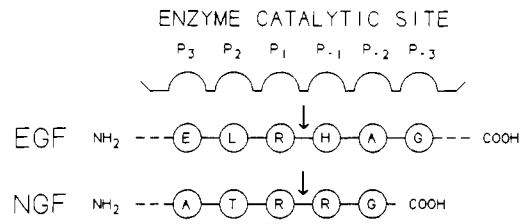

FIGURE 1: Schematic representation of the alignment of the carboxyl-terminal junction sequences of the precursors of NGF and EGF with the corresponding subsites in the catalytic centers of γ-NGF or EGF-BP.

Materials and Methods

Purification of Proteins. EGF-BP was isolated from male Swiss-Webster mouse submandibular glands according to the method of Taylor et al. (1970a). This preparation was judged to be >95% pure by Coomassie blue visualization on SDS–polyacrylamide gel electrophoresis (PAGE) analyses and by amino acid sequence analysis. The EGF-BP was identified as the mGK-9 gene product (Blaber et al., 1987a; Drinkwater et al., 1987); no other kallikreins were found complexed with EGF. This preparation was subjected to further purification by preparative isoelectric focusing followed by gel filtration chromatography on Sephadex G-100. There were no subforms resolved with isoelectric focusing. The final protein sample was characterized by its ability to form a complex with mouse EGF and was quantitated by amino acid composition analysis. An extinction coefficient of $E_{1\%}^{280\,nm} = 15.6$ (Taylor et al., 1974a) and a molecular mass of 27 500 Da were used for the calculation of protein concentration.

γ-NGF was isolated from the same tissue according to the method of Varon et al. (1968). This preparation was also subjected to additional purification by preparative isoelectric focusing. The majority (~80%) of the γ-NGF was present as the γ_3 subform (Smith et al., 1968), and this was the only subform isolated for further purification by gel filtration on Sephadex G-100. γ_3 has been characterized as being most similar to EGF-BP with regard to posttranslational modification (Burton & Shooter, 1981). The final product was characterized by amino acid sequence analysis and by the ability to reassociate with the α- and β-NGF subunits to form the 7S NGF complex. The material was quantitated by amino acid analysis. An extinction coefficient of $E_{1\%}^{280\,nm} = 15.6$ (Server & Shooter, 1976) and a molecular mass of 27 500 Da were used to calculate protein concentration.

PPK was purchased from Boehringer Mannheim Biochemicals GmbH, and BPT was purchased from Sigma Chemical Co. Both enzymes were further purified to apparent homogeneity using preparative isoelectric focusing followed by Sephadex G-100 chromatography. An extinction coefficient of $E_{1\%}^{280\,nm} = 16.6$ (Kutzbach & Schmidt-Kastner, 1972) and a molecular mass of 27 500 Da were used to calculate the protein concentration for PPK. An extinction coefficient of $E_{1\%}^{280\,nm} = 15.4$ (Benmouyal & Trowbridge, 1966) and a molecular mass of 26 600 Da were used to calculate the protein concentration for BPT.

The N-acetylated tripeptide *p*-nitroanilide substrates *N*-Ac-Glu-Leu-Arg-*p*Na (AcELR*p*Na),[2] *N*-Ac-Ala-Thr-Arg-*p*Na (AcATR*p*Na), *N*-Ac-Glu-Thr-Arg-*p*Na (AcETR*p*Na), *N*-Ac-Ala-Leu-Arg-*p*Na (AcALR*p*Na), *N*-Ac-Glu-Phe-Arg-*p*Na (AcEFR*p*Na), and *N*-Ac-Glu-Lys-Arg-*p*Na (AcEKR*p*Na) were prepared by using Merrifield solid-phase synthetic methods (Stewart & Young, 1984). *tert*-Butyloxycarbonyl-amino acids (BaChem) were activated with diisopropylcarbodiimide (Aldrich) and coupled in dichloromethane/dimethylacetamide containing 1 equiv of 1-hydroxybenzotriazole (Omni Labs). Modified 1% cross-linked polystyrene beads (Bio-Rad) possessing a novel acid-labile linker were used as the resin support which allowed direct formation of the peptide *p*-nitrophenylamide following cleavage in hydrofluoric acid/methyl ethyl sulfide/anisole (J. P. Burnier, unpublished results). The peptides were purified by preparative reversed-phase HPLC (9.4 × 500 mm, 15–20-μm Vydac C18) using a 0.25%/min H_2O/acetonitrile/0.1% trifluoroacetic acid gradient and analyzed by amino acid composition (Heinrickson & Meredith, 1984; Tarr, 1984) and fast atom bombardment mass spectrometry (Hewlett Packard 5985B/Phasor). D-Val-Leu-Arg-*p*Na [(D)VLR*p*Na] and D-Val-Leu-Lys-*p*Na [(D)VLK*p*Na] were purchased form Helena Laboratories: N^{α}-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA) was purchased from Sigma Chemical Co.

The heptapeptide Trp-Glu-Leu-Arg-His-Ala-Gly (WELRHAG), representing the EGF precursor P_4 to P_{-3} positions (Scott et al., 1983; Gray et al., 1983), was synthesized on an Applied Biosystems Model 430A peptide synthesizer and removed from the resin by HF cleavage. It was purified by reverse-phase HPLC utilizing a preparative C4 column. The peptide was characterized by sequence analysis, and concentrations were quantitated by amino acid analysis.

Determination of Kinetic Constants. Initial reaction rate kinetics were evaluated by using a Hitachi Model U-2000 spectrophotometer. Unless otherwise indicated, all analyses were performed in 50 mM Tris/0.1 mM EDTA, pH 8.5. Production of the *p*-nitroaniline chromophore was monitored at an absorbance of 405 nM; an extinction coefficient E = 9767 M^{-1} cm^{-1} (Lottenberg & Jackson, 1983) was used in calculating the rate of product formation. The substrate and enzyme samples were maintained at 4 °C prior to mixing, and the sample was then introduced to the spectrophotometer via an automated sipping device which maintained the flow cell at 37 °C. The rate of change in absorbance was automatically calculated every 15 s over a period of 3 min. Approximately 60 s was required for stabilization of the signal after introduction of sample, and it was estimated that approximately 20 s was required to raise the sample temperature to 37 °C. The initial reaction rate was determined by extrapolation to a 20-s time point from interval values between 60 and 180 s using linear regression analysis. Although the kinetic equation is hyperbolic, the short time course assured a minimal change in substrate concentration, and the coefficient of correlation for a linear fit to these time points was typically greater than 0.95. Initial analyses were performed with each enzyme and substrate to estimate approximate K_m values and to determine appropriate enzyme concentrations to maximize signal response while minimizing (<10%) depletion of substrate over the experimental time course. Enzyme concentrations varied between 1.0×10^{-9} and 6.8×10^{-8} M. Typically, a set of seven serial 1:2 substrate dilutions were analyzed in duplicate with the midpoint sample equal to the initially determined approximate K_m value. Thus, the substrate values covered a concentration range from aproximately $0.125K_m$ to $8K_m$. This general protocol had to be varied in those cases of enzyme/substrate combinations where the K_m was <10 μM or >500 μM. Practical substrate ranges were from 1.0×10^{-6} to 1800×10^{-6} M.

Values for K_m and k_{cat} were determined by computer fitting of substrate concentration vs initial reaction rates to the

[2] One-letter code for amino acids: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Table I: Kinetic Constants for EGF-BP, γ-NGF, BPT, and PPK for the Substrates (D)VLR*p*NA and (D)VLK*p*NA[a]

	substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	error[c]	ratio of k_{cat}/K_m
EGF-BP	(D)VLR*p*NA	11.2 ± 2.7	27.6 ± 4.2	2464	0.19	37
	(D)VLK*p*Na	621 ± 20	41.0 ± 8.0	66.0	0.11	
γ-NGF	(D)VLR*p*Na	1.18 ± 0.36	7.7 ± 2.1	6525	0.28[b]	3.2
	(D)VLK*p*Na	12.6 ± 0.3	25.3 ± 0.3	2008	0.02	
PPK	(D)VLR*p*Na	18.3 ± 2.2	6.6 ± 0.7	361	0.12	76
	(D)VLK*p*Na	868 ± 65	4.1 ± 0.4	4.72	0.09	
BPT	(D)VLR*p*Na	68.2 ± 9.1	34.2 ± 0.9	501	0.08[b]	17
	(D)VLK*p*Na	622 ± 17	17.9 ± 1.4	28.8	0.05[b]	

[a] Assay conditions were 50 mM Tris/0.1 mM EDTA, pH 8.5 at 37 °C. [b] Enzyme/substrate combinations displaying an initial reaction rate lag at high substrate concentrations (>500 μM). [c] Error is reported as the combined average of K_m and k_{cat} deviation from the mean.

Michaelis–Menten equation utilizing the method of the direct linear plot (Eisenthal & Cornish-Bowden, 1974). From previous kinetic analyses of both BPT and PPK, it has been shown that the acylation rate for *p*-nitroanilide substrates is the rate-limiting step (Bizzozero & Dutler, 1987). Thus, there is a close correlation between the experimentally determined value for K_m and the actual equilibrium dissociation constant (K_{EA}) for the ES complex, and with k_{cat} and the acylation rate constant (K_{23}) [nomenclature of Bizzozero and Dutler (1987)]. The kinetic data are thus presented as $K_m \sim K_{EA}$ and $k_{cat} \sim K_{23}$.

Proteolysis of the heptapeptide WELRHAG by EGF-BP, γ-NGF, and BPT was evaluated by utilizing enzyme concentrations of 10 nM and substrate concentrations of 750 μM in 50 mM Tris, pH 8.5 at 37 °C. Time points at 0, 5, 15, and 30 min were taken, and an equivalent volume of glacial acetic acid was added to halt the reaction. The extent of proteolysis of the heptapeptide substrate and production of the tetrapeptide WELR were evaluated by utilizing a Hitachi Model L-6200 HPLC, with a C18 column. A 0–30% CH_3CN linear gradient over 30 min eluted the heptapeptide and tetrapeptide at approximately 20% and 22% CH_3CN, respectively. The amounts of the two peptides were quantitated by comparison with peptide standards. The HAG tripeptide was not retained under these conditions, and thus the hydrolysis of the WELRHAG peptide was evaluated by the decrease of the heptapeptide and the appearance of the WELR tetrapeptide normalized for a total peptide sample of 750 pmol.

Results and Discussion

The mouse glandular kallikreins, EGF-BP and γ-NGF, have demonstrated distinct specificities for growth factor association yet share a high degree of sequence identity (Blaber et al., 1987a). In order to deduce the molecular bases for the observed specificities of these kallikreins, we have obtained kinetic constants for a series of substrates corresponding to the carboxyl-terminal sequences of the mature growth factors EGF and NGF. The derivation of these constants correlated with standard Michaelis–Menten kinetics except for certain enzyme/substrate combinations which displayed an initial reaction rate lag at high substrate concentrations (>500 μM). For these combinations, the calculated K_m and k_{cat} values were determined from data points at lower substrate concentrations in an effort to obtain more accurate constants. However, the reported values for K_m and k_{cat} may be lower than the actual kinetic constants. The exact nature of this rate lag is unknown but appears to be due to inhibition at substrate concentrations approaching saturation kinetics.

The error for the kinetic data is higher for those enzyme/substrate combinations yielding extremely low or high K_m values. With low substrate values (<10 μM), detection of the *p*-nitroaniline chromophore above background levels required a concentration of enzyme which significantly hydrolyzed the substrate within several minutes. Significant product formation occurs over this time, as well as significant reduction of substrate/reaction rate, and the extrapolation to the initial reaction rate thus is subject to greater error. Due to limits in the availability of substrate, those enzyme/substrate combinations displaying high K_m values (>750 μM) are subject to error because the highest concentrations utilized were 1800 μM and therefore the kinetic analyses covered a substrate range in which the highest concentration did not exceed $2K_m$ in some cases.

P_1 Position Specificity. Table I lists the kinetic parameters for EGF-BP, γ-NGF, BPT, and PPK toward (D)VLR*p*Na and (D)VLK*p*Na. These substrates, which contain the D isomer of valine with a free α-amino group in the P_3 position, were used to compare the effect of lysine vs arginine in the P_1 position. Both of the mouse kallikreins display an increased value of k_{cat} for the lysine substrates, although the overall catalytic efficiency (k_{cat}/K_m) is significantly higher for the arginine peptides, as seen for the other enzymes. This is reflected in the k_{cat}:K_m ratios greater than 1 for the arginine vs lysine substrate for each enzyme.

Of the variety of serine proteases that have been evaluated, only plasmin shares this significant increase in k_{cat} for lysine.[3] Accordingly, EGF-BP and γ-NGF were tested for potential plasmin-like activity utilizing a fibrin plate method (Walton, 1966) with and without plasmin(ogen)-depleted fibrin. The results indicate that neither enzyme has any direct plasmin-like activity (data not shown). However, in the course of this study, it was observed that γ-NGF exhibits approximately 200*x*-fold greater plasminogen activator activity than EGF-BP. This is in contrast to a previous report showing that the two kallikreins are essentially identical with regard to plasminogen activator activity (Hiramatsu et al., 1982). One explanation for the discrepancy is the concentration of plasminogen in the fibrin preparation. Under conditions of very low substrate concentration, the general nature of γ-NGF with its significantly lower K_m values for substrates with lysine in the P_1 position would allow it to display potentially higher activity than EGF-BP, if the k_{cat} values for the plasminogen substrate were similar.

The kinetic constants for the hydrolysis of N^{α}-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA) by BPT, PPK, and the mouse kallikreins are summarized in Table II. These data reflect the interaction of the arginine in the substrate P_1 position; there are no peptide contributions for L-BAPNA in the P_2 and P_3 positions.

Notably, PPK has a 10-fold lower catalytic rate for this substrate relative to the other enzymes. This has been explained in terms of the binding pocket geometry of PPK in relationship to that of BPT (Bizzozero & Dutler, 1987; Bode

[3] Information provided by the catalog of Helena Laboratories, Beaumont, TX.

Table II: Kinetic Constants for N^{α}-Benzoyl-L-arginine-*p*-nitroanilide by Mouse and Porcine Kallikreins and Bovine Trypsin[a]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	error[b]
EGF-BP	241 ± 59	1.78 ± 0.05	7.4	0.14
γ-NGF	35.7 ± 5.7	1.80 ± 0.1	50.4	0.11
BPT	368 ± 42	2.69 ± 0.31	7.3	0.11
PPK	383 ± 33	0.154 ± 0.03	0.16	0.16

[a] Assay conditions were 50 mM Tris/0.1 mM EDTA, pH 8.5 at 37 °C. [b] Error is reported as the combined average of K_m and k_{cat} deviation from the mean.

et al., 1983); in BPT, the arginine interacts with optimal geometry with its electrostatic partner Asp-189 while in PPK, the alignment of arginine is less than optimal. The K_m value for L-BAPNA with PPK (Table II) is similar to that of BPT. Thus, the interaction of the substrate arginine with PPK adversely affects the orientation of the scissile bond, decreasing k_{cat}, but not affecting the total binding energy (K_m). This has been explained by the substitution of the glycine at position 226 in BPT by a serine in PPK, and the change of residues 217–219, His-Thr-Pro, in PPK to Ser-Gly in BPT, which are postulated to alter the binding geometry of the P_1 arginine in PPK vs BPT (Bizzozero & Dutler, 1987; Bode et al., 1983).

In the mouse kallikrein, the Gly-226 observed in BPT is conserved, and the His-Thr-Pro loop (residues 217–219) found in PPK is retained in γ-NGF and appears as Phe-Thr-Pro in EGF-BP. The mouse enzymes share characteristics of both BPT and PPK within these sequences. The kinetic results suggest that positions 217–219 are less significant than Gly-226 in conferring the arginine binding geometry observed in trypsin. Comparison of the K_m values for L-BAPNA in Table II indicates that γ-NGF has a uniquely higher affinity for the P_1 arginine than the other enzymes. Improved geometry of existing contacts or introduction of additional contacts with the arginine may exist within the γ-NGF binding pocket. A comparison of the active-site structures of γ-NGF, PPK, and BPT may, therefore, suggest general ways whereby the kinetic parameters, both K_m and k_{cat}, for arginine substrates can be significantly improved.

P_2 and P_3 Position Specificity. The influence of side-chain substituents in positions P_2 and P_3 of the substrates on the kinetic constants can be seen in Table III. Although effects upon K_m and k_{cat} are not isolated events, comparing these values for the six tripeptide *p*-nitroanilide substrates tested (AcELR*p*Na, AcATR*p*Na, AcEKR*p*Na, AcEFR*p*Na, AcETR*p*Na, and AcALR*p*Na) with those for L-BAPNA indicate that, on average, the main contribution of the residues in the P_2 and P_3 positions is improvement of k_{cat}. In comparison to L-BAPNA, K_m constants for the tripeptide substrates either increase or decrease within approximately a 5-fold range. The k_{cat} values, however, all display a 10-20-fold improvement over those seen for L-BAPNA. Thus, occupancy of these positions appears to significantly improve alignment of the scissile bond in each case. Furthermore, since the K_m values for the tripeptides do not differ greatly from those observed with L-BAPNA, the contribution of P_2 and P_3 interactions only minimally extends that of the P_1 arginine, indicating the importance of the contribution of the P_1 arginine to the total binding energy and why removal of this single residue abolishes formation of the growth factor complexes.

An important characteristic of substrate specificity of the mouse kallikreins in comparison to BPT and PPK is the effect of hydrophobic side chains in the P_2 position. PPK shows a marked preference in k_{cat} for substrates with a hydrophobic residue in this position while BPT is distinctly less specific (Table III). The structural interpretation of this phenomenon suggests the interaction of Tyr-99 and Trp-215 in PPK to form a hydrophobic pocket which is sterically blocked in BPT due to a substitution of leucine for tyrosine at position 99 (Bizzozero & Dutler, 1987; Bode et al., 1983). Both EGF-BP and γ-NGF have a tyrosine at position 99, as is found in PPK; thus, considering these specific interactions, the mouse kallikreins would be expected to display a preference in k_{cat} toward substrates with a hydrophobic side chain in the P_2 position. From the data shown in Table III, this expectation is generally realized for the k_{cat}/K_m values but is less clearly manifested in the k_{cat} data, particularly with γ-NGF. Thus, other interactions beside the Tyr-99 and Trp-215 positions are af-

Table III: Kinetic Constants for EGF-BP, γ-NGF, BPT, and PPK for the Hydrolysis of a Series of Tripeptide N^{α}-Acetyl-*p*-nitroanilide Substrates[a]

	substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	error[c]
EGF-BP	AcELR*p*Na	74.1 ± 3.8	51.5 ± 1.4	695	0.04
	AcATR*p*Na	506 ± 73	17.8 ± 0.9	35.2	0.10
	AcEKR*p*Na	519 ± 73	39.2 ± 6.2	75.5	0.15
	AcEFR*p*Na	342 ± 15	47.0 ± 4.7	137	0.07
	AcETR*p*Na	806 ± 67	12.6 ± 0.1	15.6	0.05
	AcALR*p*Na	72.2 ± 5	54.9 ± 5.9	760	0.09
γ-NGF	AcELR*p*Na	23.8 ± 1.5	14.3 ± 0.2	601	0.04
	AcATR*p*Na	93.5 ± 26	29.0 ± 0.9	310	0.15
	AcEKR*p*Na	129 ± 29	10.8 ± 1.8	83.7	0.20[b]
	AcEFR*p*Na	22.0 ± 3.7	14.5 ± 1.9	833	0.15
	AcETR*p*Na	292 ± 23	11.9 ± 2.4	40.7	0.14
	AcALR*p*Na	4.50 ± 0.01	18.3 ± 1.7	4067	0.05[b]
PPK	AcELR*p*Na	849 ± 116	9.2 ± 0.4	10.8	0.09
	AcATR*p*Na	571 ± 53	0.72 ± 0.09	1.26	0.11[b]
	AcEKR*p*Na	291	0.029	0.10	
	AcEFR*p*Na	765 ± 85	6.62 ± 0.22	8.7	0.07
	AcETR*p*Na	929 ± 12	0.38 ± 0.08	0.41	0.12
	AcALR*p*Na	222 ± 10	6.74 ± 0.21	30.4	0.04
BPT	AcELR*p*Na	187 ± 2.0	42.9 ± 3.5	229	0.05
	AcATR*p*Na	52.9 ± 10.7	21.1 ± 0.5	399	0.11[b]
	AcEKR*p*Na	44.8 ± 3.5	22.0 ± 2.0	491	0.08
	AcEFR*p*Na	61.9 ± 2.9	44.0 ± 1.1	711	0.04
	AcETR*p*Na	64.5 ± 3.8	24.6 ± 2.7	381	0.08
	AcALR*p*Na	347 ± 22	23.6 ± 0.15	68.0	0.03

[a] Assay conditions were 50 mM Tris/0.1 mM EDTA, pH 8.5 at 37 °C. [b] Indicates enzymes/substrate combinations displaying an initial reaction rate lag at high substrate concentrations (>500 μM). [c] Error is reported as the combined average of K_m and k_{cat} deviation from the mean.

Table IV: Dependency on pH of K_m and k_{cat} Values for EGF-BP and γ-NGF with the Tripeptide Substrate AcELR*p*Na[a]

	pH	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
EGF-BP	8.50	74	52	695
	7.75	58	43	750
	6.75	29	21	710
γ-NGF	8.50	24	14.3	601
	7.75	2.9	6.1	2100
	6.75	5.9	3.3	555

[a] Assay conditions were 50 mM Tris (pH 8.5), 50 mM sodium phosphate (pH 7.75 and 6.75), and 0.1 mM EDTA at 37 °C.

fecting the interactions with the P_2 position in γ-NGF and perhaps to a lesser extent with EGF-BP. Nonetheless, the k_{cat}:K_m ratios, a measure of overall catalytic efficiency, indicate that the mouse kallikreins are more similar to PPK than BPT with regard to hydrophobic preference for the P_2 substrate position.

The side chain occupying position P_3 has very little effect on either k_{cat} or K_m values with EGF-BP (Table III). This is not the case with γ-NGF where alanine is much more favored in the P_3 position than glutamic acid as judged by both k_{cat} and K_m values. The environment of the P_3 position in EGF-BP is presumably "open" while in γ-NGF specific interactions can occur. In comparison to PPK and BPT, EGF-BP appears to be somewhat unique in this lack of P_3 site specificity.

The hypothesis that the kallikreins process the precursor forms of their associated growth factors, and are not just merely binding proteins, implies a preferred acylation rate for these substrates. Of the substrates tested, γ-NGF displayed the greatest k_{cat} value for the AcATR*p*Na substrate, representing the carboxyl-terminal sequence of mature β-NGF (Angeletti & Bradshaw, 1971). EGF-BP displayed an equal preference for either AcALR*p*Na or AcELR*p*Na, with AcELR*p*Na representing the carboxyl-terminal sequence of mature EGF (Savage et al., 1972). These results suggest the amino acid differences between EGF-BP and γ-NGF have led to optimization of the P_2 and P_3 interactions with their associated growth factors, resulting in preferential hydrolysis.

It is important to note that, in a general sense, γ-NGF has a lower K_m and lower k_{cat} for the tripeptide substrates than EGF-BP. The overall catalytic efficiency for the two enzymes, however, is approximately equivalent. Thus, EGF-BP and γ-NGF effectively accomplish hydrolysis of substrates in a differing manner; EGF-BP may have a reduced affinity for substrates than γ-NGF, but it has a higher rate of hydrolysis. The basis for this difference can be partially interpreted with regard to the treatment of the substrate P_1 arginine, as indicated by the kinetic data for the substrate BAPNA summarized in Table II. In comparison to EGF-BP, γ-NGF has a significantly lowered K_m value toward L-BAPNA which is reflected in all other substrates tested. The general increase in k_{cat} rates for EGF-BP, however, is enhanced by P_2 and P_3 position interactions.

Effect of pH. The association of γ-NGF with β-NGF is most stable at pH 8.5, the pH chosen for the kinetic studies (Silverman & Bradshaw, 1982). The EGF-BP/EGF complex displays stability over a broad pH range (5–8) (Taylor et al., 1974b). Since pH 8.5 is slightly outside this range, the effects of pH on the kinetic parameters for EGF-BP and γ-NGF with the AcELR*p*Na substrate were evaluated. The results (Table IV) indicate that at lower pH values both the K_m and k_{cat} values decrease for both EGF-BP and γ-NGF. These results suggest that the observed dissociation of the EGF-BP/EGF complex above pH 8.0 may in part be due to an increase in the dissociation constant of the arginine in the P_1 position. These observations do not, however, explain the disruption of the γ-NGF/β-NGF complex at pHs below 8.5. Also, even though at lower pH values, γ-NGF has a lowered K_m value for the ELR substrate; it is still unable to complex with the mature EGF molecule. The pH-dependent disruption of the growth factor complexes may involve the postulated additional binding interactions.

Growth Factor and Kallikrein Interactions. If the entire set of interactions between the kallikreins and mature growth factors is defined by the P_3 to P_1 positions, then the equilibrium dissociation constant for the complex should approximate the K_m value for the corresponding tripeptide substrate. The K_m values for AcELR*p*Na and AcATR*p*Na (Table III) with EGF-BP and γ-NGF, respectively, are significantly higher than the deduced equilibrium dissociation constants for the mature growth factor/kallikrein complexes. EGF-BP and EGF can form a complex which is stable when analyzed with a variety of techniques including chromatography on cellulose acetate strips, native gel electrophoresis, and gel exclusion chromatography (Taylor et al., 1974b; Server et al., 1976). The 7S NGF complex formation (involving the α-, γ-, and β-NGF subunits) can be demonstrated in a similar fashion (Varon et al., 1968). The interaction between γ-NGF and β-NGF subunits in the absence of the α-NGF subunit is, however, significantly weaker than the 7S NGF complex (Silverman & Bradshaw, 1982) or the EGF-BP/EGF complex. The γ-NGF/β-NGF interaction was demonstrated only with sedimentation velocity experiments (Silverman & Bradshaw, 1982) and is not stable in discontinuous gel electrophoresis experiments. The equilibrium dissociation constant for the γ-subunit from the 7S complex (in EDTA) has been estimated at 1×10^{-7} M (Bothwell & Shooter, 1978), the K_i for β-NGF with γ-NGF has been calculated at 4.5×10^{-7} M (Nichols & Shooter, 1983), and the equilibrium dissociation constant for the γ-NGF/β-NGF interaction has been reported as 1.6×10^{-6} M (Almon & Varon, 1978). Thus, the γ-NGF/β-NGF equilibrium dissociation constant in EDTA is approximately 10^{-6} M. The EGF-BP/EGF dissociation constant can been estimated to be at least 10^{-7} M as judged by the behavior of the complex on gels. It could be even lower, i.e., stronger.

From the data in Table III, it might be reasoned that γ-NGF, in comparison to EGF-BP, would preferentially complex with EGF if the entire growth factor/kallikrein interactions involved only the P_3 to P_1 positions. Likewise, BPT, as compared to γ-NGF, should preferentially complex with β-NGF. Clearly, this does not occur. Furthermore, a comparison of the range of K_m values with the equilibrium dissociation constants shows substantial differences in overall magnitude. This suggests that significant contacts between the growth factor and kallikrein exist outside these positions. The interactions of the tripeptide substrates, from the K_m values, translate to 5.7 and 5.9 kcal/M for the γ-NGF/AcATR*p*Na and EGF-BP/AcELR*p*Na interactions, respectively. Given the equilibrium dissociation constants for the complexes involving the intact growth factors, there is a minimum of about 2.8 and 4.0 kcal/M of binding energy unaccounted for by the tripeptides representing β-NGF and EGF, respectively.

The ability of EGF-BP to process the precursor form of EGF was evaluated directly by testing the ability of the heptapeptide WELRHAG (representing the carboxyl-terminal junction of the precursor P_4 to P_{-3} positions; Scott et al., 1983; Gray et al., 1983) to competitively inhibit EGF-BP. A standard substrate/reaction rate plot for the AcELR*p*Na

Table V: Cleavage of the Heptapeptide Trp-Glu-Leu-Arg-His-Ala-Gly by EGF-BP, γ-NGF, and BPT[a]

	Trp-Glu-Leu-Arg produced (pmol) in time (min)		
enzyme	5	15	30
EGF-BP	23	75	120
γ-NGF	90	180	255
BPT	165	300	473

[a] Assay conditions were 50 mM Tris/0.1 mM EDTA, pH 8.5 at 37 °C. [S] = 750 μM, [E] = 10 nM. Data were normalized for a total peptide sample of 750 pmol.

substrate with EGF-BP at fixed WELRHAG peptide concentrations of 0, 50, 100, or 200 μM displayed no inhibition of any kind (data not shown). The ability of EGF-BP, γ-NGF, and trypsin to cleave this heptapeptide was evaluated by utilizing HPLC. The results (Table V) indicate that at equal enzyme concentrations and at a substrate concentration of 750 μM, γ-NGF hydrolyzes this substrate at a rate approximately 56% that of trypsin, while EGF-BP hydrolyzes it at a rate approximately 21% that of trypsin.

Several explanations may account for this relatively poor cleavage by EGF-BP. First, while EGF in solution appears to have little if any defined structure in the carboxy-terminal four or five amino acids (Montelione et al., 1987), the precursor molecule likely has a specific conformation which promotes efficient proteolysis by EGF-BP. Second, a significant binding interaction between EGF and EGF-BP outside of the P_3 to P_1 positions is indicated. The effect of such a site(s) would be to significantly lower the equilibrium dissociation constant for the growth factor precursor substrate. It should be noted that the free α-amino group of the substrate P_4 tryptophan or the α-carboxyl group of the P_{-3} glycine may adversely affect interactions within the enzyme binding pocket, thus lowering the catalytic efficiency. Finally, it may also be the case that despite the obvious preference for the tripeptide representing the appropriate growth factor substrate, the function of EGF-BP may not be to process the EGF precursor but simply to complex with the mature growth factor after processing is complete.

The ability of EGF-BP and γ-NGF to efficiently cleave the AcEKR*p*Na substrate (Table III) is evidence that the kallikreins may display additional processing functions. Dibasic sequences are present at a number of precursor protein processing sites including the amino-terminal site of the β-NGF precursor (Ullrich et al., 1983). The results suggest that both γ-NGF and EGF-BP may process these dibasic sequences. The k_{cat} for this substrate is similar to the rates observed for the other tripeptide substrates and is considerably higher than that for L-BAPNA. This indicates that the hydrolysis is of the intact tripeptide and not the residual Arg-*p*Na that would exist if significant cleavage was occurring after the lysine residue. Thus, the data reflect a dibasic processing event and not two sequential single basic cleavages. The recent observation that γ-NGF can cleave the amino-terminal dibasic processing site of the β-NGF precursor is in agreement with these findings (Edwards et al., 1988).

EGF-BP and γ-NGF are subject to posttranslational proteolytic processing at three internal sites (positions 83, 87, and 140) of the mature protein (Silverman & Bradshaw, 1982; Isackson et al., 1987; Blaber et al., 1987). All three positions involve cleavages after basic residues and are thus potential autolysis sites. The cleavage at position 140 in both mouse kallikreins may be particularly susceptible to autolysis owing to preferred P_2 site residues. In γ-NGF, the P_3 to P_1 sequence at the site of processing is Pro-Thr-Lys, and in EGF-BP, it

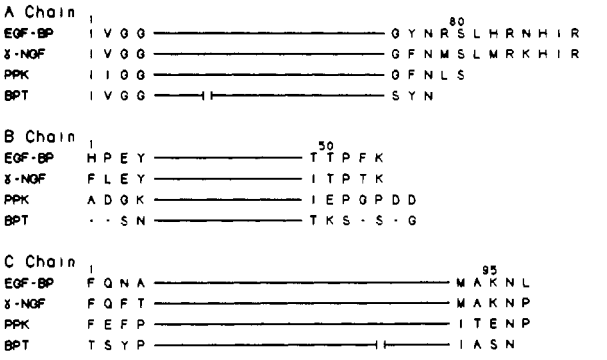

FIGURE 2: Three-chain numbering system useful for maintaining register of homologous amino acid positions of the mouse kallikreins EGF-BP (Blaber et al., 1987a) and γ-NGF (Ullrich et al., 1984; Thomas et al., 1981) with PPK and BPT (Bode et al., 1983). One-letter code for amino acids defined in footnote 2.

is Pro-Phe-Lys. Thus, these internal sittes of processing are intriguingly similar in sequence to the carboxyl-terminal sequences of the associated growth factors.

Implications for Molecular Modeling. The ultimate purpose of this work is to explain the kinetic data in terms of the physical structure of EGF-BP and γ-NGF. A structure/function interpretation for these molecules may allow an understanding of growth factor complex formation, the mechanism whereby EGF-BP and γ-NGF accomplish their significantly different (k_{cat} vs K_m) approaches to catalysis, and the preferred substrate P_2 and P_3 specificities. Neither molecule has been crystallized to date; thus, comparative molecular modeling utilizing the structures of related enzymes is the only available methodology. Therefore, the kinetic parameters for the same series of substrates were evaluated for the related enzymes BPT and PPK. Prior to these kinetic analyses, it was postulated that PPK would be a better model on which to base the mouse kallikreins than BPT (Blaber et al., 1987b). The basis for this decision was that PPK shares 60% identity with the mouse kallikreins whereas BPT is only approximately 40% identical. Additionally, BPT requires a number of gap penalties when attempting to align the amino acid sequence with the mouse kallikreins.

The kinetic results indicate that the mouse kallikreins fall somewhere between BPT and PPK; they display the optimal P_1 arginine alignment observed in BPT, but they also display a general P_2 hydrophobic preference similar to PPK. Therefore, either PPK or BPT can rationally be utilized in model building of the mouse kallikreins. However, PPK needs fewer deletions than BPT when aligning these amino acid sequences with the mouse kallikreins (Bode et al., 1983). When these amino acid sequences are compared, the problems of insertions and deletions lead to difficulty in the register of homologous amino acid positions throughout the length of the protein (Bode et al., 1983). An alternative numbering system, useful for comparative molecular modeling of these proteases and which greatly simplifies alignment of these sequences, is shown in Figure 2. It is based on the observation that the major regions of insertions and deletions between these enzymes occur at surface loops of the molecules which delineate two potential sites of posttranslational proteolytic processing common to the serine family of proteases. The molecules are thus numbered as if they existed as a three-chain molecule; chain A begins with the isoleucine of the amino terminus of the mature protease (Ile-16; Bode et al., 1983), chain B begins with Ala-Y95 (Bode et al., 1983) of PPK, or His-88 (Blaber et al., 1987a) of EGF-BP, and chain C begins with Phe-149 (Bode et al., 1983) or PPK or Phe-141 (Blaber et al., 1987a)

of EGF-BP. The insertions and deletions between the mouse kallikreins and PPK then exist simply as extensions of the various chains, and homologous amino acids, including the active-site residues, are in register. This greatly simplifies comparative molecular modeling for those regions of heterogeneity where coordinates do not exist. Whether starting with BPT or PPK, the validity of the resulting mouse kallikrein model would seem to rest on the correlation between computer-derived binding energies and geometries for the various tripeptide substrates and the experimentally determined kinetic values. Such analyses are in progress.

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