Actinidin Hydrolysis of Substituted-Phenyl Hippurates: A Quantitative Structure–Activity Relationship and Graphics Comparison with Hydrolysis by Papain

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Received October 14, 1983

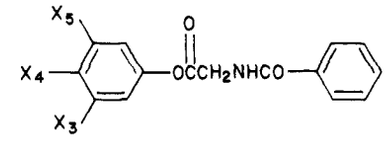
The hydrolysis of 29 phenyl hippurates ($XPhOCOCH_2NHC(=O)C_6H_5$) by the cysteine protease actinidin has been studied and a quantitative structure–activity relationship (QSAR) has been formulated: $\log 1/K_m = 0.74\sigma + 0.50\pi'_3 + 0.24MR_4 + 2.90$. In this expression K_m is the Michaelis constant, σ is the Hammett constant, π'_3 is the hydrophobic parameter for the more hydrophobic of the two meta substituents, and MR_4 is the molar refractivity of para substituents. The QSAR for actinidin is compared with a similar one obtained for another cysteine plant protease papain. A color stereo computer graphics model constructed from the X-ray crystallographic coordinates of actinidin is compared with those of our previously reported models for papain.

The problem of understanding how drugs react with receptors, although extremely complex and difficult, is central to the development of medicinal chemistry. We are terribly limited in the study of receptors by the fact that we do not know their detailed structure. However, in some instances enzymes are drug receptors and we do have X-ray crystallographic structure on many enzymes. Thus the study of enzyme–ligand interactions is one of the best ways to develop our understanding of how drugs react with macromolecules. For this reason we have inititated a series of studies in which we attempt to correlate quantitative structure–activity relationships (QSAR) of enzyme–substrate and enzyme–inhibitor interactions with the X-ray crystallographically determined structure of the enzyme, using computer graphics molecular modeling. Our goal is to understand QSAR at the atomic level of enzyme–ligand interaction using molecular models of the enzyme–ligand complex. Recent studies on papain[1] and dihydrofolate reductase[2,3] demonstrated the potential of this approach and resulted in some understanding of the QSAR of enzyme–ligand interaction when the ligand structure is varied and the enzyme held constant. The inverse problem of understanding the molecular basis of the structure–activity relationships when the enzyme structure is varied and the *inhibitor* held constant is considerably more difficult and has led us to study the closely related enzymes papain and actinidin.

The extensive amino acid sequence homology (48%) and kinetics similarities between papain and actinidin[4,5] suggested similar mechanisms of action and similar active-site structures. The three-dimensional structure of papain has been determined by X-ray crystallography by Drenth[6,7] and that of actinidin has been established by Baker,[8,9] demonstrating that they have remarkably similar structures with nearly identical active sites, which differ by only a few amino acid residues. Therefore, these two enzymes provide an ideal system for studying and understanding QSAR at the atomic level and, in particular, for delineating the role of different amino acid residues in otherwise identical active sites in determining enzyme–substrate specificity.

Actinidin is a cysteine protease isolated from the fruit of *Actinidia chinensis* (Kiwi fruit) similar to the other plant cysteine proteases papain, ficin, and bromelain.[5] Papain, bromelain, and ficin have been studied by several groups,[1,4-7,12-15,17] but few studies have appeared on actinidin.[16-19] Papain and actinidin are the only cysteine proteases whose structures have been solved by X-ray crystallography.

In our previous study on the papain-catalyzed hydrolysis of esters I, the QSAR of eq 1 was formulated. In this



I

$$\log 1/K_m = 1.03\ (\pm 0.25)\ \pi'_3 + 0.57\ (\pm 0.20)\ \sigma + 0.61\ (\pm 0.29)\ MR_4 + 3.80\ (\pm 0.17) \quad (1)$$

$$n = 25,\ r = 0.907,\ s = 0.208$$

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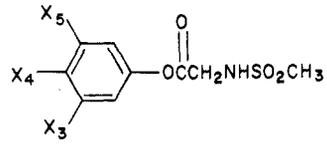
expression, n represents the number of data points used to derive the equation, r is the correlation coefficient, s is the standard deviation from the regression, and the figures in parentheses are for construction of the 95% confidence intervals. $1/K_m$ is considered as an approximate binding constant since K_{cat} is essentially constant for this class of substrates.

The hydrophobic parameter π'_3 refers to the more hydrophobic of the two meta substituents on I; only this substituent is assumed to play a hydrophobic binding role in the formation of the enzyme–substrate complex. This hypothesis was subsequently found to be consistent with a computer graphics molecular model[1] that showed that one meta substituent contacts a hydrophobic pocket of the enzyme, while the other meta substituent is forced into the surrounding aqueous phase.

The Hammett constant σ in eq 1 represents the sum of σ for substituents in all three positions. Its positive coefficient indicates that electron withdrawal by substituents promotes formation of the enzyme–substrate complex, probably due to the interaction of the electron-rich cysteine SH group with the carbonyl carbon of the ester.

The MR term (molar refractivity) applies only to substituents in the 4-position of I. MR (scaled by 0.1 to make it more nearly equiscalar with π) is a measure of the molar volume and polarizability of a substituent.[20] The positive coefficient with this term in eq 1 indicates that the larger and more polarizable the 4-substituent, the more it promotes formation of the enzyme–substrate complex. Since the two vectors π and MR are reasonably orthogonal and MR gives a better correlation than π for 4-substituents, we proposed that the interaction of these substituents with the active site of papain occurs in a nonhydrophobic region. This view was supported by molecular models of the enzyme–substrate complex formed by papain and I,[1] which showed that X_4 most likely contacts the hydrophilic side chain of Gln-142.

In order to extend our analysis to larger, more hydrophobic X groups, we studied the papain hydrolysis of the more water-soluble *N*-(methylsulfonyl)glycinates II, resulting in the QSAR of eq 2.[21] The parameters and statistics in eq 2 have the same connotation as in eq 1.



II

$$\log 1/K_m = 0.61\ (\pm 0.09)\ \pi'_3 + 0.55\ (\pm .20)\ \sigma + 0.46\ (\pm 0.11)\ MR_4 + 2.00\ (\pm 0.12) \quad (2)$$

$$n = 32,\ r = 0.945,\ s = 0.178$$

Due to the difference in the coefficients of π'_3 in eq 1 and 2, we concluded that X_3 of I must bind differently to the active site than X_3 of II, although the interaction is still hydrophobic. The coefficient of about 1 with π'_3 in eq 1 suggests complete desolvation of the substituents such as that which occurs in the transfer of a solute from water to octanol (π substituent constants are derived from octanol/water partition coefficients).[20] The smaller coefficient of π'_3 in eq 2 indicates less complete desolvation of the substituent on II and therefore poorer interaction of the substituent with the protein.

Since the $NHSO_2CH_3$ moiety of II is very hydrophilic ($\pi = -1.18$) compared to the hydrophobic $NHCOC_6H_5$ ($\pi = 0.49$) group of I, the interaction of these two groups with the large hydrophobic portion of the active site to which they bind must be quite different.[1] This is evident from a comparison of the intercepts of eq 1 and 2, which shows that, other factors being equal, congeners I bind about 25 times more strongly to papain than congeners II.

In the present study we derive QSAR for the actinidin-catalyzed hydrolysis of congeners I, model the interaction of I with actinidin using computer graphics, and compare the results with our previous studies on papain.

Results and Discussion

From the data in Table I we have derived the QSAR of eq 3–5.

$$\log 1/K_m = 0.64\ (\pm 0.27)\ \sigma + 3.17\ (\pm 0.14) \quad (3)$$

$$n = 27,\ r = 0.699,\ s = 0.289,\ F_{1,25} = 23.9$$

$$\log 1/K_m = 0.73\ (\pm 0.16)\ \sigma + 0.45\ (\pm 0.14)\pi'_3 + 2.99\ (\pm 0.10) \quad (4)$$

$$n = 27,\ r = 0.908,\ s = 0.172,\ F_{1,24} = 46.1$$

$$\log 1/K_m = 0.74\ (\pm 0.15)\ \sigma + 0.50\ (\pm 0.13)\ \pi'_3 + 0.24\ (\pm 0.21)\ MR_4 + 2.90\ (\pm 0.12) \quad (5)$$

$$n = 27,\ r = 0.927,\ s = 0.158,\ F_{1,23} = 5.70$$

The collinearity between variables is quite low as the following r^2 values show:

	σ	π'_3	MR_4
σ	1	0.03	0.00
π'_3		1	0.12

The experimental conditions and parameters used to derive eq 3–5 are comparable to those of eq 1. Equation 3 is the most significant one-variable equation, eq 4 is the best two-variable result, and eq 5 is the best overall equation.

In the development of these equations it was decided after considerable study that two congeners 4-SO_2NH_2 and 3,5-$(OCH_3)_2$ are outliers and do not fit our model. These data points were not used to derive eq 3–5. Equation 5 differs from eq 1 in a number of significant ways, the most striking of which is the coefficient with the π'_3 term. The hydrophobic effect resembles that of eq 2 rather than eq 1, suggesting less effective desolvation of the 3-substituents of I with actinidin than papain. In our model of eq 1 it was proposed that X_3 binds in a hydrophobic pocket so that it is completely desolvated. In the case of congeners II it is postulated that binding occurs on a hydrophobic surface with only partial desolvation. For the papain equation the most important variable is π'_3, while for the actinidin equation the σ term is more important. The role of MR_4 in binding to an actinidin is less important than in the case of papain.

The much lower intercept of eq 5 compared with that of eq 1 reveals that on the average papain binds congeners I about 8 times more strongly than actinidin. Actually the intercepts and the coefficient with π'_3 in eq 5 resemble those of eq 3. It would seem that actinidin has a hydrophobic surface available for X_3 of I very similar to that encountered by X_3 of congeners II.

The poorer affinity of actinidin for ligands may explain to some extent the higher coefficient with σ and its greater importance than in the case of papain. In the study of congeners I their hydrolysis was also carried out in the same buffer used to determine K_m in the absence of en-

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Table I. Physicochemical Parameters[a] Used in the Derivation of Equations 3–5 for the Actinidin-Catalyzed Hydrolysis of $XC_6H_4OCOCH_2NHCOC_6H_5$ at 25 °C, pH 6.0

no.	X	log $1/K_m$ obsd	log $1/K_m$ calcd[b]	$\lvert\Delta \log 1/K_m\rvert$	σ	π'_3	MR_4
1	H	2.77	2.92	0.15	0.00	0.00	0.10
2	3-F	3.01	3.24	0.23	0.34	0.14	0.10
3	3-Cl	3.63	3.55	0.08	0.37	0.71	0.10
4	3-Br	3.64	3.64	0.00	0.39	0.86	0.10
5	3-I	3.93	3.74	0.19	0.35	1.12	0.10
6	3-CH_3	3.26	3.15	0.11	−0.07	0.56	0.10
7	3-*t*-Bu	3.66	3.84	0.18	−0.10	1.98	0.10
8	3-CF_3	3.47	3.68	0.21	0.43	0.88	0.10
9	3-CN	3.08	3.33	0.25	0.56	0.00	0.10
10	3-NO_2	3.53	3.44	0.09	0.71	0.00	0.10
11	3-$NHCOCH_3$	3.18	3.08	0.10	0.21	0.00	0.10
12	3-$CONH_2$	3.15	3.13	0.02	0.28	0.00	0.10
13	3-SO_2NH_2	3.42	3.26	0.16	0.46	0.00	0.10
14	4-F	2.72	2.96	0.24	0.06	0.00	0.09
15	4-Cl	3.04	3.21	0.17	0.23	0.00	0.60
16	4-CH_3	2.95	2.91	0.04	−0.17	0.00	0.56
17	4-$COCH_3$	3.47	3.54	0.07	0.50	0.00	1.12
18	4-CN	3.62	3.54	0.08	0.66	0.00	0.63
19	4-NO_2	3.80	3.65	0.15	0.78	0.00	0.74
20	4-OCH_3	2.87	2.89	0.02	−0.27	0.00	0.79
21	4-NH_2	2.55	2.54	0.01	−0.66	0.00	0.54
22	4-$CONH_2$	3.47	3.40	0.07	0.36	0.00	0.98
23	4-SO_2NH_2	3.22[c]	3.62	0.40	0.57	0.00	1.23
24	3,5-$(CH_3)_2$	3.37	3.09	0.28	−0.14	0.56	0.10
25	3-CH_3-5-Et	3.52	3.33	0.19	−0.14	1.02	0.10
26	3,5-$(OCH_3)_2$	3.60[c]	3.09	0.51	0.24	−0.02	0.10
27	3,5-Cl_2	3.84	3.82	0.02	0.74	0.71	0.10
28	3,5-$(NO_2)_2$	3.90	3.83	0.07	1.42	−0.28	0.10
29	3,4,5-Cl_3	4.01	4.12	0.11	0.97	0.71	0.60

[a] Physicochemical constants are from ref 20. [b] Calculated by using eq 5. [c] Not used in the derivation of eq 3–5.

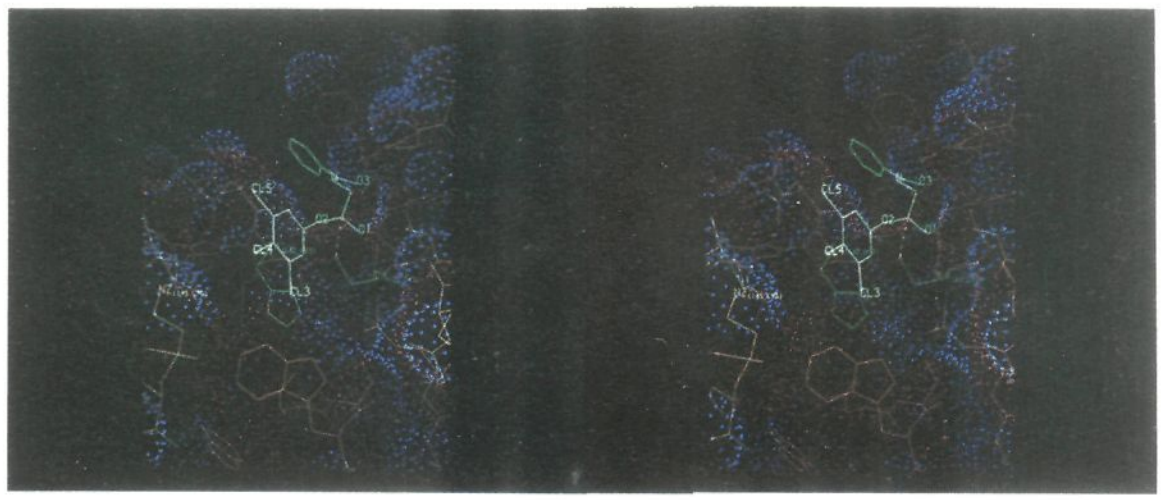

Figure 1. The white "wire model" is the phenyl hippurate (3,4,5-Cl_3-$C_6H_2OCOCH_2NHCOC_6H_5$) in the cleft of the actinidin active site. O_1 and O_2 are the oxygen atoms of the ester, and O_3 is the oxygen of the amide linkage. The blue dots represent the effective surface of the polar atoms of the protein (oxygen and nitrogen) while the red dots code for hydrophobic surface carbon). The yellow dotted lines show the path of approach of the SH of Cys-125 to the carbonyl of the hippurate ester. The essential His-162 is also shown in green. NZ represents the terminal NH_2 of Lys-145. Much of the large hydrophobic surface for the binding of large 3-X is provided by the indole ring shown in yellow in the lower center portion of the picture. No attempt has been made to represent the solvation of the NH_2 of Lys-145.

zyme.[1] From these rates constants a Hammet equation with $\rho = 1.91$ was obtained. This is much higher than ρ in eq 1 and shows that effective catalysis by papain obviates the electronic effect of substituents. Poorer intrinsic and hydrophobic binding by actinidin forces more of the burden to fall on the electronic effect of the substituents.

Graphics Model

In the color stereo view I of the 3,4,5-Cl_3 congener, the surface placed on the enzyme (calculated by using the program MS, written by Michael Connolly) is coded so that red dots represent hydrophobic surface (carbon) and blue dots represent hydrophilic areas (oxygen and nitrogen). The essential hydrolytic apparatus (Cys-25, His-162) is colored green and the yellow dotted line indicates the path of attack by the SH moiety of Cys-25 on the carbonyl group of the ester part of I. Coordinates for the actinidin structure were obtained from the Protein Data Bank.[22] The rotation matrix and translation vector derived from a least-squares fit[23] of the α-carbon atoms of the papain active site (55 residues included) onto the actinidin active site (55 residues included) (0.735 Å root mean square error) were applied to the coordinates of I from the papain I model[1] to produce an approximate fit of I into the actinidin active site. The fit of I was then optimized slightly by using the interactive computer graphics modeling program MIDAS.[24]

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One of the major differences between actinidin and papain is that a Lys residue replaces Gln-142 of papain in actinidin. The QSAR and graphics analysis of papain suggest[1] that contact between Gln-142 and X_4 accounts for the presence of the MR_4 term in the QSAR. Molecular modeling of the actinidin–I complex shows that a hydrated $^+NH_3$ group of the lysine side chain is likely to contact X_4 of I. The apparent function of Lys-145, like that of Gln-142 in papain, is to buttress the ligand at the 4-substituent. Possibly the greater flexibility of the lysine side chain renders it less effective in binding the ligand as indicated by the lower coefficient of the MR term in eq 5 compared to that in eq 1 (0.24 vs. 0.61).

The coefficient of 0.50 with π in eq 5 suggests that meta substituents are not completely desolvated in binding to the enzyme. The situation is similar to that for congeners II interacting with papain. Therefore, in adjusting the position of the substituted phenyl ring, it has been placed so that a meta substituent makes contact with the red hydrophobic surface created by a tryptophan residue (outlined in yellow) in such a way that it is partially open to solvent. That is, we consider only the side of the substituent in contact with the enzyme surface to be desolvated. When one meta substituent is forced to contact the enzyme, the other must fall in aqueous space and hence receives no parameterization except that for σ in eq 5. There is a more pocketlike hydrophobic region to the left of where X_3 contacts the surface. This pocket corresponds closely to one in papain where we believe the X_3 binds with a slope of 1.0π.[1] For some reason that is not apparent, we believe that X_3 does not bind in this region of actinidin.

Another interesting difference between the two enzymes is that in actinidin Met-211 projects into the hydrophobic pocket into which the unsubstituted phenyl ring of I binds. This reduces the size of the hydrophobic cleft compared to papain (compare view I with view II of Smith et al.).[1]

Two data points 4-SO_2NH_2 and 3,5-$(OCH_3)_2$ were omitted in deriving eq 3–5. If these two points are included, essentially the same final equation is obtained but with a poorer fit ($r = 0.883$, $s = 0.158$). The great similarity between papain and actinidin is no doubt connected with the fact that these same two congeners are poorly fit by eq 1 and were not used in deriving it. The lower activity of actinidin and the relatively low solubility of congeners I restricted our inquiry. We had hoped to explore much more hydrophobic groups in position 3 and bulkier groups in position 4. The following congeners proved to be impossible to test because of their small $\Delta\epsilon$ and/or their poor solubility: 4-I, 4-$C(CH_3)_3$, 3-C_6H_5, 3-$OCH_2C_6H_5$, 3-OCH_2-2′-naphthyl. These results are particularly unfortunate with respect to X_4. The largest MR_4 is for 4-$COCH_3$; hence the coefficient of the weakest term in eq 6, MR_4, is the least firmly established.

Equation 5 has been developed by using largely 3- and 4-monosubstituted I. Nevertheless it predicts well the log $1/K_m$ for four disubstituted and one trisubstituted phenyl hippurate. The 3,4,5-Cl_3 derivative is the most active congener and its activity is very well predicted by eq 5 as is the activity of the least active congener 4-NH_2.

In order to more thoroughly explore substituent space into which the substituents falls, we shall have to devise a *much* more hydrophilic moiety to replace the phenyl of the benzamide unit. But since this group binds in a very hydrophobic cleft, modification will have to be made with some care. Our previous graphics study of papain made clear that a hydrophilic meta substituent on the amide phenyl group could remain free of the enzyme and in the aqueous phase. This would allow the phenyl group its usual hydrophobic contact and thus produce congeners of high intrinsic binding capacity. It is toward this objective that we are now working.

(24) Huang, C.; Ferrin, T. E.; Gallo, L.; Langridge, R. Molecular Graphics Laboratory, School of Pharmacy, University of California, San Francisco.

Table II. Analytical and Physical Properties of $XC_6H_4OCOCH_2NHCOC_6H_5$ and Spectral Data for Their Actinidin-Catalyzed Hydrolysis[a]

X	mp, °C	cryst solvent	λ,[b] mm	Δε,[c] L M^{-1} cm^{-1}
3-Br	143–144	acetone	274	1613
3-$C(CH_3)_3$	109–110	methanol/water	269.5	1358
3-$OCH_2C_6H_5$	134–135	methanol	279	*d*
3-OCH_2-2′-naphthyl	155–156	chloroform	293	2333
4-I	181–182	benzene	281	637
4-$C(CH_3)_3$	154–155	benzene	272	1132
3,4,5-Cl_3	186–187	chloroform	293	1476

[a] At pH 6.0, in 0.1 M sodium phosphate aqueous solution at 25.0 °C. [b] Optimum wavelength for spectrometric study of ester hydrolysis; when two absorption maxima were present, the one having the longer wavelength was chosen. [c] Change in molar absorptivity on complete hydrolysis at optimum wavelength (average of two runs). [d] Not reported because the value was too small to be accurately measured.

Conclusions

The QSAR model brings out the fact that actinidin acts on the phenyl hippurates in much the same way that papain acts on (methylsulfonyl)glycinates[21] and not the way papain acts on phenyl hippurates.[1] This difference in mechanism of action can be rationalized via molecular graphics. However, the reason for the two types of hydrophobic interaction are as yet unclear.

Comparison of the graphics and mathematical models provide us two independent means for the evaluation of the π and MR parameters used in structure–activity studies. We have assumed from correlation equations that when π is the significant parameter interaction is occurring in macromolecular hydrophobic space, but when MR is operative then interaction occurs in nonhydrophobic space (i.e., polar space). It is satisfying that the independent graphic conformation of these assumptions is observed. This provides confidence for the use of these two parameters in exploring via QSAR the interactions of drugs with receptors whose structures are unknown.

Studies on isolated enzymes of known structure followed by studies on enzymes in living cells[25,26] should better prepare us for drug design starting with isolated receptors.

When X-ray crystallographic coordinates are available, the comparison of QSAR and molecular graphics built models is a powerful means for developing our understanding of ligand–macromolecular interactions.

Experimental Section

The syntheses of most of the substituted-phenyl hippurates used in this study have been previously reported.[1] The new analogues have been prepared in the same fashion with the appropriate phenol and hippuric acid as the starting material. Their physical and analytical properties are listed in Table II.

3-(Benzyloxy)phenol and 3-(2-naphthalenylmethoxy)phenol were prepared as previously described.[21]

Melting points (Büchi capillary apparatus) are uncorrected. Microanalyses were performed by C. F. Geiger of Ontario, CA.

IR spectra (Nujol mull, Perkin-Elmer 297 spectrophotometer) and ^1H NMR spectra ($CDCl_3$, 1% Me_4Si, Varian FT-80 spec-

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trophotometer) were fully consistent with the chemical structures.

Isolation and Purification of Actinidin. Actinidin was prepared by the method of Boland[16] with notes kindly supplied by Professor Baker of the Chemistry Department of Massey University in New Zealand. The pulp of 1 kg of fresh Kiwi fruit was mashed with 1 L of extraction medium (1 g/L of cysteine, 1 mM Na_2EDTA). The homogenate was centrifuged at 13000*g* for 30 min. All centrifugations were in a Sorvall RCS-B centrifuge at 0 °C. The precipitate was discarded and the enzyme was then precipitated by slowly bringing the supernatant to 50% saturation with respect to ammonium sulfate (313 g/L), followed by stirring at room temperature for 1 h. The suspension was then centrifuged at 13000*g* for 30 min and the precipitate was resuspended in 100 mL of buffer (1 mM Na_2EDTA, 10 mM sodium tetrathionate, 0.2 M sodium phosphate, pH 6.8). This was then dialyzed for 24 h against 2 × 5 L of the same solution, the dialysate was centrifuged at 28000*g* for 30 min, and the precipitate was discarded. The supernatant was introduced onto a DEAE-cellulose column (25 mm × 40 cm) which had previously been equilibrated with 0.25 M sodium phosphate buffer, pH 6.8, and the protein washed in. Actinidin was eluted at room temperature from the column using a gradient of 0.2–0.6 M sodium phosphate buffer at a flow rate of 1–1.5 mL/min. Fourteen-milliliter fractions were collected. The elution of the protein from the column was followed by checking the absorbance at 280 nm while activity was followed by using the standard assay conditions (see below). Fractions with specific activity greater than 15 were pooled, and actinidin was precipitated by the slow addition of ammonium sulfate to a saturation of 50% and collected after centrifugation of this solution at 13000*g* for 30 min. The precipitate was resuspended in 0.1 M, pH 6.0 sodium phosphate buffer to give a 1% solution and dialyzed against buffer containing 20% ammonium sulfate until crystallization appeared to be complete. The crystals were isolated by centrifugation at 28000*g* for 30 min and they were finely ground and suspended in 0.1 M sodium phosphate buffer, pH 6, which contained 20% ammonium sulfate.

Analysis of the actinidin preparation was made by polyacrylamide gel electrophoresis. Only one spot was detected.

Standard Actinidin Assay. The enzyme was assayed by measuring the rate of hydrolysis of $H_2N(CH_2)_4CH(NHCOOCH_2C_6H_5)COOC_6H_4$-4′-$NO_2$ to give an equilibrium mixture of *p*-nitrophenol and the *p*-nitrophenolate ion, which both have extinction coefficient of 5400 L mol^{-1} cm^{-1} at 348 nm. The enzyme solution (50 μL) and 50 μL of dithioerythritol (15 mg/mL of distilled water) were mixed to reduce the enzyme to its active form. Of this solution 50 μL was then mixed in a cuvette containing 2.85 mL of buffer (0.1 M phosphate, pH 6.0) and 0.1 mL of substrate was added (6 mg/5 mL of deionized distilled water). The hydrolysis at 25 °C was followed at 348 nm by using a Gilford 2400S UV spectrophotometer. It was assumed that the initial slope was equal to V_{max} and a value of k_{cat} = 29 s^{-1} (Boland[16]) was used to obtain $[E_0]$ via $V_{max} = k_{cat}[E_0]$. Total protein was determined on a Gilford spectrophotometer using absorbance at 280 nm and a value of $E_{1cm}^{0.1\%}$ = 2.12 L g^{-1} cm^{-1}.[13] Specific activity (μmol of substrate transformed min^{-1} cm^{-1} mg of $protein^{-1}$) was used as a measure of actinidin purity. The specific acitivity of our preparation was found to be 64.

Enzymic Assay. The optimum wavelength (λ) for use in the spectrophotometric determination of the hydrolysis rates and the change in molar absorptivity ($\Delta\epsilon$) that results from complete hydrolysis of the esters at 25 °C in the aqueous buffer solution (0.1 M sodium phosphate) have been summarized in Table II for the new substrates. Values not in Table II have been reported previously.[1] For the kinetic measurements the stock suspension of actinidin was first diluted (1:3) with 0.1 M sodium phosphate buffer pH 6.0 and then activated by adding an equal volume of an aqueous solution of DTE (~15 mg/mL). The activated enzyme solution was further diluted with the same buffer (0.5 ml/28.5 mL) and used for the kinetic work. The initial rate of enzyme hydrolysis of a substrate was determined by mixing 0.10 mL of a CH_3CN solution of known concentration of hippurate with 2.90 mL of enzyme–buffer solution already equilibrated at 25 °C in the cell compartment of the spectrophotometer. Initial rates for at least eight different concentrations were measured for each substrate. K_m and V_{max} values were obtained by applying the method of least squares to a Lineweaver–Burk plot. V_{max} was essentially constant for the compounds in Table I. For 25 of the 29 congeners the mean and standard deviations for V_{max} are 3.96 (±0.90) × 10^{-5} mol L^{-1} min^{-1}. Three congeners [4-SO_2NH_2, 3,5-$(OCH_3)_2$, 3.5-Cl_2] have significantly greater values (~7 × 10^{-5}), and 4-$CONH_2$ has a value of ~2 × 10^{-5}.

Acknowledgment. This research was supported by NIH Grant GM-30362 and by a fellowship for J. M. Blaney from the American Foundation for Pharmaceutical Education. We thank Professor Sharon Panasenko, Pomona College, for helpful advice in the isolation of the actinidin. We are indebted to Professor Robert Langridge of the University of California at San Francisco for the use of the Molecular Graphics Laboratory for our modeling experiments.

Registry No. 1, 2979-54-6; **2**, 81592-10-1; **3**, 57461-26-4; 4, 91631-74-2; **5**, 81592-17-8; **6**, 66895-70-3; **7**, 91631-75-3; 8, 81592-20-3; **9**, 81592-12-3; **10**, 2979-53-5; **11**, 81592-09-8; **12**, 81592-11-2; **13**, 81592-14-5; 14, 29736-22-9; **15**, 2979-52-4; **16**, 29736-20-7; **17**, 29736-18-3; **18**, 81592-05-4; **19**, 3101-11-9; **20**, 29736-21-8; **21**, 30022-13-0; **22**, 81592-07-6; **23**, 81592-06-5; **24**, 81592-15-6; **25**, 81592-19-0; **26**, 81592-16-7; **27**, 81592-18-9; **28**, 81592-13-4; **29**, 91631-76-4; actinidin, 39279-27-1; papain, 9001-73-4.