# Secondary Enzyme–Substrate Interactions: Kinetic Evidence for Ionic Interactions between Substrate Side Chains and the Pepsin Active Site†

Jan Pohl and Ben M. Dunn*

*Department of Biochemistry and Molecular Biology, College of Medicine, University of Florida, Gainesville, Florida 32610*

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ABSTRACT: The possibility that pig pepsin has a cation binding specificity in its secondary binding subsites has been examined by the pepsin-catalyzed hydrolysis of a series of synthetic octa- to undecapeptide substrates. These chromophoric substrates are cleaved by pepsin in the phenylalanyl–*p*-nitrophenylalanyl (Phe–Nph) bond. Lys and Arg residues were placed into seven different positions in the substrates, and their effect on $k_{cat}$ and $K_m$ was examined between pH 2.8 and pH 5.8 ($I$ = 0.1 M, 37 °C). Kinetic evidence indicates the existence in the enzyme binding subsites S4, S3, S2, S3′, S4′, and S5′ of a group(s) which become(s) negatively charged at higher pH. For most substrates, the magnitude as well as the pH dependence of $k_{cat}$ was unaffected by the presence of Lys or Arg in these peptides. In constrast, changes up to 5 orders of magnitude were observed for $K_m$, depending on the number of basic residues and on their positions in the sequence. $K_m$ for a group of substrates at pH >5.5 was lower than 50 nM. Values for $k_{cat}/K_m$ for some substrates exceed the level of $10^8$ $M^{-1}$ $s^{-1}$. Therefore, the free energy derived from ionic interactions in secondary binding sites influences mostly the binding step on the reaction pathway. This result is in contrast to the previous observations that the length and the hydrophobic character of the substrate residues in some positions influence $k_{cat}$ with little effect on $K_m$ toward shorter substrates of pepsin [Fruton, J. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol. 44*, 1–36].

The preference of vertebrate pepsins for the cleavage of peptide bonds formed by hydrophobic and aromatic residues has long been recognized. From studies of Fruton and colleagues (Fruton, 1970, 1971), it became known that the catalytic activity of pepsin and of related aspartic endopeptidases (Blum et al., 1985) is greatly dependent on the length of the peptide and on the nature of the amino acid residues relatively remote from the scissile peptide bond. The pepsin active site can accommodate at least an octapeptide substrate in its productive mode. The term "secondary specificity" was used for these effects (Fruton, 1970; Sachdev & Fruton, 1969). Prolongation of a pepsin dipeptide substrate on both sides of the scissile peptide bond by *neutral* residues (e.g., by Ala) improved $k_{cat}$[1] by several orders of magnitude, without a comparable large effect on $K_m$ [Sachdev & Fruton, 1975; Medzihradszky et al., 1970; see also Blum et al. (1985)]. Pepsin preference for hydrophobic residues in P2′ and P3′ [see Schechter and Berger (1967) for nomenclature] was also manifested largely by the improvement of $k_{cat}$ with little change in $K_m$. For many pepsin substrates, $K_m$ was shown to approximate $K_s$, the substrate binding constant (Clement, 1973; Fruton, 1970; Sachdev & Fruton, 1975).

In this paper, the effects of *basic* residues which are placed into several positions in oligopeptide substrates on the kinetic parameters of peptide cleavage by pig pepsin are examined. The experimental rationale for this was to examine the possibility that some pepsin carboxylates [the enzyme is very acidic and contains 13 Glu and 30 Asp residues in its sequence (Tang et al., 1973)] may be present in the individual enzyme binding subsites and form ion pairs with substrate basic residues. Such interactions should influence the kinetics of cleavage and should be pH dependent between pH 2 and 6, where carboxylates are expected to undergo dissociation. Previous studies (Dunn et al., 1987) have provided kinetic evidence for hydrogen-bonding interactions between substrate side chains and the S3 and S2 subsites of the active site by comparing pH effects on catalysis by porcine pepsin, calf chymosin, and *Endothia parasitica* proteinase. In this study, we have determined the pH dependence for $k_{cat}$ and $K_m$ for a more extensive variety of substrates and porcine pepsin.

This study was partly conducted in an attempt to identify the binding mode of propart peptides to pepsin. These peptides are released from pig pepsinogen during its activation to pepsin, form complexes with pepsin above pH 4.5, and are rich in Lys and Arg as well as hydrophobic amino acid residues (Dunn et al., 1978).

Table I: Pepsin Substrates Used in This Work

| substrate | sequence |
|---|---|
| 1 | Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu |
| 2 | Lys-Pro-Leu-Glu-Phe-Nph-Arg-Leu |
| 3 | Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu |
| 4 | Lys-Pro-Lys-Glu-Phe-Nph-Arg-Leu |
| 5 | Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu |
| 6 | Lys-Lys-Ala-Lys-Phe-Nph-Arg-Leu |
| 7 | Lys-Pro-Lys-Lys-Phe-Nph-Arg-Leu |
| 8 | Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu-Arg-$NH_2$ |
| 9 | Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu-Arg-Lys |
| 10 | Lys-Pro-Ala-Lys-Phe-Nph-Arg-Leu-Arg-Lys-Lys-$NH_2$ |
| 11 | Lys-Pro-Ala-Lys-Phe-Nph-Arg-Lys-Arg-$NH_2$ |

## Materials and Methods

*Pepsin.* Crystalline porcine pepsin was purchased from Sigma, St. Louis, MO (lot 64F8080), and was used without further purification. Pepsin was dissolved in distilled/deionized water (1 mg/mL) at 0 °C and centrifuged, and the supernatant was stored in aliquots frozen at −20 °C and was stable for months. The working pepsin solution was prepared fresh each day, by diluting pepsin solution with ice-cold water, and was stable at 0 °C until used. The protein concentration of pepsin in solution was calculated from its absorption spectrum

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[1] For the definition of $k_{cat}$ and $K_m$, see Fruton (1976).

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($\epsilon_{278}$ = 51 000 $M^{-1}$ $cm^{-1}$). The active-site concentration of pepsin (90–95% active) was determined by the titration of 100 nM pepsin with a standardized solution of isovalerylpepstatin using Lys-Pro-Ala-Glu-Phe-Nph-Arg-Leu[2] (**1**) (see Table I) as substrate at pH 3.5, 37 °C.

*Buffers.* The buffers used were as follows: (a) pH 2.9–5.8, sodium acetate, 0.1 M in acetate, 0.1 mM in EDTA, ionic strength adjusted to 0.1 M with NaCl; (b) pH 2.4–4.5, sodium formate, 0.1 M in formate, 0.1 mM in EDTA, ionic strength adjusted to 0.1 M with NaCl. The pH of buffers was measured by using a glass electrode at 25 °C and was not corrected for its temperature dependence. Immediately before kinetic measurements, all buffers were deaerated at 37 °C and ultrafiltrated using a 0.45-μm Waters solvent clarification kit (Waters, Milford, MA).

*Peptide Synthesis and Purification.* All peptide syntheses were performed on an Applied Biosystems 430A peptide synthesizer using the solid-phase methodology and Boc-protected L-amino acids (Applied Biosystems, Foster City, CA, or Bachem, Torrance, CA). Boc-Arg(Tos), Boc-Glu(Bzl), and Boc-Lys(Cl-Z) were used for the incorporation of Arg, Glu, and Lys, respectively. Peptides with a free C-terminal carboxyl group were synthesized on Pam-polystyrene resins (Applied Biosystems). Peptides with a C-terminal amide group were synthesized on *p*-Me-BHA-polystyrene resins (Applied Biosystems). Solvents used for syntheses were from Applied Biosystems or from American Burdick & Jackson (Muskegon, MI) and were used without further purification. The manufacturer's standard synthetic program (Applied Biosystems, Inc., Foster City, CA) using single couplings of preformed symmetric anhydrides of Boc-amino acids was used without modifications. Arg and Asn were double-coupled via the hydroxybenzotriazole esters utilizing the manufacturer's standard protocol. Lys and Nph residues were incorporated by a double-coupling procedure using the manufacturer's standard recoupling protocol. Boc-Nph was incorporated by using the protocol specified for Boc-Phe. After final Boc-group deprotection and neutralization, the peptides were cleaved from the resin and deprotected in liquid HF/anisole (9:1, 0 °C, 60 min). The resins were washed with ethyl acetate/diethyl ether (1:1, 20 mL/1 g of resin), and the peptides were extracted into 1 M acetic acid and lyophilized. The peptides were purified by ion-exchange chromatography on a column (1.5 × 60 cm) of CM-Sepharose (fast flow, Pharmacia, Uppsala, Sweden) in a gradient of ammonium acetate, pH 4.5. Depending on the peptide charge, up to 2 M buffer was used to elute the peptides. After lyophilization, if necessary, the peptides were purified to final homogeneity by reverse-phase HPLC on C18 Radial Pack cartridges (Waters), using a linear gradient of methanol or 2-propanol in 0.05 M ammonium acetate, pH 4.5. The amino acid composition of all peptides was confirmed by amino acid analysis of the hydrolysates of peptides (6 M HCl, 110 °C, 20 h) on a Beckman 6300 amino acid analyzer. The purity of peptides was also confirmed by amino acid sequencing on a 0.2–1.0-nmol scale on an Applied Biosystems Model 470A gas-phase sequencer. PTH-amino acids were identified by HPLC on a Waters Model 6000A instrument with a WISP 710B automatic sample injector, a 730 data module, and a 721 controller. The mobile phase was a mixture of solvent A (5% aqueous tetrahydrofuran/0.1 M sodium acetate, pH 4.1) and solvent B (acetonitrile). An octadecyl silica column (Brownlee) was developed at 55 °C with the following gradient at a flow rate of 1 mL/min: 8–19% B, 2 min; 19–51% B, 14 min; 51% B isocratic, 6 min. Detection was at 254 nm with a Waters 440 detector.

*Kinetic Measurements.* Peptide stock solutions (5–20 mg/mL) were in water, 0.1 mM in EDTA, ultrafiltered through a 0.45-μm filter, and were stored at −20 °C. The concentration of peptides was determined by amino acid analysis using norleucine as an internal standard. Initial rates of peptide cleavage were measured in a Varian Cary Model 210 or 2200 spectrophotometer with a thermostated cell compartment. The hydrolyses were followed in either 1-cm or 10-cm (for substrates with $K_m$ < 5 μM) light-path quartz cells thermostated at 37 °C with the use of a Haake E52 thermostated water bath. When measurements were done in a 10-cm cell, the reaction mixture (10 mL) was preincubated and triggered outside the cell compartment and was rapidly transferred to the jacketed cell with the use of glass Pasteur pipet (the use of plastic pipet tips led to significant losses of enzyme activity). This was necessary to eliminate bubble formation in order to minimize subsequent disturbances in absorbance readings at scales 0.05–0.01. Pepsin concentrations used in these experiments were lower (10–300 pM) to avoid significant hydrolysis of substrate during sample transfer and temperature reequilibration. The kinetic parameters ($k_{cat}$ and $K_m$) determined for some of the substrates both in 1-cm and in 10-cm cuvettes were found to be the same within experimental error (±10% in 1-cm cuvette, ±20% in 20-cm cuvette) of our measurements. For substrates with $K_m$ < 1 mM, the rates of hydrolysis were recorded at 310 nm as described (Dunn et al., 1984). For substrates with $K_m$ > 1 mM, the rates were measured at 330 nm in order to decrease the background substrate absorbance. The magnitude of the differential extinction coefficient, $\delta\epsilon$, was determined for every combination of substrate, pH, and wavelength, and these values were used to calculate the reaction rates and the $k_{cat}$ values. Above pH 5.2, $K_m$ for substrate **10** dropped below 50 nM and was difficult to measure by spectrophotometry (see Results). At these pH values, we used **10** as an alternate substrate to **1**, and its inhibition of substrate **1** hydrolysis was measured. In principle, the assays were conducted at a fixed concentration of **1** in the presence of various concentrations of **10** which were so low that the absorbance changes due to hydrolysis of **10** were negligible as compared to the changes from hydrolysis of **1**. The magnitudes of the initial rates were used to determine the $I_{50}$ values for inhibition by **10**. The $K_i$ values for **10** were calculated by using the formula for competitive inhibition: $K_i = I_{50}/(1 + [S]/K_m)$, where [S] and $K_m$ are the concentration of **1** and its Michaelis–Menten constant, respectively, and $I_{50}$ is a concentration of **10** at which 50% inhibition of the reaction rate was observed. For all substrates, $K_m$ and $k_{cat}$ values were determined by using double-reciprocal plots of 1/initial velocity vs 1/[substrate]. The correlation coefficients of those plots were 0.98 or higher.

*Product Analysis.* The substrates (50–100 μM) were hydrolyzed by pepsin (5–100 nM) at 37 °C in a cuvette, and the decrease of absorbance at 310 nm was recorded. At the end of hydrolysis, the products were isolated by reverse-phase HPLC on C18 Radial Pack cartridges in a linear gradient of either methanol or acetonitrile in 0.05 M ammonium acetate, pH 4.5. A Model 2140 Rapid Spectral Detector (LKB,

[2] Abbreviations: Ac, acetyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Cl-Z, 2-chlorobenzyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid disodium salt; HPLC, high-performance liquid chromatography; [LySta], (4*S*,3*S*)-4,8-diamino-3-hydroxyoctanoic acid; Nph, *p*-nitrophenylalanine; OEt, ethyl ester; Pam, (phenylacetamido)methyl; *p*-Me-BHA, *p*-methylbenzhydrylamine; [Sta], (4*S*,3*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; Tos, *p*-toluenesulfonyl; Z, benzyloxycarbonyl; CM, carboxymethyl; PTH, phenylthiohydantoin; Iva, isovaleryl; all amino acids used in this work have the L configuration.

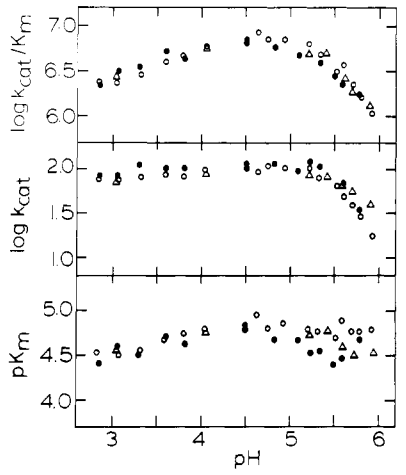


FIGURE 1: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for hydrolysis of **1**, **2**, and **3**. Assays were performed in sodium acetate/NaCl buffer ($I$ = 0.1 M), at pepsin concentrations of 0.5–1.5 nM at 37 °C: (○) **1**; (●) **2**; (△) **3**. The units in which the kinetic parameters are expressed in this and in the following figures are $k_{cat}$ in $s^{-1}$, $k_{cat}/K_m$ in $M^{-1}$ $s^{-1}$, and $K_m$ in M.

Bromma, Sweden) was used to monitor the column eluate. The wavelength of the absorbance maximum changes from 278 nm for the substrate to 272 nm for the product Nph–Arg–Leu or variants of this sequence. The products were identified by amino acid analysis. The product analysis was performed for every substrate at several pH values between 2.5 to 5.9.

*pH Dependence Studies.* The kinetic measurements were limited to the pH interval 2.5–5.8. Above pH 6, pig pepsin spontaneously denatures (Edelhoch, 1958), and, therefore, the analysis could not be completed on the alkaline limb of the pH profiles for some substrates. Below pH 2.8, for most substrates, $K_m$ values were 1 mM or above. Collection of sufficient data to define $K_m$ under those conditions was prohibitively expensive in terms of synthetic peptide substrates.

## RESULTS

The peptide substrates used in this work are listed by sequence in Table I. With the exception of **4**, only the Phe–Nph bond was cleaved by pepsin in all peptides at pH 2.5–6.0. The kinetics of cleavage of substrates strictly followed the Michaelis–Menten scheme over the pH range of 2.5–5.8.

*pH Dependence of $k_{cat}$ and $K_m$.* Substrates of the type Lys-Pro-Xxx-Glu-Phe-Nph-Arg-Leu where Xxx is Ala, Leu, and Ile (**1–3**) were used as the reference substrates for the evaluation of the effect of replacement of amino acid residues in different positions by Lys or Arg on the shape of the pH dependence of $k_{cat}$ and $K_m$. It is seen in Figure 1 (substrates **1–3**) that above pH 4, $k_{cat}$ and $k_{cat}/K_m$ are controlled by dissociation of a group with p$K_a$ 5–5.5 and that $K_m$ is pH independent. The same profile for $k_{cat}$ has been found for most of the substrates in Table I (see below). The value of $k_{cat}/K_m$ decreased and $K_m$ increased 2–3-fold between pH 3 and 4 as an enzyme group (p$K_a$ 4, free enzyme) is protonated. These effects are, however, minimal compared to changes seen upon replacement and addition of Lys and Arg into the Lys-Pro-Xxx-Glu-Phe-Nph-Arg-Leu sequence.

*Lys → Xxx Replacement.* Substrate **4** was designed to evaluate the effect of substitution of a neutral residue in P3 by Lys. However, unlike other substrates listed in Table I, **4** is split by pepsin in *two* peptide bonds, i.e., between Phe–Nph (an expected cleavage site) and between the Glu–Phe bond (the new cleavage site). Quantitative HPLC analysis of peptic cleavage products of **4** shows (Table II) that about 50% of

Table II: Quantitative HPLC Analysis of Products of Peptic Hydrolysis of **4** at Different pH Values

| | product (nmol) (% of total) | |
|---|---|---|
| pH | Nph-Arg-Leu | Phe-Nph-Arg-Leu |
| 2.0 | 152 (90.3) | 17 (9.7) |
| 2.4 | 88 (87.9) | 12 (12.1) |
| 3.1 | 131 (78.2) | 37 (21.8) |
| 4.0 | 102 (60.9) | 66 (39.1) |
| 5.0 | 81 (47.6) | 89 (52.4) |
| 5.6 | 47 (47.5) | 52 (52.5) |
| 5.8 | 44 (48.4) | 47 (51.6) |

[a] Peptide **4** (100–200 μM) was hydrolyzed with pepsin (15 nM) at 25 °C, $I$ = 0.1 M. The reaction was followed to completion (4–8 h) by analytical HPLC of 10-μL aliquots of the soultion. Aliquots of 50–200 μL of the reaction mixture corresponding to 5–10 nmol of original **4** were withdrawn, and the split products were separated by HPLC (see Materials and Methods), collected, and quantitated by amino acid analysis. The values reported are the amounts present in the total sample.

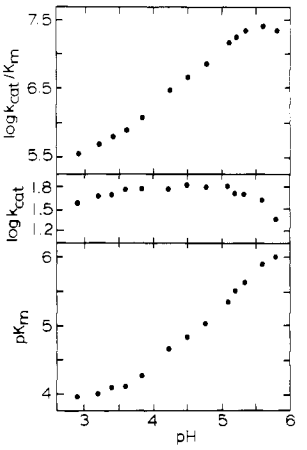


FIGURE 2: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for pepsin hydrolysis of **5**. The conditions were the same as in the legend for Figure 1.

substrate is cleaved through an alternate pathway at pH >4.5, whereas at lower pH the expected cleavage pathway predominates. This result indicated a strong preference of pepsin for Lys bound in subsite S2. In order to obtain cleanly quantitative data for the kinetics of substrate cleavage, Glu (P2) had to be replaced by Lys in **1** in the rest of the peptide substrates synthesized to give substrates that generally bind very well to the pepsin active site.

*Lys in P2.* This substrate (**5**) is cleaved exclusively at the Phe–Nph bond at all pH values examined. The replacement of Glu by Lys in **5** has no significant effect on the log $k_{cat}$ vs pH profile (compare Figures 1 and 2). However, a significant change in the pH dependence of $k_{cat}/K_m$ and $K_m$ is seen. The magnitude of both parameters is controlled by the dissociation of a group with p$K_a$ > 5.8 (free enzyme), and consequently, the slopes of the Dixon plots were changed from zero to +1. Since the $K_m$ values for **5** dropped to low micromolar levels at higher pH, Lys was kept in P2 in other substrates in order to secure specific binding and unambiguous cleavage by pepsin.

*Lys in P4.* The replacement of Pro by Lys (**6**) did not influence the log $k_{cat}$ vs pH profile significantly nor did it have any significant effect on the magnitude of $k_{cat}$ (Figure 3). It resulted, however, in a change of the log ($k_{cat}/K_m$) vs pH and p$K_m$ vs pH profiles. Compared to **5**, the maximum slopes of both parameters were increased by +0.8, to give slopes of +1.8 in each case.

*Lys in P3.* The quantitative evaluation of the effect of Lys in P3 was possible with **7**. **7** is a poor substrate of pepsin at all pH values. It is seen in Figure 4 that the slope of p$K_m$ vs

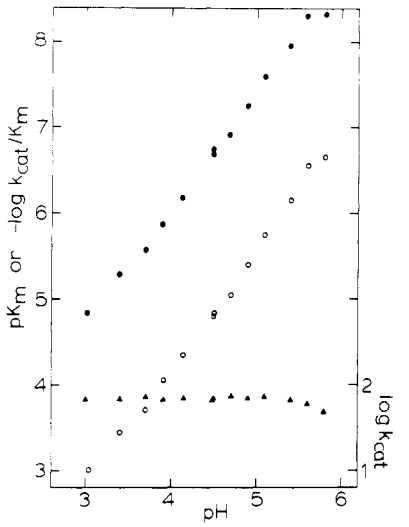


FIGURE 3: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for hydrolysis of **6** by pepsin: (●) log ($k_{cat}/K_m$); (▲) log $k_{cat}$; (○) p$K_m$. The conditions were the same as in the legend for Figure 1.

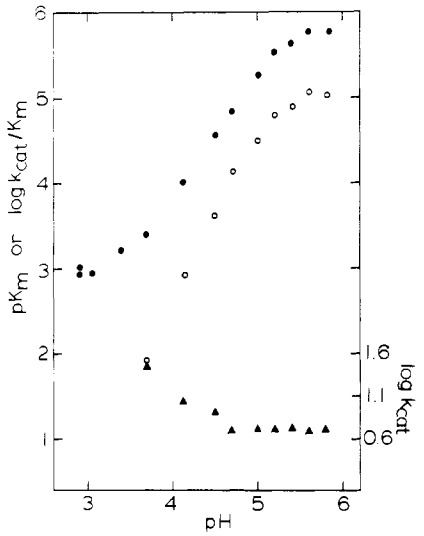


FIGURE 4: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for hydrolysis of **7** by pepsin: (●) log ($k_{cat}/K_m$); (▲) log $k_{cat}$; (○) p$K_m$. The conditions were the same as in the legend for Figure 1.

pH was increased by +1 relative to substrate **5**, however, the absolute magnitude of $K_m$ was 1–2 orders of magnitude higher than for **5**. For these reasons, the separate determination of $k_{cat}$ and $K_m$ for **7** was not technically possible below pH 3.5 due to high background substrate absorbance at 325 nm, and only $k_{cat}/K_m$ values were determined below pH 3.5. Unlike other substrates studied in this work, the pH dependence of $k_{cat}$ shows a unique profile. Its magnitude is lowered by dissociation of a group with p$K_a$ 4.5 (enzyme–substrate complex), and $k_{cat}$ reaches a plateau level 10–20 times lower in magnitude than $k_{cat}$ for other substrates in this pH region. The change in the slope from 0 to −1 around pH 5 seen for $k_{cat}$ with other substrates was not detected for **7** up to pH 5.8.

*Arg in P4′.* In **8**, an extra Arg residue was added into P4′. It is seen in Figure 5 that the log $k_{cat}$ vs pH profile was not changed (slopes 0 and −1). The value of p$K_a$ of the break was shifted by approximately 1 pH unit toward lower pH. However, the absolute magnitude of $k_{cat}$ in the plateau region was not influenced significantly. It is also apparent from Figure 5 that the log ($k_{cat}/K_m$) vs pH and p$K_m$ vs pH profiles were changed. The maximum slopes of the Dixon plot were increased by approximately +0.8 unit when compared with **5**, to again give slopes of +1.8.

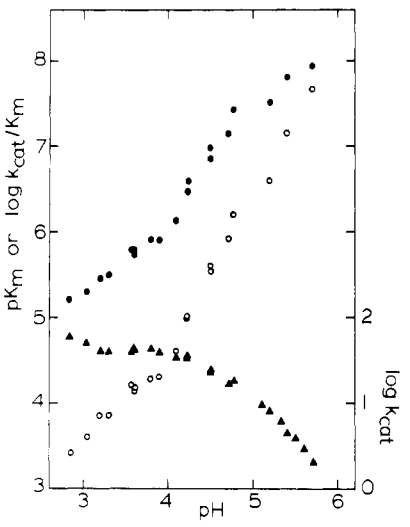


FIGURE 5: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for hydrolysis of **8** by pepsin: (●) log ($k_{cat}/K_m$); (▲) log $k_{cat}$; (○) p$K_m$. The conditions were the same as in the legend for Figure 1.

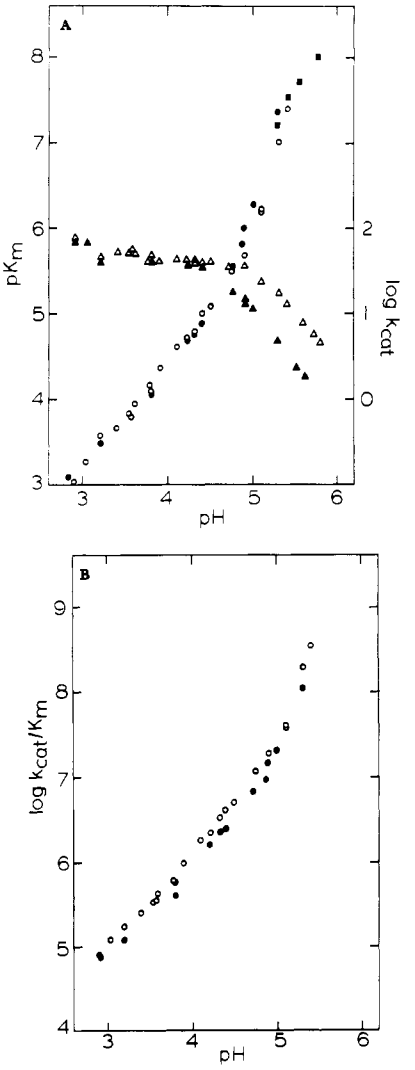


FIGURE 6: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for hydrolysis of **9** and **10** by pepsin. (A) log $k_{cat}$ vs pH: (△) **9**; (▲) **10**. p$K_m$ vs pH: (○) **9**; (●) **10**; the $K_i$ values for **10** (■) were determined at a single concentration of substrates **4** and **3** close to saturation (5–10 mM). (B) log ($k_{cat}/K_m$) vs pH: (○) **9**; (●) **10**. The conditions were the same as in the legend for Figure 1.

*Lys in P5′ and P6′.* Substrate **8** was further prolonged by one and two lysines on its C-terminus (substrates **9** and **10**).

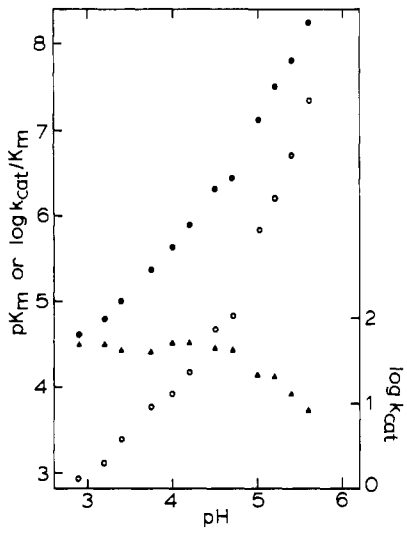


FIGURE 7: pH dependence of log ($k_{cat}/K_m$), log $k_{cat}$, and p$K_m$ for pepsin hydrolysis of **11**: (●) log ($k_{cat}/K_m$); (▲) log $k_{cat}$; (○) p$K_m$. The conditions were the same as in the legend for Figure 1.

It is seen in Figure 6A that the prolongation did not influence significantly the log $k_{cat}$ vs pH profile for both substrates, except for the difference in several tenths of a pH unit seen in the value of the break point of slopes 0 and −1. It is also apparent that the addition of the lysines did not change significantly the log ($k_{cat}/K_m$) vs pH and p$K_m$ vs pH profiles below pH 5 (Figure 6A,B) as compared to **8**. However, above this pH, a further change in slope of approximately +1 is seen. It is also evident that no significant difference was found in the profiles between substrates **9** and **10** in the pH interval between pH 2.8 and 5.6, indicating that Lys in P6′ provided no additional influence on the kinetic parameters at all. The analysis could not be done at pH up to 5.8 as $K_m$ rapidly dropped below the 0.05 μM level and the changes in absorbance at 310 nm were too low to measure in 10-cm light-path cell with accuracy. In a separate experiment, however, **10** was employed as an alternate substrate. Under the conditions used (Figure 6A), **10** acts as a competitive inhibitor of the hydrolysis of substrate **1**. At pH 5.3, the $K_i$ value (70 ± 20 nM) for **10** determined in the alternate substrate assay and the $K_m$ value (40 ± 20 nM) determined in the direct assay with **10** were found to be very close in magnitude. At pH 5.8, $K_i$ measured for **10** was as low as 10 nM. These values are plotted in Figure 6A, as the closed squares.

*Lys in P3′*. The effect of Lys in P3′ was determined by comparison of profiles for **11** (Figure 7) and **8** (Figure 5). This replacement has no significant effect on the absolute magnitude of $k_{cat}$ and on the log $k_{cat}$ vs pH profile. It is, however, seen that above pH 5, the maximum slope is changed for **11** by +0.8 as compared to **8** in the log ($k_{cat}/K_m$) vs pH and p$K_m$ vs pH profile. Above pH 5.6, the analysis was not finished for the same technical reasons as in the case of **9** and **10**.

*Comparison of Changes in $K_m$'s*. Table III summarizes the differences in p$K_m$ (measured at the boundary values of the pH interval) and in the slopes of the Dixon plot for p$K_m$ vs pH as the result of the presence of Lys or Arg in different positions. Due to the technical difficulties described above, the absolute magnitudes of the limiting values of $K_m$ could not be determined for some substrates. In an effort to estimate the change in binding induced by the addition of one or more basic residues, the values of $K_m$ at the extremes of the pH profiles accessible to experiment have been used. These values, therefore, must give a *minimum* estimate of the effects. Assuming that for these substrates $K_m = K_s$ (see Discussion),

Table III: Difference in Binding to Pepsin as a Consequence of the Addition of Lys or Arg at Various Positions in the Substrate

| Lys or Arg in position N | $pH_1$ | $pH_2$ | $pK^1_m - pK^2_m$ | $-\Delta G_s$[a] (kcal/mol) | max slope of plot of p$K_m$ vs pH |
|---|---|---|---|---|---|
| "control substrates"[b] | 2.8 | 5.8 | 0.2 | 0.3 | 0 |
| P2 | 2.8 | 5.8 | 2.0 | 2.8 | +1.0 |
| P4,P2 | 3.0 | 5.8 | 3.7 | 5.3 | +1.8 |
| P3,P2 | 3.7 | 5.8 | 3.2 | 4.5 | +2.0 |
| P2,P4′ | 2.9 | 5.7 | 3.6 | 5.1 | +1.8 |
| P2,P4′,P5′ | 2.7 | 5.4 | 4.4 | 6.2 | +3.0 |
| P2,P4′,P5′,P6′ | 2.9 | 5.3 | 4.4 | 6.2 | +3.0 |
| P2,P3′,P4′ | 2.9 | 5.6 | 4.5 | 6.4<br>2.4[c] | +3.0 |

[a] This estimate of the value of an additional basic residue of 2.4 kcal/mol, on average, is an underestimate since in several cases it is not possible to cover a large enough range of pH to observe the maximum change in binding. [b] Substrates **1**, **2**, and **3** of Table I. [c] Average $\Delta G_s$ per substrate basic residue.

the minimum average difference in the free energy of binding of a positively charged residue to pepsin, $\delta G_s$, is 2.4 kcal mol$^{-1}$ residue$^{-1}$.

## Discussion

There is only limited information on the effect of basic residues on catalysis by aspartic proteinases in general. It has been known for some time (Hofmann, 1974; Sodek & Hofmann, 1970) that microbial pepsins have the ability to hydrolyze -Lys-Xxx- bonds in some proteins, i.e., trypsin-like specificity. In the case of penicillopepsin, it has been concluded that this is due to an ionic interaction of the ε-amino group of the substrate Lys and the side-chain carboxylate of Asp-77 of the enzyme, as seen in the refined X-ray structure of the penicillopepsin/Iva-Val-Val-[LySta]-OEt complex (James & Sielecki, 1985). Iva-Val-Val-[LySta]-OEt was bound to penicillopepsin 20 times more tightly than the parent Statine-containing derivative (Salituro et al., 1987). Importantly, the $K_m$ values for the specific substrate, Ac-Ala-Ala-Lys-Nph-Ala-Ala-$NH_2$, and penicillopepsin (cleavage occurs between Lys–Nph) decreased by 2 orders of magnitude from pH 2 to 6 (Hofmann et al., 1984). For pig pepsin substrates cleaved in the Phe–Phe bond, a difference in the shape of pH dependence between pH 2.5 and 4.5 was noted for substrates Z-His-Phe-Phe-OEt and Z-Gly-His-Phe-Phe-OEt as compared to Z-His-Gly-Phe-Phe-OEt (Holland et al., 1969). The former substrates showed a decrease of severalfold in $K_m$, with little change in $k_{cat}$. For the latter one, only a slight decrease of $K_m$ with increasing pH was seen over this pH range.

For many pepsin substrates, evidence has been obtained that $K_m$ closely approximates $K_s$ (Clement, 1973; Inouye & Fruton, 1968; Raju et al., 1972; Sachdev & Fruton, 1975; Fruton, 1976; Dunn & Fink, 1983). This is assumed to be valid also for the substrates reported here, and it is supported by the fact that (i) for certain pH intervals and substrates, $K_m$ varied by several orders of magnitude without any measurable effect on $k_{cat}$; (ii) $K_m$ for **1**–**3** at $4 < pH < 5.5$ was constant, whereas $k_{cat}$ was controlled by dissociation of a group with p$K_a$ 5–5.5 (Martin, 1984; Dunn et al., 1987); (iii) for **10**, we find an agreement between $K_m$ and $K_i$ when **10** acts as an alternate substrate. It is, therefore, obvious that $k_{cat}$ does not measurably contribute to the magnitude of $K_m$ over the pH interval of 2.8–5.8 and, thus, $k_{cat} \ll k_{-1}$ where $k_{-1}$ refers to the rate constant of dissociation of the enzyme–substrate complex. For most of our substrates, the log ($k_{cat}/K_m$) vs pH effects seen as the result of the presence of a basic residue(s) originate in $K_m$ effects whereas $k_{cat}$–pH profiles were affected only slightly

or not at all. In several cases, $K_m$ dropped to the nanomolar level. The magnitude of $k_{+1}$ for our substrates must be close to the diffusion limit value as the values for $k_{cat}/K_m$, the second-order rate constant, for some substrates are on the order of $10^8$ $M^{-1}$ $s^{-1}$. In fact, the $k_{+1}$ values for pepsin and dansylated tetrapeptide substrates were found to be too high ($>10^6$ $M^{-1}$ $s^{-1}$) to be measured in a stopped-flow apparatus (Sachdev & Fruton, 1975). Therefore, the dissociation rate constant of the enzyme–substrate complex, $k_{-1}$, is the most likely pH-dependent step that controls the magnitude of $K_m$ for our substrates.

We assume that most of the effects seen on substrate affinity have their origin in the ionic interactions of substrate basic residues with side-chain carboxylates present in individual binding subsites. Pepsin is a very acidic protein, and most of its carboxyl groups would undergo dissociation in the pH range of our measurements. The finding that the maximal values of the slopes were reached at different pH values would indicate that there are significant differences in the group p$K_a$'s for these carboxyl groups in individual binding subsites. We consider it, therefore, less likely that the p$K_m$ vs pH effects would reflect differences in substrate affinity caused by gross conformational change of pepsin in the pH interval of interest. First, it has been shown that the general folding patterns of the core of pepsin (crystallized at pH 2; Andreeva et al., 1985) and of the pepsin part of pig pepsinogen (pepsinogen was crystallized at pH 6.5; James et al., 1986) are similar and differ mostly in the orientation of the N-terminal pepsin segment, which is reoriented during the zymogen activation. In addition, there are movements of some surface loops which are likely to contribute to specificity (see below). Also, the binding of neutral pepsin substrates as measured by $K_m$ and of their diastereomeric inhibitors (D-Phe-substituted peptides) as measured by $K_i$ is not influenced between pH 0.2 and 6 (Knowles et al., 1969). Lastly, $K_m$ for **1–3** showed little dependence on pH. Pig pepsin is irreversibly denaturated above pH 6; therefore, our analysis was limited up to pH 5.8 where it is fully stable.

An anomalous pH profile was obtained when binding subsite S3 was occupied by lysine in **4** and **7**. The fact that **4**, but not **1–3** is cleaved in two peptide linkages[3] indicates high preference of pig pepsin for Lys when this residue is bound in S2 as opposed to Ala, Leu, and Ile in the case of **1–3**. The interaction of Lys (P2) with enzyme overcame the otherwise very favorable (Fruton, 1970) binding of Phe-Nph dipeptide to the primary binding site. The quantitative data for **7** show that pepsin has a low affinity for a basic residue in S3 [see also Fruton (1970) and Dunn (1986)], and this certainly contributes to the secondary cleavage pathway of **4**. It is clear that this is due to the positive charge of Lys and not solely due to its bulky character, as almost no effect was seen on $K_m$ for **1–3** in which P3 residues are Ala, Leu, and Ile. Previously, kinetic evidence has been presented for the presence in the subsite S3 of a carboxyl group (suggested to be Glu-13) which can hydrogen bond to the P3 residue of a substrate if it is a potential hydrogen-bond donor (Dunn et al., 1987). In addition, the increase in slope seen when substrate **7** is compared to **5** also strongly indicates a favorable interaction in the S3 subsite. Despite this apparent improvement in binding due to the putative electrostatic interaction with the side chain of residue 13 of the enzyme, the maximal binding is weaker for substrates with Lys in the P3 position as compared to neutral residues. This phenomenon probably arises due to the partially hydrophobic character of the residues in subsite S3 and the fact that in the homologous proteinase endothiapepsin (Blundell et al., 1985; Foundling et al., 1987) the S3 site is more of a depression rather than a deep pocket. The extended Lys side chain with a strong charge at the end probably has difficulty fitting into that environment. Increasing the pH which causes Glu-13 to lose a proton and become negatively charged can improve the binding of substrates with Lys in P3, but they cannot overcome the unfavorable situation created by the length of the side chain in the first place.

Subsite S3 is the only one to which placing the Lys residue changes the log $k_{cat}$ vs pH profile. It is quite possible that formation of an ion pair of Lys (P3) with an enzyme group of p$K_a$ 4.5 in S3 (detected in the log $k_{cat}$ vs pH profile) substantially decreases pepsin catalyic efficiency. It has been noted earlier (Powers et al., 1977) that the presence of a basic residue in P3, e.g., of Lys in -Lys-Xxx-Yyy-Zzz-, lowers significantly the probability of hydrolysis of the Yyy–Zzz bond in protein substrates of pepsin. Obviously, the correct explanation for the low cleavage probability is that this is due to the poor binding of these sequences to pepsin and not to the poor catalysis ($k_{cat}$) at low pH as most of the experiments analyzed by Powers et al. were carried out near pH 2.

In the case of subsites S4′ and S5′, the control substrates possessing a neutral residue in P4′ and P5′ were not examined. We consider any large effect of a neutral residue on the pH profile as very improbable. As an argument in favor of this, we indicate that, for example, no difference in the p$K_m$ vs pH profile was seen with **9** and **10** as compared to **8** below pH 5.

If our interpretations of the p$K_m$ vs pH effects are valid, then pig pepsin is unique among endopeptidases in possessing the cation binding specificity in subsites S4, S3, S2, S3′, S4′, and S5′. This report of improved binding due to electrostatic interactions in S4′ and S5′ was not anticipated from previous studies of pepsin specificity (Antonov, 1977; Powers et al., 1977). These earlier correlations utilized data obtained from low-pH cleavage studies and so would not be sensitive to the effects reported here at pH >4.5. These effects are in direct contrast with the earlier results of Fruton and co-workers, who found that, in the case of pepsin, large improvements in $k_{cat}$ and not in $K_m$ can be achieved when the *length* of pepsin substrate is increased, e.g., from dipeptide to hexapeptide (Fruton, 1976), with neutral amino acid residues. We have eliminated the "length problem" by using peptides at least eight residues long. On the basis of these results and on the recent X-ray crystallographic study of complexes of aspartic proteinases with strong inhibitors (Foundling et al., 1987; Seguna, 1987b), it would appear that the separate effects on $k_{cat}$ and $K_m$ are derived from different types of interactions. The $k_{cat}$ effects likely arise from the additional hydrogen bonds formed by the extra peptide bonds of longer substrates with backbone and side-chain groups of the enzyme (Pearl, 1985; Blum et al., 1985). The $K_m$ effects seen here and earlier (Dunn et al., 1987) are likely due to the electrostatic interactions of substrate *side chains* with enzyme *side chains*. The observation of improved binding due to electrostatic interactions at subsites S4′ and S5′ is also relevant to previous suggestions (Cumin et al., 1987; Stammers et al., 1987) that part of the exquisite specificity of enzymes like renin is due to interactions between the enzyme and large protein substrate at sites very remote from the cleavage position. In addition, Visser et al. (1987)

[3] In our earlier experiments with **4** (Dunn et al., 1986), only the cleavage of the Phe–Nph bond by pepsin was reported. Under the conditions of those experiments, the secondary cleavage in the Glu–Phe bond does not significantly influence the kinetic parameters for the primary reaction pathway.

have discussed the role of electrostatic interactions between the related aspartic proteinase chymosin arising from His residues at positions 98, 100, and 102 of its natural substrate casein.

From a mechanistic point of view, the identity of the pepsin groups which participate in substrate binding remains an open question. The crystal structures of pig pepsin and pig pepsinogen, as well as of three microbial pepsins, have been solved at high resolution (Andreeva et al., 1984; Pearl & Blundell, 1984; Foundling et al., 1987; Suguna et al., 1987a; James & Sielecki, 1984). The binding subsites for their specific inhibitors have been also depicted (Foundling et al., 1987; Suguna et al., 1987b). On the basis of an examination of the binding of the homologous inhibitor Pro-Thr-Glu-Phe-$\psi$[$CH_2NH_2$]Phe-Arg-Glu to *Endothia parasitica* proteinase (Foundling et al., 1987), we can suggest that the loop involving residues 295–300 will be near enough to the P2 residue so that the Glu in position 297 of pig pepsin could interact with a Lys in that position of these substrates. There are several other loops that come near the P4 and P2 positions of the bound inhibitor and could serve as candidates for interactions of the electrostatic type detected in the current study. These include the loops containing Glu-244, Tyr-274, Asp-Asp-Asp (280–282), and Asp-290. Potential residues capable of providing electrostatic interaction on the "prime" side of the substrate, i.e., at P3′, P4′, and P5′, are more difficult to select since the binding of all inhibitors at that end of the active-site cleft is less well-defined (Foundling et al., 1987; Seguna et al., 1987b). Residue 297 will also be near the P3′ position and is the only candidate that can be identified on the basis of this model. However, the X-ray coordinates of pig pepsin and pepsinogen at high resolution are not available in the Brookhaven Protein Data Bank. Once the X-ray coordinates of pig pepsin are available, those data can be used as the basis for a site-directed mutagenesis/kinetic approach (Knowles, 1987; Craik et al., 1985; Cronin et al., 1987). As an example, the ion pairing between substrate lysine and two subtilisin side-chain carboxylates has been studied in greater detail (Wells et al., 1987). In their work, the electrostatic free energy of 1.8 kcal/mol in one case or 2.3 kcal/mol in the second case was ascribed to these ion pairs. Our data in Table III show that the free energy contribution of Lys or Arg residue to binding at the pepsin active site is a minimum of 2.4 kcal $mol^{-1}$ $charge^{-1}$ in the range expected for a solvated ion pair (Fersht, 1972).

While the preceding discussion has indicated considerable enthusiam for specific electrostatic interactions associated with the individual subsites of the active-site cleft, the data presented can also be interpreted to indicate a more general electrostatic attraction between the enzyme and positively charged substrates. Pig pepsin is well-known to be an extremely acidic protein, and increasing pH in the range studied here will generate more negative charge on the protein surface. It is possible that this general electrostatic attraction will increase the concentration of the substrate in the vicinity of the active-site cleft and will result in an apparent lowering of the observed $K_m$ value. However, the analysis of substrate **10** with an extra positive charge revealed no additional interaction when compared with substrate **9**. Furthermore, the fact that the control substrates **1–3**, which contain lysine in P5 and Arg in P2′, show a flat pH profile for $K_m$ over the same pH range suggests that a general electrostatic mechanism is only possible when the substrate contains a net charge of 3+ or more. Again, a definitive choice between specific interaction or general electrostatic attraction will be possible through double site-directed mutagenesis that maintains the overall net charge on the protein.

In summary, by placement of Lys and Arg into proper position, it is possible to prepare very sensitive substrates which far surpass in affinity and specificity the known peptide substrates for pepsin. According to the kinetic approach, the cationic binding specificity in six secondary binding subsites of pepsin was discovered. The data obtained in the study of subsite specificity are used in our attempt to identify the binding mode of propart peptide inhibitors of pepsin and may serve as the basis of construction of more powerful inhibitory compounds.

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# Kinetics of Acylglycerol Sequential Hydrolysis by Human Milk Bile Salt Activated Lipase and Effect of Taurocholate as Fatty Acid Acceptor†

Chi-Sun Wang,* Jean A. Hartsuck, and Deborah Downs

*Lipoprotein and Atherosclerosis Research Program and Protein Studies Program, Oklahoma Medical Research Foundation, and Department of Biochemistry and Molecular Biology, College of Medicine, University of Oklahoma, Oklahoma City, Oklahoma 73104*

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ABSTRACT: The simplest reaction scheme for the conversion of trioleoylglycerol to glycerol catalyzed by human milk bile salt activated lipase can be described by consecutive first-order reactions: triacylglycerol $\xrightarrow{k_1}$ diacylglycerol $\xrightarrow{k_2}$ monoacylglycerol $\xrightarrow{k_3}$ glycerol. In these equations, $k_1$, $k_2$, and $k_3$ represent the pseudo-first-order rate constants for the indicated reactions. The results from this study show that although the relative ratio of $k_2/k_1$ or $k_3/k_1$ may change somewhat, depending on the reaction conditions, the enzyme has a reactivity with the order of dioleoylglycerol > trioleoylglycerol > monooleoylglycerol. The incomplete equilibration of the intermediary diacylglycerol and monoacylglycerol with the bulk of the substrate during sequential lipolysis of triacylglycerol provides a means for their efficient lipolysis and minimizes the effect of partial acylglycerol as competitive substrates for intact triacylglycerol lipolysis. Taurocholate functions both as an activator of the enzyme and also as fatty acid acceptor to relieve product inhibition. In the presence of sufficient taurocholate, bovine serum albumin is no longer required as a fatty acid acceptor for the in vitro lipolysis.

Bile salt activated lipase is found in the milk of a limited number of mammals including humans (Hernell & Olivecrona, 1974; Wang, 1981; Olivecrona & Bergtsson, 1984) and a number of primates (Freudenberg, 1966), as well as in dogs and cats, as recently found by Freed et al. (1986). Previously, we have utilized the monoacid long-chain triacylglycerols as substrates of this enzyme to show that, in the absence of the activator, the enzyme can interact only with trioctanoylglycerols and shorter chain monoacid triacylglycerols. The presence of bile salt is required for the lipolysis of longer chain triacylglycerols (Wang & Lee, 1985). The initial interaction

† Address correspondence to this author at the Oklahoma Medical Research Foundation, 825 N.E. 13th St., Oklahoma City, OK 73104. This study was supported by NIH Grant HL-23181 and by the resources of the Oklahoma Medical Research Foundation.

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