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A KINETIC STUDY ON THE EFFECT OF COUPLING DISTANCE BETWEEN INSOLUBLE TRYPSIN AND ITS CARRIER MATRIX*

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SUMMARY

Trypsin (EC 3.4.4.4) was covalently attached by its carboxyl groups through peptide chains of various length to an insoluble polystyrene matrix. The peptide chains ranged from one to ten amino acid residues in length composed entirely of glycine residues, except for a terminal alanine bound to the polystyrene matrix in chains of two to ten residues. Using tosyl-L-arginine methyl ester as substrate, the observed catalytic rate, k_{cat}, is independent of the chain length or column flow rate. However, the apparent Michaelis constant is dependent upon both parameters. These results suggest that the contributions from "unreactive" molecules and electrostatic interactions are negligible for this case. The data are discussed in terms of effects of the unstirred layer and the microenvironment of the surface.

INTRODUCTION

Several studies have been published on water-insoluble trypsin (EC 3.4.4.4). Axén and Porath[1] bound trypsin to cross-linked dextran. Habeeb[2] prepared active water-insoluble derivatives of trypsin by using glutaraldehyde to conjugate trypsin to aminoethyl cellulose. Hayes and Walsh[3] adsorbed trypsin as a monolayer onto colloidal silica particles. Mosbach[4] used carbodiimide to attach trypsin to acrylic polymers. Glassmeyer and Ogle[5] have studied several properties of trypsin bound to aminoethyl cellulose.

In an effort to examine the effects of distance between the bound enzyme and the peripheral boundary of the insoluble matrix, we synthesized peptide chains on a

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; Tos-Arg, *p*-tosyl-L-arginine; Tos-Arg-OMe, *p*-tosyl-L-arginine methyl ester; Boc-Ala, *tert*-butyloxycarbonyl-alanine; Boc-Gly, *tert*-butyloxycarbonylglycine.

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neutral hydrophobic polystyrene matrix by the solid-phase peptide synthesis method[6,7]. The chains ranged from 1 to 10 amino acid residues in length, being composed completely of glycine except for an initial residue of alanine on chains 2–10. The trypsin was covalently attached to each respective chain length by the carbodiimide method[8]. Each insoluble trypsin derivative was placed in a column and allowed to react with a low molecular weight synthetic substrate, *p*-tosyl-L-arginine methyl ester. The kinetic parameters were determined from the integrated Michaelis–Menten equation[9].

EXPERIMENTAL PROCEDURE

Materials

The chloromethylstyrene divinylbenzene copolymer, Bio-Beads S.X-2, 0.72 mequiv/g, 200–400 mesh, was obtained from Bio-Rad Laboratories. The *N*,*N*-dicyclohexylcarbodiimide used in solid phase synthesis was purchased from Regis. 1-Ethyl-3-(3-dimethylaminopropylcarbodiimide (EDC) hydrochloride was purchased from Pierce Chemical Co. Lyophilized trypsin, 2 times crystallized, was purchased from Worthington Biochemical Corp. *tert*-Butyloxycarbonylalanine (Boc-Ala), *tert*-butyloxycarbonylglycine (Boc-Gly), *p*-tosyl-L-arginine methylester·HCl (Tos-Arg-OMe), M.A., lot No. u2516 and lot No. v3011, and *p*-tosyl-L-arginine (Tos-Arg), M.A., were purchased from Mann Research Laboratories. "Trizma Base", trishydroxymethylaminomethane, was obtained from Sigma Chemical Co. All other chemicals were reagent grade.

Solid phase synthesis

The 1 M HCl–acetic acid method[7] was used to manually synthesize the amino acid chains on the polymer beads. For chain lengths 2–10, alanine polymer was used as the base onto which glycine residues were added to attain each desired chain length. An amino acid analysis[10] was performed on a sample from each chain length after 24-h acid cleavage, in a refluxing toluene bath.

Attachment of trypsin

Trypsin was covalently bound to the polymer beads by activating its carboxyl groups with EDC in the presence of the derivatized beads. Dry beads were placed in ethanol, then dialyzed against distilled water to hydrate them. The wet polymer was weighed by transferring it to filter paper and then into a covered weighing vessel. The percent dry polymer was determined on a set of wet samples by difference in weight before and after drying. Hence, the dry polymer weight equalled 0.69 ($\pm$0.02 at 95% confidence) multiplied by the wet weight. A typical reaction contained 0.6 g wet polymer, 0.011 g trypsin, and 0.1 g EDC in a total volume of 2 ml of 0.05 M sodium phosphate buffer at pH 4.5. The average mole ratios for the reactants was: EDC/chain sites = 6.69, EDC/trypsin = 1194, and chain sites/trypsin = 182. The EDC was added to the mixture last. The reaction mixture was stirred at 2 °C for 12 h, then washed with 1 l of 1 mM HCl.

Determination of trypsin activity

The spectrophotometric method of Hummel[11] was used to determine the activity of trypsin. Tos-Arg-OMe (lot No. u2516) concentrations were prepared in 0.046 M Tris–

HCl buffer with 0.1 M $CaCl_2$ at pH 8.1 ± 0.05. Three samples at each Tos-Arg-OMe concentration were taken after the prior elution of 3 ml to insure the column's effluent had reached equilibrium. The concentration of Tos-Arg was calculated from the average absorbance and experimentally determined molar extinction coefficients at 247 nm. For measurement of the molar extinction coefficient, each Tos-Arg-OMe concentration was prepared just prior to reading to minimize spontaneous hydrolysis. The values obtained were $\varepsilon_{\text{Tos-Arg}} = 1786.7 \pm 7.8\ M^{-1} \cdot cm^{-1}$, $\varepsilon_{\text{Tos-Arg-OMe}}$ (lot No. u2516) $= 1276 \pm 18.9\ M^{-1} \cdot cm^{-1}$ and $\varepsilon_{\text{Tos-Arg-OMe}}$ (lot No. v3011) $= 1219 \pm 11.7\ M^{-1} \cdot cm^{-1}$.

The individual substrate solutions were prepared from a stock solution of Tos-Arg-OMe. For each series of experiments the concentration of the stock Tos-Arg-OMe solution was corrected for spontaneous hydrolysis. Spontaneous hydrolysis of Tos-Arg-OMe was determined at 2 °C, 3 °C, and 25 °C. Rate constants obtained from the linear portions of the curves were 3.09 ± 1.45, 8.89 ± 1.41, and 84.8 ± 7.15 ($\times 10^{-4}$) h^{-1} at 2 °C, 3 °C, and 25 °C, respectively.

Determination of kinetic parameters of bound trypsin

For an enzyme obeying Michaelis–Menten kinetics the integrated rate equation is

$$c_P = K_m \ln(1-F) + k_2 E\beta/Q \qquad (1)$$

as derived by Lilly *et al.*[9], where c_p is the product concentration, F is the fraction of the initial substrate hydrolyzed, K_m = Michaelis constant, k_2 = rate constant for the breakdown of the enzyme–substrate complex to product, E = total amount of enzyme (in moles) in the column, β = ratio of the void volume to total bed volume, and Q = the flow rate. A plot of $\ln(1 - F)$ *vs* c_p gives a straight line with slope K_m and the c_p axis intercept gives $k_2\beta E/Q$, and thus k_2.

Each trypsin–polymer was placed in a jacketed, 0.6-cm (inside diameter) column, maintained at 25 °C, through which substrate was pumped at either 58.4 ml/h or 114.6 ml/h by a Milton–Roy piston pump. For chain lengths 1 and 2 the maximum Tos-Arg-OMe concentration was $5.0 \cdot 10^{-3}$ M. The maximum Tos-Arg-OMe concentration for the remaining chain lengths was $2.5 \cdot 10^{-3}$ M. During each test the substrates were maintained at 25 °C. At the end of the experiment the column's bed height was measured and then the polymer was removed, dried, and the number of moles of trypsin per dry polymer weight determined by amino acid analysis after a 48-h acid hydrolysis.

RESULTS

Solid phase synthesis

Since each chain length, except for the shortest, contained one alanine, it was possible to determine the chain length homogeneity by amino acid analysis, using the following equation:

$$C_n = \frac{\text{experimental ratio Gly/Ala}}{\text{theoretical ratio Gly/Ala}}$$

$$C_n = \frac{a_1 + a_2a_1 + \ldots + a_n \ldots a_2a_1}{n} \qquad (2)$$

TABLE I

RESULTS OF AMINO ACID ANALYSIS OF THE PEPTIDE CHAINS SYNTHESIZED ON THE POLYSTYRENE MATRIX

Chain length	*Theoretical ratio Gly/Ala*	*Experimental ratio Gly/Ala*	*Experimental ratio / Theoretical ratio*
1	—	—	—
2	1	0.86	0.86
3	2	1.72	0.86
4	3	2.66	0.89
5	4	2.54	0.64
6	5	4.03	0.81
7	6	5.83	0.91
8	7	5.56	0.79
9	8	6.97	0.87
10	9	6.73	0.75

where a_i is the coefficient of binding for each position on the chain and n denotes the number of glycine residues on the chain after the residue in question was bound. The assumption was made that previously inactive sites of attachment would remain inactive.

If there was a difference in the binding (a_i) for each residue position, then one would obtain a different C_n for each chain length. This was not the case as shown in Table I. The value for C_n was 0.83 ± 0.07 at 95% confidence. This meant that a_1 was 0.83 and a_2 through a_9 was unity. Therefore, each polymer derivative contained only two chain forms, that of unreactive alanine and the respective synthesized Ala–Gly chain length.

The amino acid analysis of Boc-Ala polymer gave 0.247 mmoles Ala/g dry Boc-Ala polymer. Thus, the resins with chain lengths 2–10 had 0.204 mmole chain sites/g dry Boc-Ala polymer. The amino acid analysis of Boc-Gly polymer gave 0.187 mmole Gly/g dry Boc-Gly polymer.

Attachment of trypsin

As shown in Table II the ratio of free trypsin to bound trypsin was approximately constant, 105, indicating that only one out of every 100 molecules of free trypsin was bound. However, more chain sites were required in chain lengths 1 and 2 and fewer were required in chain lengths 9 and 10 to bind 1 mole of trypsin (Table II). Also, the moles of bound trypsin/g polymer indicates that chain length is a factor in determining the amount of trypsin bound. Presumably, this phenomenon was due to an increase in the accessibility of the chain sites as the chain length was increased, as indicated by the calculation of the moles of chain sites on the surface/(g of polymer), *i.e.* exclusive of chain weight. In order to calculate moles of chain sites on the surface of a given weight of polymer, we made the following assumptions: (1) all polymer beads were the same size, (2) the beads were solid spheres in water, and (3) the chain sites were evenly distributed throughout the beads' volume. The moles of chain sites on the surface/(g of polymer), S, equalled $4/3\pi N\varrho(\bar{r}^3_n - \bar{r}^3_{n-a})$, where N is the number of beads/(g of polymer), ϱ is the moles of chain sites/unit volume, or, operationally, [moles chain sites/g polymer in column] [g polymer in column/(total volume — void

volume)], $\bar{r}_n$ is the mean radius of the dry polymer spheres and $\bar{r}_{n-a}$ is the mean radius less the thickness of the surface element, a. For $a = 1$ amino acid residue, *i.e.* 3.64 Å, using the *trans* configuration[12] $S = [1.592 \cdot 10^8$ beads/(g polymer)] $[(4/3)\ (3.14)\ (1.859 \cdot 10^{-4}$ moles chain sites/cm^3)] $[(12.049 \cdot 10^{-4}$ cm$)^3 - (12.048636 \cdot 10^{-4}$ cm$)^3] = 1.96 \cdot 10^{-8}$ moles chain sites/(g polymer). Hence, it appears that chain sites in a surface element of thickness of one amino acid residue could account for the amount of trypsin bound as expressed in the moles bound/g resin column in Table II.

TABLE II

ATTACHMENT PARAMETERS FOR THE COVALENT COUPLING OF TRYPSIN TO THE PEPTIDYL POLYSTYRENE MATRIX

Chain length	*Chain sites*	*moles free trypsin*	*moles bound**	*moles bound**
	mole trypsin bound ($\times 10^{-4}$)	*moles bound trypsin* ($\times 10^{-2}$)	*g dry resin*** ($\times 10^8$)	*column* ($\times 10^9$)
1	2.30	1.05	0.84	3.43
2	2.18	1.10	0.89	3.06
3	1.77	0.94	1.21	3.27
4	1.91	1.08	1.12	2.78
5	1.90	1.06	1.13	3.18
6	1.92	1.05	1.12	3.07
7	2.07	1.12	1.07	3.04
8	2.07	1.17	1.03	3.81
9	1.41	0.84	1.52	4.28
10	1.45	1.10	1.47	3.24

* Based on 21.5 moles Asp/mole trypsin and 24 000 molecular weight for trypsin[18].
** Exclusive of trypsin and chain weights.

Determination of kinetic parameters of bound trypsin

The columns had an average bed height of 1.89 cm and an average substrate retention time of 11.7 s for the 58.4 ml/h flow rate and 5.9 s for the 114.6 ml/h flow rate. A value of 0.371 was obtained for β. All columns exhibited piston type flow as determined by the F diagram method[9].

In agreement with earlier work[13], we found that Tos-Arg-OMe undergoes spontaneous hydrolysis at alkaline pH. By using each initial Tos-Arg-OMe concentration as the blank it was possible to negate the Tos-Arg absorbance due to spontaneous hydrolysis in the determination of enzymatically produced Tos-Arg. However, for kinetic analysis, the initial Tos-Arg-OMe concentration had to be corrected for spontaneous hydrolysis by computation of the respective loss of Tos-Arg-OMe due to the time spent at each storage temperature before use. The rate constants calculated previously were used to make these corrections.

Two typical plots of the integrated Michaelis–Menten equation are shown in Fig. 1. The resultant k_{cat} and K_m(app) values for each trypsin–polymer type examined are shown in Figs 2 and 3, respectively.

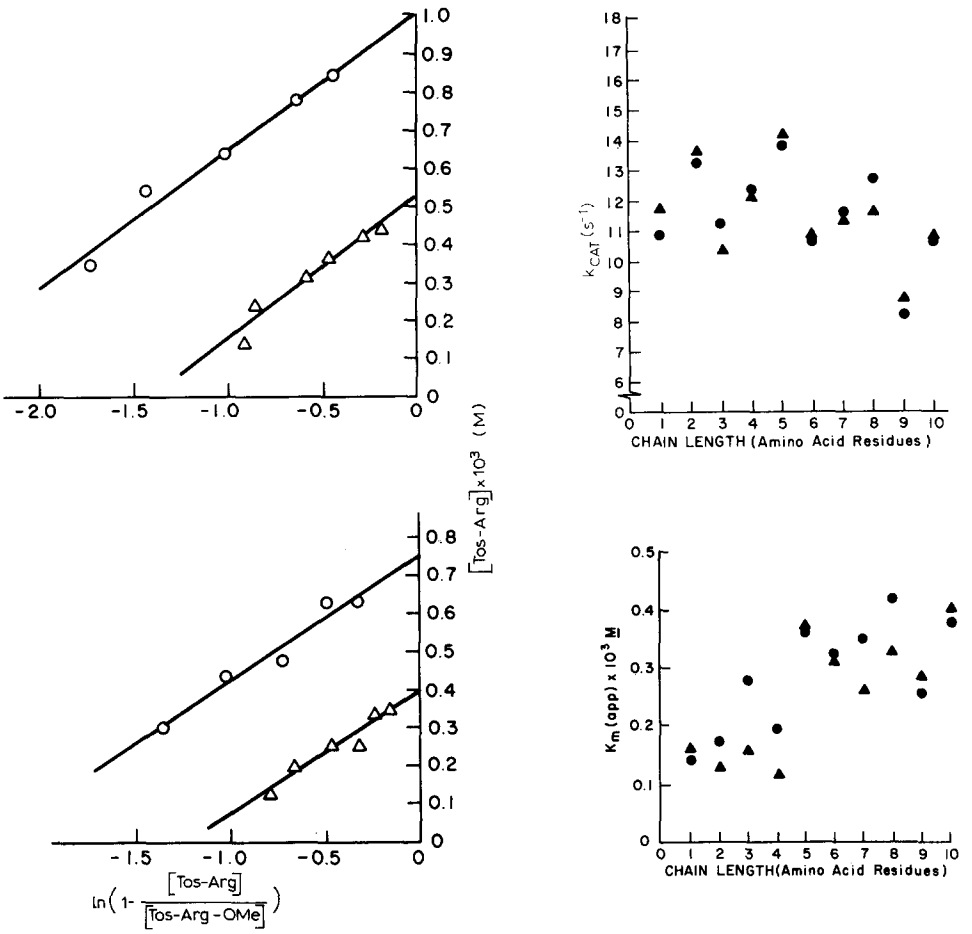

Fig. 1. Plots of $\ln(1-F)$ *vs* c_P for chain lengths of 5 (upper) and 6 (lower) amino acid residues ○, values at 58.4 ml/h and △, values at 114.6 ml/h column flow rate.

Fig. 2. The catalytic constant as a function of the amino acid chain length. ●, k_{cat} for 58.4 ml/h and ▲, 114.6 ml/h column flow rate.

Fig. 3. The apparent Michaelis constant as a function of amino acid chain length. ●, K_m (app) for 58.4 ml/h and ▲, 114.6 ml/h column flow rate.

DISCUSSION

Attachment of trypsin

There are several potentially adverse reactions inherent in attempting to covalently attach trypsin through its carboxyl groups. The first is the possibility of inactivation by attachment through an essential carboxyl group. Eyl and Inagami[14] have found that those carboxyl groups involved in binding and catalysis possess no increase in reactivity towards EDC under their modification conditions of pH 4.75 and 25 °C. In fact, they found that Asp 182 reacted more slowly with EDC than did other side chain carboxyl groups. Hence, their evidence suggests that EDC may react randomly with all available carboxyl groups of trypsin.

The second area for an adverse effect stems from the side reactions of EDC. One such reaction is the formation of an unreactive (under conditions used) *O*-arylisourea complex with tyrosine as reported by Carraway and Koshland[15]. However, in concurrence with the data of Eyl and Inagami[14], the formation of the *O*-arylisourea complex with tyrosine does not impair trypsin's activity. A second reaction is the rearrangement of an *O*-acylisourea derivative to the inactive *N*-acylurea form in which the EDC becomes permanently bound to the carboxyl groups of trypsin; however, under the conditions used, the extent of this reaction is negligible[16].

The third possibility for adverse reaction is the creation of inactive, bound polymerized trypsin. This case has been investigated by Mosbach[4] using 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide at pH 6.5 and pH 4 for 12 h. He found no evidence in the schlieren pattern from the ultracentrifuge to indicate the formation of higher molecular weight forms such as a dimer or oligomer.

Kinetic parameters of bound trypsin

Bechet and Yon[17] and Trowbridge *et al.*[18] have shown that Tos-Arg-OMe activates trypsin at high concentrations, while Howard and Mehl[19] found that Tos-Arg also activates trypsin under comparable conditions. In order to use the Michaelis–Menten equation for this system one has to consider high and low concentrations separately. The K_m(app) thus obtained is comparable to the K'_m(app) obtained from the high or low concentration asymptote of the rectangular hyperbola formed by Eadie plots (v *vs* v/S) from the Tos-Arg-OMe activation studies cited. All the Tos-Arg-OMe concentrations in our study were in the linear nonactivated portion of the cited Eadie plots, i.e. low Tos-Arg-OMe concentration region. Further confirmation of Michaelis–Menten kinetics was established by the linearity of all integrated kinetic plots (*e.g.* see Fig. 1). If activation had occurred, the integrated Michaelis–Menten plot would have been curvilinear as shown by the theoretical and experimental investigation of Self *et al.*[20] for inhibiting systems.

The kinetics of matrix-bound enzymes have been examined by several workers[21–27]. Several factors, other than changes in conformation of the active site, affect the kinetic behavior of immobilized enzymes, *viz.* (1) the presence of "unreactive" enzyme molecules due to inclusion in portions of the matrix impenetrable to substrate, (2) difference in substrate concentration at the particle surface in comparison to the bulk phase as a result of electrostatic or other specific interactions of the substrate with the surface, (3) difference in proton concentration between the surface and the bulk phase due to electrostatic interactions with a charged surface, and (4) the presence of an unstirred layer adjacent to the surface resulting in a substrate concentration gradient at the interface.

As the polystyrene matrix should not be swollen under the attachment reaction conditions, it may be presumed that the enzyme is bound only to the "surface" of the beads; that is, "inaccessible" or "buried" enzyme should not be present. Electrostatic interactions are also believed to be negligible since calculations (based on the assumption that the beads are not swollen) indicate that most of the attached peptide chains bind enzyme so the surface should be essentially neutral. This interpretation is supported by the experimental observation that K_m(app) increases with increasing chain length, whereas the opposite effect would result from electrostatic repulsion of the substrate, since the charge density and hence the potential should decrease with in-

creasing chain length. Therefore, the major contributions to changes in the kinetic parameters for this system are presumed to be specific interactions, other than electrostatic, between the substrate and the bead surface and the effect of the unstirred layer.

If substrate is being removed at the surface by an enzyme obeying Michaelis–Menten kinetics, and if the diffusional flux is considered as only perpendicular to the surface, then in the steady state in which the diffusional flux equals the chemical flux the folowing relation obtains[23,27],

$$D_s \frac{S_b - S_o}{\delta} = \frac{V'_m S_o}{K_m(\text{app}) + S_o} \tag{3}$$

by application of the Nernst approximation, where D_s is the subtrate diffusion coefficient, S_b the bulk solution substrate concentration, S_o the substrate concentration at the surface, δ the thickness of the unstirred layer, V'_m the maximum velocity, and $K_m(\text{app})$ the observed Michaelis constant. It is to be noted that diffusion through the unstirred layer should not affect the maximum velocity and consequently k_{cat} should not vary with chain length or with the flow rate even though the value of δ is inversely proportional to the flow rate. As shown in Fig. 2, k_{cat} appears to be constant at 11.4 s^{-1}, within experimental error, for all chain lengths and substrate flow rates examined. This observation also supports the above conclusion that all of the enzyme bound is located on the surface of the beads since the active site concentration was not affected by flow rate. The value for k_{cat} obtained from a double-reciprocal or Eadie plot of the data from solution kinetics for the same lot of commercial enzyme was 44 s^{-1}. The considerations above suggest that the lower value obtained for the bound enzyme results from the binding of inactive enzyme.

The surface substrate concentration, S_o, may be eliminated from Eqn 3 giving a quadratic whose solution is[23]

$$v = \frac{V_m' S_b}{K'_m(\text{app}) + S_b} \tag{4}$$

where

$$K'_m(\text{app}) = K_m(\text{app}) + \frac{\delta V'_m}{D_s} \tag{5}$$

Thus the apparent Michaelis constant is influenced by the thickness of the unstirred layer and the surface concentration of the enzyme, the latter being reflected in the value of V'_m. Since the thickness of the unstirred layer is directly related to the column flow rate, comparison of the apparent Michaelis constants for the same column at different flow rates should parallel changes in the value of δ. Accordingly, the apparent Michaelis constants for chain lengths 1–4 and 5–10 (see Fig. 3) were averaged for the 60 and 120 ml/h flow rates and the change in δ calculated by the relation

$$\Delta\delta = D_s \frac{K_m(\text{app})_{60} - K_m(\text{app})_{120}}{k_{cat} E^\circ_s} \tag{6}$$

where E_s° is the surface concentration in moles/cm^2. For chain lengths 1–4 these calculations indicate a decrease of 55 μm in the thickness of the unstirred layer when the flow rate is doubled, whereas, for chain lengths 5–10 the decrease in thickness is 22

μm. These results are consistent with the positioning of the enzyme in a volume element subject to increased stirring at the longer chain lengths.

Studies of the same lot of enzyme in solution yielded a K_m(app) of 0.05 mM. Calculation of δ based on this value, using Eqn 5 gave a value of 150 μm for chain lengths 1–4 at the fast flow rate. This value is unreasonably large suggesting that factors in addition to an unstirred layer are affecting the kinetics. Furthermore, if the unstirred layer were the only factor involved, the K_m(app) should decrease with increasing chain length instead of increasing. The accepted mechanism for "serine proteinases" is[28]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \overset{k_2}{\rightarrow} ES + P_1 \overset{k_3}{\rightarrow} E + P_2$$

where k_2 and k_3 are the rate constants for acylation and deacylation, respectively. Hence, in terms of the experimentally obtained parameters, one can show that

$$k_{cat} = \frac{k_2 k_3}{k_2 + k_3} \text{ and } K_m(\text{app}) = \frac{k_{-1} + k_2}{k_1} \cdot \frac{k_3}{k_2 + k_3} \tag{7}$$

If the deacylation step is rate-limiting ($k_2 \gg k_3$) as has been suggested for esters[29], then changes in k_2 or k_3 would affect the value for K_m(app). Also, a change in k_3 should be reflected in the value for k_{cat} since $k_{cat} \approx k_3$. However, as the data in Fig. 2 indicate, a systematic variation in k_{cat} was not observed. Thus, the increase in K_m(app) over that for the native enzyme is apparently due to a change in the substrate's binding constant. The decrease in K_m(app) for the short chain lengths may reflect on actual change in the substrate's binding constant due to the hydrophobic microenvironment at the surface or to specific interaction of the substrate with the surface resulting in its higher concentration near the surface than in the bulk solution. The rate of trypsin-catalyzed hydrolysis of Tos-Arg-OMe in solution has been shown to increase with a small decrease in dielectric constant[30]; however, the effect on K_m was not reported.

Manecke[31] has reported a destabilization of proteins bound to polystyrene, presumably due to hydrophobic interactions with the surface. Destabilization was not observed in this study, probably a result of the interposition of peptide chains between the surface and the enzyme.

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