Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1331–1336

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Activation and Stabilization of Chymotrypsin in Microdomains of Poly(ethylenimine) Derivatives. A Model of *in vivo* Environment

Junghun Suh,*,† Young-sam Lee,† and Sanghwa Han‡

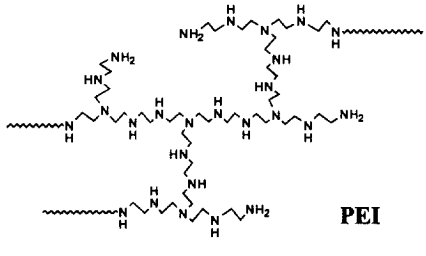
†Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-*742*, and ‡Department of Biochemistry, Kangwon National University, Chuchon 200-701, Korea

Received 17 March 1998; accepted 20 April 1998

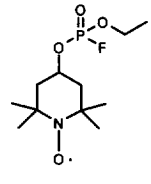
Abstract: Both activity and stability of α-Chymotrypsin (ChT) are substantially enhanced in the microdomains of laurylated or benzylated derivatives of poly(ethylenimine). EPR data revealed that the enhancement in activity of ChT is due to increase in the polarity of the microenvironment of Ser-195 caused by complexation of ChT to the polymer derivatives.

Properties of the *in vivo* environments for enzymatic actions are mostly quite different from those of aqueous buffer solutions. In many *in vitro* studies, however, enzymes are examined in aqueous buffer solutions. For example, α-chymotrypsin (ChT) has been studied intensively in aqueous buffer solutions, producing many pieces of mechanistic information.[1] To what extent the behavior of ChT characterized in these solutions represents that in its biological environments, however, has not been systematically investigated.

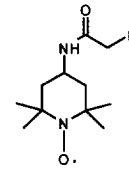
Branched poly(ethylenimine) (PEI)[2] is a synthetic polymer containing ethylamine as the monomeric unit. The typical PEI has the average M.W. of 60000, corresponding to 1400 monomers. Among the amino groups, ca. 25 % are primary amines, ca. 50 % are secondary amines, and the remaining 25 % are tertiary amines. The tertiary amines represent branching points on PEI. PEI has, therefore, a highly branched and globular structure.



PEI



1



2

0960-894X/98/$19.00
PII: S0960-894X(98)00211-X

Microdomains on PEI derivatives can be considered as a model of the *in vivo* media for biological reactions. When hydrophobic pendants are attached to PEI, the microdomains on PEI contain hydrophobic groups as well as ionic and polar groups. By adding various structural elements to PEI, the microenvironments of the polymer can be tuned to mimic various kinds of biological media. Unlike micelles, reverse micelles, or vesicles, the amphiphilic structure of PEI derivatives is stabilized by covalent linkages. PEI derivatives, therefore, can create various kinds of stable microenvironments in water that mimic the *in vivo* environments of many biological reactions.

In this study, PEI was laurylated or benzylated to prepare Lau_nPEI and Ben_nPEI (n; content of the pendant expressed in terms of residue mol %), respectively, according to the method previously reported.[3] 4-(Ethoxyfluorophosphinyloxy)-2,2,6,6-tetramethyl-1-piperidinyloxy radical (**1**) and 4-(2-iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy radical (**2**) were attached to ChT as described in the literature[4] as probes to obtain information on ChT located in the microdomains of the PEI derivatives.

Long-chain aliphatic hydrocarbons attached to PEI form micelle-like clusters. A ^{19}F NMR study indicated that 10,10,10-trifluorodecanoyl groups attached to PEI reside mostly in micelle-like structure.[5] Decarboxylation of 6-nitrobenzisoxazole-3-carboxylate is considerably enhanced in the microdomains of laurylated PEI derivatives, indicating the apolar nature of the microenvironments on the polymers.[6] As the content of lauryl group on PEI increases, the decarboxylation is catalyzed better, indicating increased hydrophobicity of the microenvironment. Although less information is available for the benzylated PEI derivatives, the benzyl groups would also aggregate together to minimize contact with water.

Analysis with HPLC of products obtained from the reaction of *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA) with ChT in the presence of PEI derivatives revealed that the hydrolysis products of BTNA were obtained in 1:1 molar ratios. Deacylation of BTNA by the PEI derivatives in the absence of ChT was negligible compared with the enzymatic reaction. The effect of PEI derivatives on the activity of ChT toward BTNA was examined by measuring the initial velocity (v_o).[7] The value of v_o/E_o increased as the concentration of the laurylated or benzylated PEI derivative was raised and approached limiting values as illustrated in Figs. 1 and 2. Concentrations of the PEI derivatives ($[Pol]_o$) are expressed in terms of molar concentration

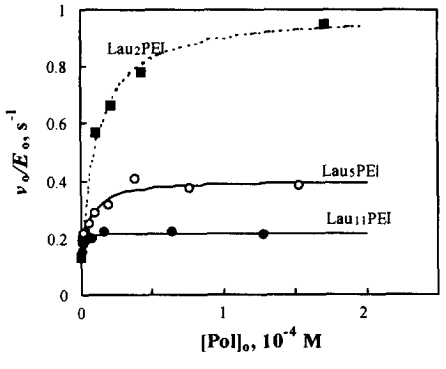


Fig. 1. Plot of v_o/E_o for hydrolysis of BTNA (1.20×10^{-4} M) catalyzed by ChT (2.57×10^{-6} M) in the presence of various amounts of laurylated PEI derivatives at pH 7.50 and 25°C.

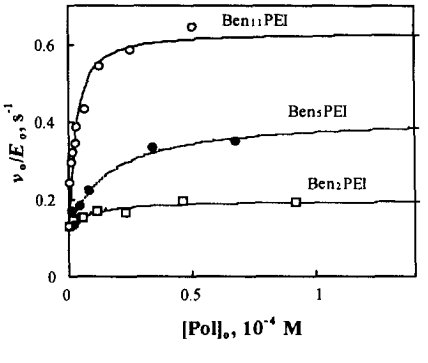
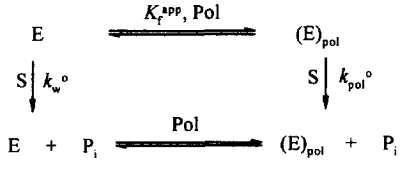


Fig. 2. Plot of v_o/E_o for hydrolysis of BTNA (1.20×10^{-4} M) catalyzed by ChT (2.57×10^{-6} M) in the presence of various amounts of benzylated PEI derivatives at pH 7.50 and 25°C.

calculated by using M.W. 60000 for PEI. Dialysis (cutoff M.W. 12000) of solutions containing BTNA (3.5×10^{-4} M) and a PEI derivative (1.1×10^{-4} M Lau_2PEI or 5.5×10^{-4} M Ben_2PEI) at pH 7.50 and 25 °C revealed that BTNA was not noticeably complexed to the PEI derivatives. Thus, the results of Figs. 1 and 2 can be taken to indicate that ChT is complexed to the PEI derivatives at sufficiently high $[Pol]_o$ concentrations and that the complexed ChT exhibits greater reactivity compared with the uncomplexed ChT. The isoelectric point of ChT is 8.5[8] and ChT is cationic at pH 7.5. Thus, the complexation between polycationic[9] PEI derivatives and ChT appears to be driven mainly by hydrophobic interaction rather than electrostatic interaction between the PEI and ChT.[10]

The saturation curves of Figs. 1 and 2 were analyzed in terms of Scheme 1, from which eq 1 is derived. The saturation curves of Figs. 1 and 2 indicate that ChT is almost completely complexed to the PEI derivatives when $[Pol]_o$ is much greater than $1/K_f^{app}$. The values of k_w^o, k_{pol}^o, and K_f^{app} estimated for the PEI derivatives are summarized in Table 1.

Scheme 1



$$v_o/E_o = (k_w^o + k_{pol}^o K_f[Pol]_o)/(1 + K_f[Pol]_o) \quad (1)$$

Table 1. Values of k_{pol}^o and K_f^{app} Estimated from the Kinetic Data of Figs. 1 and 2[a]

polymer	k_{pol}^o (s^{-1})	K_f^{app} (10^5 M^{-1})
Lau_2PEI	0.996 ± 0.043	0.837 ± 0.150
Lau_5PEI	0.401 ± 0.017	1.98 ± 0.60
$Lau_{11}PEI$	0.220 ± 0.005	15.4 ± 5.1
Ben_2PEI	0.199 ± 0.003	1.18 ± 0.55
Ben_5PEI	0.414 ± 0.034	0.622 ± 0.218
$Ben_{11}PEI$	0.632 ± 0.031	5.14 ± 1.54

[a]The value of k_w^o is 0.131 ± 0.009 s^{-1}.

Kinetic behavior of the ChT-catalyzed hydrolysis of BTNA taking place in the microdomains of the PEI derivatives was examined in details by varying S_o ($E_o = 2.57 \times 10^{-6}$ M, $S_o = 1.42$~9.96×10^{-5} M) under the conditions of [S] >> [ES]. To ensure complete complexation of ChT by each PEI derivative, $[Pol]_o$ was chosen as the highest value used for collection of data points of Figs. 1 and 2. The Lineweaver-Burk plot of E_o/v_o against $1/S_o$ in the presence of a sufficiently high $[Pol]_o$ concentration produced the values of kinetic parameters summarized in Table 2. The ChT-catalyzed hydrolysis of BTNA in the microdomains of Lau_2PEI was studied at various pHs. The bell-shaped pH profiles of k_{cat} and k_{cat}/K_m were analyzed to estimate the pK_a values associated with the acidic and the basic limbs. The values of pK_{a1} and pK_{a2} thus obtained are summarized in Table 3, together with those[11] measured for the ChT-catalyzed hydrolysis of BTNA in the absence of the PEI derivatives.

Table 2. Values of k_{cat}, K_m, and k_{cat}/K_m Measured for ChT-Catalyzed Hydrolysis of BTNA in Microdomains of Various PEI Derivatives at pH 7.50 and 25°C

polymer	k_{cat}, s^{-1}	K_m, 10^{-5} M	k_{cat}/K_m, 10^3 $s^{-1}M^{-1}$
none	0.161 ± 0.015	3.02 ± 0.21	5.33± 0.62
Lau_2PEI	1.20 ± 0.077	2.32 ± 0.43	51.7 ± 10.1
Lau_5PEI	0.473 ± 0.040	4.40 ± 0.34	10.8 ± 1.2
$Lau_{11}PEI$	0.297 ± 0.026	6.44 ± 0.47	4.61 ± 0.52
Ben_2PEI	0.290 ± 0.018	4.15 ± 0.23	6.98 ± 0.58
Ben_5PEI	0.421 ± 0.023	2.76 ± 0.35	15.3 ± 2.1
$Ben_{11}PEI$	0.630 ± 0.066	2.87 ± 0.57	22.0 ± 4.9

Table 3. Values of pK_{a1} and pK_{a2} Estimated from Bell-Shaped pH Profiles of ChT-Catalyzed Hydrolysis of BTNA in the Presence and Absence of Lau_2PEI at 25°C

polymer	parameter	pK_{a1}	pK_{a2}
none[a]	k_{cat}	6.60 ± 0.09	9.26 ± 0.11
	k_{cat}/K_m	6.45 ± 0.07	9.11 ± 0.07
Lau_2PEI	k_{cat}	6.56 ± 0.28	9.00 ± 0.30
	k_{cat}/K_m	7.02 ± 0.14	8.87 ± 0.14

[a]from ref 11.

Analysis of the enzymatic kinetic data obtained with ChT complexed to the PEI derivatives indicate that the activation of ChT is mainly due to the increase in k_{cat} and k_{cat}/K_m whereas K_m is little affected (Table 2). If acylation of ChT with BTNA is the rate-determining step as generally observed with anilide substrates,[1c] k_{cat} and k_{cat}/K_m represent the reactivities of the Michaelis complex and the free enzyme, respectively.[1d] Thus, the enhancement in the activity of ChT in the microdomains of the PEI derivatives originates from the improved reactivity of both the free enzyme and the enzyme-substrate complex.

The pK_a values estimated from the acidic and the basic limbs of the pH dependence of k_{cat} or k_{cat}/K_m for ChT-catalyzed reactions are generally assigned to His-57 and the *N*-terminal Ile, respectively.[1a] For BTNA, the pK_a values deduced from the pH profiles of k_{cat} and k_{cat}/K_m would reflect ionization of the Michaelis complex and the free enzyme, respectively, as discussed above. The pK_a values are affected by up to 0.6 pK units upon addition of Lau_2PEI (Table 3). Interpretation of this difference is not straightforward due to the lack of information on the effective pH and K_w in the microdomains of Lau_2PEI relative to those of the bulk media. Nevertheless, the pH profiles indicate that full activity is manifested at pH 7.5 both in the presence and absence of the polymer.

The EPR spectra of ChT labeled with **1** were taken in the presence of sufficiently high $[Pol]_o$ concentrations (the highest concentrations indicated in Figs. 1 and 2) and are illustrated in Figs. 3 and 4 and the values[4,12] of A_{zz} estimated therefrom are summarized in Table 4. For ChT labeled with **2**, little difference was observed between the EPR spectra measured in the microdomains of various PEI derivatives and that in the bulk solution. It is known that **1** and **2** specifically modify Ser-195 and Met-192 residues, respectively, in the active site of ChT.[4]

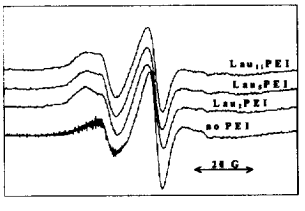


Fig. 3. EPR spectra of ChT labeled with **1** in the microdomains of laurylated PEI derivatives and in bulk solution.

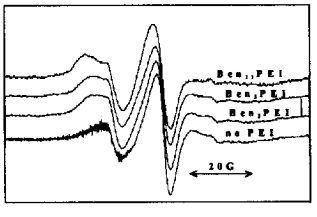


Fig. 4. EPR spectra of ChT labeled with **1** in the microdomains of benzylated PEI derivatives and in bulk solution.

Table 4. Values of A_{zz} Measured for ChT Labeled with **1** in the Microdomains of Various PEI Derivatives at pH 7.50 and 25°C

polymer	A_{zz} (G)
none	23.6
Lau_2PEI	27.9
Lau_5PEI	26.8
Lau_{11}PEI	26.0
Ben_2PEI	25.7
Ben_5PEI	26.3
Ben_{11}PEI	27.4

As the reporter radical becomes more mobile, the three peaks of EPR spectrum becomes more symmetrical.[12] When the ratio of high field to center field line heights is used as a measure of the relative degree of mobility,[4a,12b] the EPR spectra illustrated in Figs. 3 and 4 reveal that the reporter attached to Ser-195 is somewhat less mobile when ChT is located in the microdomains of the PEI derivatives than in bulk solution. The greater values of A_{zz} (Table 4) reflect the more polar microenvironment of the reporter radical.[12] Among laurylated PEI derivatives or benzylated derivatives, polymers manifesting greater k_{cat} or k_{cat}/K_m values are associated with larger values of A_{zz}.[13] Thus, the microenvironment of the reporter radical is more polar for more active ChT.

Among the PEI derivatives examined in the present study, Lau_2PEI manifested the largest activation of

ChT in terms of both k_{cat} and k_{cat}/K_m. Judging with k_{cat}/K_m, ChT is activated by 10 and 4 times, respectively, in the microdomains of Lau_2PEI and $Ben_{11}PEI$. Among the benzylated PEI derivatives, degree of activation of ChT increases as the content of the apolar pendant is raised, presumably due to the enhanced hydrophobicity. For the laurylated derivatives, however, the reverse is observed. The clustering of the apolar pendant groups would place strong constraints on the type of conformation that the hydrophobic PEI derivatives can assume in aqueous solutions.[5] This may be related to the results observed for the activation of ChT by the laurylated PEI derivatives.

To check whether the activity of ChT is also enhanced in the microdomains of the PEI derivatives toward natural substrates, ChT (2.23×10^{-6} M)-catalyzed hydrolysis of bovine serum albumin (3.26×10^{-5} M) was examined in the presence of Lau_2PEI (3.50×10^{-4} M) at pH 7.5 and 25°C by analyzing the concentration of albumin with HPLC. The amount of unhydrolyzed albumin was estimated after denaturation of ChT in the presence 4 M guanidinium chloride. The concentration of albumin decreased according to the pseudo-first-order kinetics through proteolysis by ChT with half-lives of 1.2 $\pm$ 0.1 min or 3.2 $\pm$ 0.2 min, respectively, in the presence or absence of Lau_2PEI. The activity of ChT is substantially enhanced toward the natural substrate as well as BTNA, an unnatural anilide substrate, in the presence of Lau_2PEI. Although interactions among three macromolecules (ChT, albumin, and the PEI derivative) complicate analysis of the kinetic data, ChT hydrolyzes albumin faster in the microdomains of Lau_2PEI than in the bulk solution.

Rates for thermoinactivation of ChT (1.50×10^{-6} M) at 50 °C were examined in the presence and absence of Lau_2PEI (1.71×10^{-4} M) or $Ben_{11}PEI$ (5.07×10^{-5} M). The residual activity of ChT after incubation at 50°C was determined by assay with BTNA at pH 7.50 and 25 °C. Thus, the degree of irreversible thermoinactivation of ChT was measured in this experiment. Electrophoresis (SDS-PAGE with silver staining)[14] of ChT indicated that the thermoinactivation of ChT does not involve cleavage of the polypeptide backbone.[15] ChT resisted thermoinactivation considerably in the microdomains of the PEI derivatives. The half-lives was estimated as 5.3 $\pm$ 0.1 min in the microdomains of Lau_2PEI, 18 $\pm$ 1 min in the microdomains of $Ben_{11}PEI$, and 2.3 $\pm$ 0.3 min in the bulk solution.

The microdomains of the PEI derivatives containing apolar pendants enhance the stability of ChT against thermoinactivation. Since peptide cleavage is not observed for inactivation of ChT, autoproteolysis is not responsible for the inactivation. Thus, the stabilization is not due to suppression of the autoproteolysis by compartmentalization of ChT in the microdomains of the polymer. Instead, the microdomains of the PEI derivatives appear to stabilize the three-dimensional structure of ChT, resulting in greater resistance to irreversible thermoinactivation.

ChT performs its biological functions in the paste containing organic materials and water. It is very likely that ChT manifests optimal activity and stability in its *in vivo* microenvironments. The PEI derivatives provide hydrophobic microenvironments in water that improve the activity and stability of ChT considerably. In addition, various types of mechanistic studies can be readily performed with ChT complexed to the PEI derivatives. The microdomains of the PEI derivatives, therefore, can be exploited as a model of the *in vivo* environments. Moreover, a variety of PEI derivatives containing several structural features can be prepared by modification of PEI and utilized as models of various other biological media.

Acknowledgment. This work was supported by grants from Basic Research Institute Program (1997), Ministry of Education, and from KOSEF through Center for Molecular Catalysis at SNU.

References and Notes

1. (a) Bender, M. R. In *Mechanisms of Homogeneous Catalysis from Protons to Proteins*; Wiley: New York 1971; pp 505-514. (b) Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145. (c) Fink, A. L. In *Enzyme Mechanisms*; Page, M. I.; Williams, A., Ed.; Royal Society of Chemistry: London, 1987; Chapter 10. (d) Schowen, R. L. In *Mechanistic Principles of Enzyme Activity*; Liebman, J. F.; Greenberg, A., Ed.; VCH: New York, 1988; Chapter 4. (e) Dugas, H. *Bioorganic Chemistry*, 3rd ed.; Springer-Verlag: New York, 1996; pp 184-204.
2. (a) Klotz, I. M. In *Enzyme Mechanisms*; Page, M. I.; Williams, A., Ed.; Royal Society of Chemistry: London, 1987; Chapter 2. (b) Suh, J. *Acc. Chem. Res.* **1992**, *25*, 273. (c) Suh, J. In *Polymeric Materials Encyclopedia*; Salamone, J. C. Ed.; CRC Press: Boca Raton, 1996; 4210. (d) Suh, J.; Cho, Y.; Lee, K. J. *J. Am. Chem. Soc.* **1991**, *113*, 4198. (e) Suh, J.; Lee, S. H.; Zoh, K. D. *J. Am. Chem. Soc.* **1992**, *114*, 7916.
3. Takagishi, T.; Klotz, I. M. *Biopolymers* **1979**, *18*, 2497.
4. (a) Morrisett, J. D.; Broomfield, C. A. *J. Am. Chem. Soc.* **1971**, *93*, 7297. (b) Kosman, D. *J. Mol. Biol.* **1972**, *67*, 247. (c) Levashov, A. V.; Klyachko, N. L.; Bogdanova, N. G.; Martinek, K. *FEBS Lett.* **1990**, *268*, 238. (d) Creagh, A. L.; Praushitz, J. M.; Blanch, H. W. *Enzym. Microb. Technol.* **1993**, *15*, 383. (e) Timmins, G. S.; Davies, M. J.; Gilbert, B. C. *J. Chem. Soc. Faraday Trans.* **1994**, *90*, 2643. (f) Suzawa, V. M.; Khmelnitsky, Y. L.; Giarto, L.; Dordick, J. S.; Clark, D. S. *J. Am. Chem. Soc.* **1995**, *117*, 8435.
5. Johnson, T. W.; Klotz, I. M. *Macromolecules* **1974**, *7*, 618.
6. Suh, J.; Scarpa, I. S.; Klotz, I. M. *J. Am. Chem. Soc.* **1976**, *98*, 7060.
7. Initial velocity was estimated with a computer program based on the statistical method reported in Booman, K.; Niemann, C. *J. Am. Chem. Soc.* **1956**, *78*, 3642.
8. In *Biology Data Book*, 2nd ed.; Altmann, P. I.; Dittmer, D. S. Ed.; The Federation of American Society for Experimental Biology: Bethesda, 1976; vol. 1, p 372.
9. Suh, J.; Paik, H.-j.; Hwang, B. K. *Bioorg. Chem.* **1994**, *22*, 318.
10. Below pH 6.5, complexation of ChT to Lau_2PEI was too weak to position ChT exclusively on Lau_2PEI, hampering the measurement of k_{cat} and k_{cat}/K_m for ChT in the microdomains of Lau_2PEI. Apparently, the increased cationic characters of both ChT and Lau_2PEI weaken the complexation.
11. (a) Suh, J.; Hwang, B. K. *Bioorg. Chem.* **1992**, *20*, 223. (b) Suh, J.; Cho, Y.; Kwag, G. *Bioorg. Chem.* **1992**, *20*, 236.
12. (a) Lassman, G.; Ebert, B.; Kuznetov, A. N.; Damerau, W. *Biochim. Biophys. Acta* **1973**, *310*, 298. (b) Morrisett, J. D. In *Spin Labeling: Theory and Applications*; Berliner, L. J. Ed.; Academic Press: New York, 1976; Chap. 8.
13. The A_{zz} measured in the microdomains of Lau_2PEI is greater than that measured in water by 18 %. For the same ChT derivative examined in reverse micelles, changes up to 10 % in A_{zz} have been observed upon variation of the water content of the reverse micelles.[4e] In this regard, the differences in A_{zz} summarized in Table IV are significant.
14. (a) Lameli, U. K. *Nature* **1970**, *227*, 680. (b) Humes, B. D. In *Gel Electrophoresis of Proteins*; Hames, B. D.; Rickwood, D. Ed.; IRL Press: New York, 1990; Chap. 1.
15. Results of the electrophoresis were the same as that reported in Suh, J.; Oh, S. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1067.