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Chymotrypsin Linked to Poly(ethylenimine) Derivatives: Perturbation of Ionization of Active Site Groups

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α-Chymotrypsin (ChT) is covalently linked to various poly(ethylenimine) (PEI) derivatives by using a carbodiimide as a coupling reagent. The PEI derivatives contain cationic or anionic microenvironments. In addition, hydrophobic microdomains are created on the PEI backbones in conjunction with the ionic environments. Stabilization of the tertiary structure of ChT by the cross-linking PEI derivatives is reflected by much greater resistance of the PEI-bound ChTs to thermoinactivation. Depending on the structural elements incorporated into the PEI derivatives, the activity of ChTs linked to PEI derivatives in either/both acidic or/and basic pH ranges is much greater than that of native ChT. The nature of microdomains introduced to the globular backbone of PEI affects the pK_a values of the active-site groups of ChT sensitively. Cationic microenvironments created on the PEI backbone retards protonation of His-57 more than that of Ile-16. Anionic microenvironments of the PEI backbone stabilize the ammonium ion of Ile-16 more than the imidazolium ion of His-57. Moreover, substrate binding by the active site appears to render His-57 more sensitive to the change in the microenvironments.

INTRODUCTION

Improvement of enzymatic properties such as reactivity, specificity, or compatibility with extreme conditions is closely linked to the applicability of the enzymes in biotechnology (*1–6*), and various methods have been developed for this purpose (*5, 7–17*). As a new chemical method for enhancing the durability of enzymes, we have reported cross-linking of α-chymotrypsin (ChT) with various derivatives of poly(allylamine) (PAA) to produce soluble enzyme–polymer conjugates (*18*). PAA is a linear polyamine with molecular weight of ca. 100,000. The remarkable stability against denaturing conditions and thermoinactivation manifested by ChT linked to the PAA derivatives was accounted for in terms of a multiple attachment of the enzyme to the polymer and the consequent stabilization of the tertiary structure of the enzyme.

Polyamines contain large numbers of amino groups that can be readily modified by organic reactions such as alkylation, acylation, or imine formation. Thus, several additional structural elements can be incorporated into the polyamine-bound enzymes. As the next step toward development of a general methodology for the improvement of enzymatic properties through cross-linking with poly-

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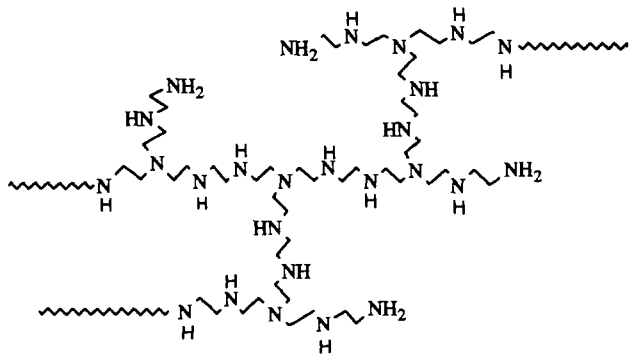


PEI

amines, we have tested poly(ethylenimine) (PEI) (*19, 20*) as the polymer skeleton. The molecular weight of PEI used in this study is about 60,000. In PEI, 25% of the nitrogens are primary amines, 50% are secondary amines, and 25% are tertiary amines. The tertiary amino nitrogens are the branching points on the PEI backbone, and PEI is a globular polyamine. The globular structure of PEI stands in contrast to the linear structure of PAA. In this article, cross-linking of ChT with various PEI derivatives and the consequent stabilization of the enzyme is described together with the sensitive response of p*K* of active-site groups to the structural elements incorporated into the PEI portions.

EXPERIMENTAL PROCEDURES

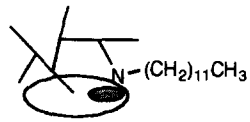
Preparation of PEI Derivatives

PEI (MW 50,000~60,000) was obtained from commercial sources (Dow, Aldrich, or Sigma). Since PEI was used after passing through an Amicon PM-30 membrane, the average molecular weight of PEI used in this study is estimated to be about 60,000. Laurylation of PEI was performed according to the literature (*21*) to obtain $Lau_{0.1}$ PEI in which 10% of the amines of PEI are laurylated. Succinylation of PEI to obtain SucPEI was carried out according to the method described in the literature (*22*). The content of succinyl group in SucPEI was estimated as 65% of the monomeric residues of PEI (*22*). Ni(II)-template condensation of PEI with butanedione was carried out as reported previously to obtain Ni(II)[PEI-BD] (*20*) in which the content of macrocyclic centers was ca. 10% of the monomeric residues of the polymer.

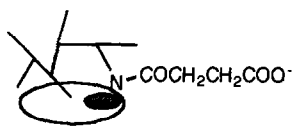
Cross-Linking of ChT with PEI Derivatives

ChT was cross-linked with PEI derivatives according to the procedure described previously for the preparation of ChTs linked to PAA derivatives (*18*). The concentration of the active site of ChT linked to a PEI derivative was determined by

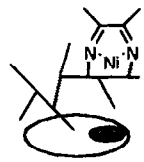
PEI-ChT



$Lau_{0.1}$PEI-ChT



SucPEI-ChT



Ni(II)[PEI-BD]-ChT

CHART 1

spectral titration with cinnamoyl imidazole according to the literature (*23*). The ChT derivatives prepared by this method are PEI-ChT (ChT linked to PEI), $Lau_{0.1}$PEI-ChT (ChT linked to $Lau_{0.1}$ PEI), and Ni(II)[PEI-BD]-ChT (ChT linked to Ni(II)[PEI-BD]).

PEI-ChT (2.0×10^{-5} M, 70 ml) was succinylated to produce SucPEI-ChT, by reacting with succinic anhydride (1 g, 10 mmol) at pH 7.7 (0.2 M Hepes or sodium phosphate buffer, pH was maintained at 7.7 during the succinylation by addition of 2 N NaOH) and 4°C followed by purification by dialysis.

Kinetic Measurements

Measurements of thermoinactivation and kinetic studies to construct the pH profiles of k_{cat} and k_{cat}/K_m were carried out as described previously (*18*).

RESULTS

Structural elements incorporated into the PEI skeleton in the derivatives of ChT linked to various forms of PEI are schematically presented in Chart 1. Here, the large ellipses symbolize ChT and the shaded ellipses the active site. Since many of the amino nitrogen atoms of PEI are protonated at ambient pHs, the PEI backbones contain many monopositive cationic sites, providing cationic microenvironments. In $Lau_{0.1}$PEI-ChT, about 10% of the nitrogen atoms on PEI contain lauryl groups, providing hydrophobic microenvironments. Succinylation of PEI converts amino nitrogens into amide nitrogens, destroying the positive charges on the amino nitrogens. Thus, microenvironments of SucPEI-ChT would be predomi-

TABLE 1

Half-Lives of ChTs Linked to Various PEI Derivatives during Thermoinactivation at 25 and 50°C

Enzyme	E_0 (10^{-5} M)	Temperature (°C)	Half-life
ChT	1.0	25	300 ± 30 h
PEI-ChT	1.0	25	620 ± 100 h
Ni(II)[PEI-BD]-ChT	1.0	25	>1000 h[a]
SucPEI-ChT	1.0	25	>1000 h[b]
ChT	1.0	50	3.0 ± 0.4 min
PEI-ChT	1.0	50	61 ± 6 min
Ni(II)[PEI-BD]-ChT	1.0	50	63 ± 6 min
SucPEI-ChT	1.0	50	150 ± 10 min

[a] Activity decreased by less than 20% in 500 h.
[b] Activity decreased by less than 10% in 500 h.

nantly anionic. On the other hand, the macrocyclic metal centers of Ni(II)[PEI-BD]-ChT would provide fixed dipositive cationic microdomains.

The thermal stability of the ChTs linked to the PEI derivatives was measured by following the enzyme activity toward BTNA during incubation at 25 and 50°C. Except for the initial portions corresponding to 10~20% activity decreases, the thermoinactivation did not deviate considerably from pseudo-first-order kinetics. The half-lives thus estimated are summarized in Table 1.

Kinetics of the hydrolysis of BTNA catalyzed by the ChT derivatives were measured under the conditions of $S_0 \gg E_0$. Analysis of the initial velocity data according to the Lineweaver–Burk plot produced the values of k_{cat}, K_m, and k_{cat}/K_m. Typical pH profiles of k_{cat} and k_{cat}/K_m are illustrated in Figs. 1 and 2. The pH profiles of k_{cat} and k_{cat}/K_m were analyzed according to Scheme 1 and Eqs. [1] and [2] (*24*) by the nonlinear regression method. Values of pK_{E1}, pK_{E2}, pK_{ES1}, pK_{ES2}, k_{cat}^{o}, and $(k_{cat}/K_m)^{o}$ thus estimated are summarized in Table 2. The pH profiles were also measured for native ChT in the presence of various derivatives of PEI. Values of pK_{E1}, pK_{E2}, pK_{ES1}, pK_{ES2}, k_{cat}^{o}, and $(k_{cat}/K_m)^{o}$ derived from these data (Table 3) indicate that the derivatives of PEI do not affect the kinetic behavior of ChT appreciably unless they are linked covalently to ChT.

$$k_{cat} = k_{cat}^{o}/(1 + [H^+]/K_{ES1} + K_{ES2}/[H^+]) \quad [1]$$

$$k_{cat}/K_m = (k_{cat}/K_m)^{o}/(1 + [H^+]/K_{E1} + K_{E2}/[H^+]) \quad [2]$$

DISCUSSION

The thermal stability of ChT is enhanced considerably upon attachment to the PEI derivatives. In fact, the degree of thermal stabilization is comparable to that achieved by cross-linking with various PAA derivatives. The intrinsic stability of the polypeptide chain of ChT is, therefore, considerably enhanced upon attach-

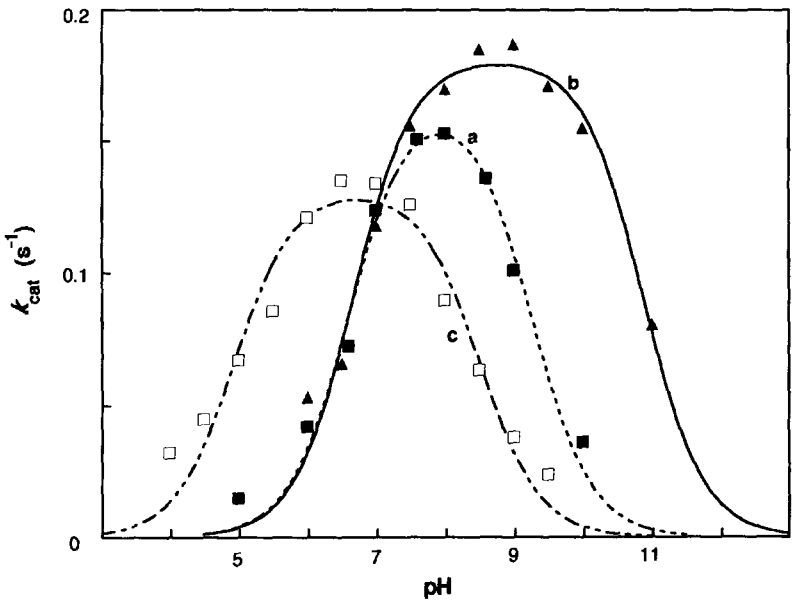


FIG. 1. Typical pH profiles of k_{cat} for various ChT derivatives at 25°C. Curve a (■) for ChT, curve b (▲) for SucPEI-ChT, and curve c (□) for Ni(II)[PEI-BD]-ChT.

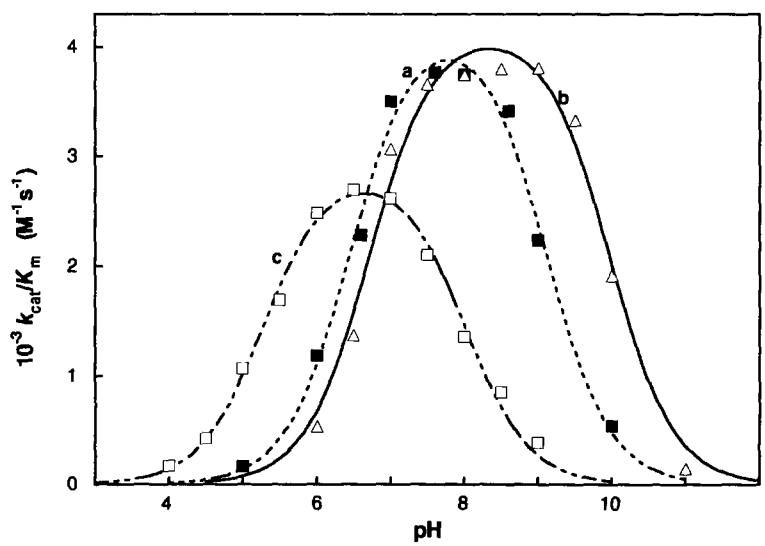
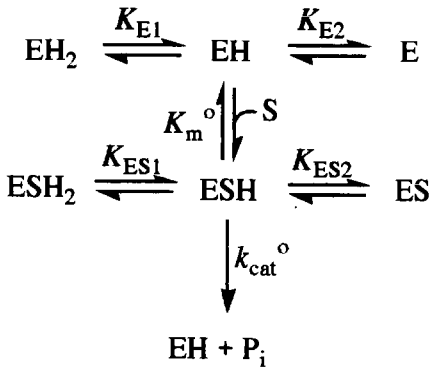


FIG. 2. Typical pH profiles of k_{cat}/K_m for various ChT derivatives at 25°C. Curve a (■) for ChT, curve b (△) for SucPEI-ChT, and curve c (□) for Ni(II)[PEI-BD]-ChT.



SCHEME 1

ment to the PEI derivatives. It appears that a multiple attachment of ChT to the polyamine support is also achieved with PEI derivatives, resulting in the suppression of the unfolding of tertiary structure. Cross-linking with PEI derivatives does not significantly damage the active site of ChT since the k_{cat}/K_m values at optimum pHs for the PEI-bound ChTs are 75–95% of that of native ChT.

Upon attachment to various derivatives of PEI, the pH dependence of the enzyme activity is significantly affected. As illustrated in Figs. 1 and 2 and as indicated by the parameter values listed in Table 2, some PEI-ChT derivatives exhibit much greater activity at high pHs or low pHs and some show wider optimum pH ranges compared with native ChT. By applying the kinetic parameters summarized in Table 2 to Eqs. [1] and [2], the values of k_{cat} and k_{cat}/K_m are calculated for each ChT derivative at various pHs. Constant k_{cat} represents the maximal rate ($V_{max} = k_{cat}E_o$) attained under the conditions of $S_o \gg K_m$ and constant k_{cat}/K_m represents the pseudo-first-order rate constant ($k_o = k_{cat}/K_mE_o$) measured under the conditions of $S_o \ll K_m$. At pH 4 and 5, $Lau_{0.1}$PEI-ChT and Ni(II)[PEI-BD]-ChT are about 20–30 times more active than ChT in terms of k_{cat} and Ni(II)[PEI-BD]-ChT is about 10 times more active than ChT in terms of k_{cat}/K_m. At pH 11 and 12, SucPEI-ChT is about 25~40 times more active than ChT in terms of k_{cat} and about 7 times more active than ChT in terms of k_{cat}/K_m.

For the ChT-catalyzed hydrolysis of BTNA, the rate-determining step is acylation of the enzyme (*25*). The pH profiles of k_{cat}/K_m and k_{cat}, therefore, reflect pK_a values of functional groups in the free enzyme (E) and the noncovalent ES complex, respectively (*26*). The pK_{E1}/pK_{ES1} and pK_{E2}/pK_{ES2} values obtained from the pH dependence for ChT-catalyzed reactions are generally assigned to ionization of the imidazole group of His-57 and the amino group of Ile-16, respectively (*27*).[2]

In ChT derivatives linked to PEI such as PEI-ChT, $Lau_{0.1}$PEI-ChT, Ni(II)[PEI-

[2] Different pK_a values observed for different substrates may sometimes be accounted for by different numbers of protons involved in the rate-controlling transition states (*28*). Different pK_a values observed for the hydrolysis of BTNA by different ChT derivatives in this study, however, are better attributed to the altered ionization constants in various microenvironments.

TABLE 2

Kinetic Parameters Estimated from Analysis of the pH Profiles of k_{cat} and k_{cat}/K_m for the Hydrolysis of BTNA by PEI-Bound ChTs

Enzyme	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}	$(k_{cat}/K_m)^o$ (10^3 s^{-1} M^{-1})	k_{cat}^o (10^{-1} s^{-1})
ChT	6.45 ± 0.07	9.11 ± 0.07	6.60 ± 0.09	9.26 ± 0.11	4.24 ± 0.15	1.67 ± 0.09
PEI-ChT	5.65 ± 0.07	8.98 ± 0.07	5.31 ± 0.14	8.77 ± 0.14	3.05 ± 0.09	1.38 ± 0.08
$Lau_{0.1}$PEI-ChT	5.67 ± 0.11	8.43 ± 0.11	4.90 ± 0.16	8.70 ± 0.16	2.69 ± 0.16	1.29 ± 0.08
SucPEI-ChT	6.71 ± 0.08	9.97 ± 0.09	6.67 ± 0.14	10.88 ± 0.01	4.17 ± 0.25	1.82 ± 0.05
Ni(II)[PEI-BD]-ChT	5.27 ± 0.06	8.01 ± 0.06	4.94 ± 0.14	8.51 ± 0.12	2.89 ± 0.09	1.32 ± 0.07

TABLE 3

Kinetic Parameters Estimated from Analysis of the pH Profiles of k_{cat} and k_{cat}/K_m for the Hydrolysis of BTNA by ChT in the Presence of Equimolar Amounts of PEI Derivatives

Added polymer	pK_{E1}	pK_{E2}	pK_{ES1}	pK_{ES2}	$(k_{cat}/K_m)^o$ (10^3 s^{-1} M^{-1})	k_{cat}^o (10^{-1} s^{-1})
None	6.45 ± 0.07	9.11 ± 0.07	6.60 ± 0.09	9.26 ± 0.11	4.24 ± 0.15	1.67 ± 0.09
PEI	6.42 ± 0.09	9.12 ± 0.09	6.54 ± 0.12	9.22 ± 0.15	3.96 ± 0.18	1.65 ± 0.10
$Lau_{0.1}$PEI	6.37 ± 0.06	9.17 ± 0.07	6.36 ± 0.09	9.50 ± 0.11	3.26 ± 0.11	1.45 ± 0.06
SucPEI	6.54 ± 0.06	9.19 ± 0.08	6.63 ± 0.08	9.48 ± 0.11	4.06 ± 0.15	1.66 ± 0.07
Ni(II)[PEI-BD]	6.39 ± 0.06	9.03 ± 0.06	6.41 ± 0.10	9.31 ± 0.12	4.00 ± 0.12	1.57 ± 0.08

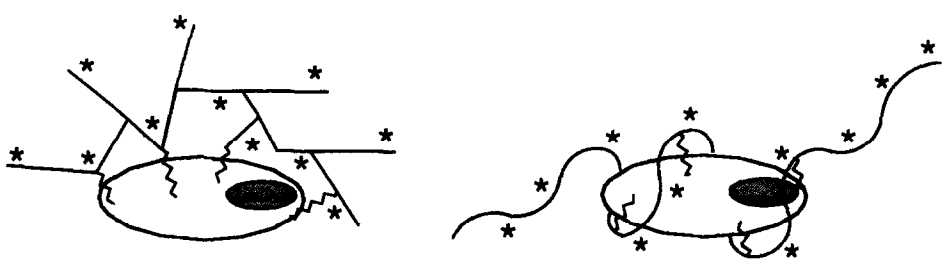
BD]-ChT, the PEI moiety would provide ChT with cationic microenvironments. This is reflected in decreases in pK_{E1}, pK_{E2}, pK_{ES1}, and pK_{ES2}. The decrease in pK_{E1} or pK_{ES1} is greater than that in pK_{E2} or pK_{ES2}, respectively. Thus, protonation of His-57 is retarded more sensitively than that of Ile-16 by introduction of the cationic environment. This may be related to the fact that the ammonium cation of Ile-16 interacts with the carboxylate anion of Asp-194 (*27*).

Hydrophobicity and cationic character are combined by attachment of lauryl groups to PEI-ChT to produce $Lau_{0.1}$PEI-ChT. The electrostatic effects exerted by the cationic media could be greater in a more hydrophobic environment. This is reflected in smaller values of pK_{E2} and pK_{ES1} for $Lau_{0.1}$PEI-ChT compared with PEI-ChT. Dipositive fixed metal centers are created in Ni(II)[PEI-BD]-ChT, and the cationic character exerted by the metal centers would be much greater than those by the ammonium ions of the PEI backbone. This results in the smaller values of pK_{E1}, pK_{E2}, pK_{ES1}, and pK_{ES2} for Ni(II)[PEI-BD]-ChT compared with the other ChT derivatives containing cationic PEIs.

An anionic microenvironment is created in SucPEI-ChT. The anionic media would facilitate protonation of His-57 or Ile-16, as reflected in increases in the pK values. The increase in pK_{E2} or pK_{ES2} is greater than that in pK_{E1} and pK_{ES1}, respectively. Thus, the ammonium ion of Ile-16 is stabilized more than the imidazolium ion of His-57 by introduction of carboxylate groups on the PEI backbone. It is possible that Ile-16, which interacts with the carboxylate anion of Asp-194, further interacts with the carboxylate anion attached to the PEI backbone.

Protonation (pK_{ES1}) of His-57 of the ES complex is more suppressed than that (pK_{E1}) of the uncomplexed enzyme (E) by the cationic or anionic microenvironment. Apparently, binding of the substrate by the active site renders His-57 more sensitive to the change in microenvironments.

In the case of ChTs linked to various PAA derivatives (*18*), obvious trends are not seen in the effects of the cationic, anionic, or hydrophobic microenvironments of the polymers on the pK of His-57 or Ile-16, in contrast with the ChTs attached to the PEI derivatives. This difference may be attributed to the globular structure of PEI and the linear structure of PAA. As schematically illustrated by A and B (stars symbolize



A **B**

structural features introduced to the polymer backbone), the active site of ChT may be affected by microenvironments created over a wide area of PEI (A), whereas it may be affected by a narrower portion of the polymer when ChT is linked to PAA (B). The nature of microdomains introduced to the globular polyamine, therefore, result in greater perturbation of the ionization behavior of active-site groups of ChT.

ACKNOWLEDGMENT

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