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Microenvironments of 3-[2-Imidazolylazo]-benzoyl-poly(ethylenimine): High Affinity for Ni(II) and 9-Anthracenecarboxylate Ions

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The 3-[2-imidazolylazo]benzoyl (Imaben) group was introduced to PEI to obtain Imaben-PEI. Imaben-PEI manifested pronounced affinity toward Ni(II) ion by binding one Ni(II) ion for three Imaben moieties. Compared with laurylated PEI (lauPEI), Imaben-PEI formed much stronger complexes with 9-anthracenecarboxylate (AC). Deacylation of 2-nitro-1-naphthyl acetate (NNA) by Imaben-PEI was far slower than that by lauPEI or PEI. These results suggest that Imaben residues of Imaben-PEI form aggregates. This can lead to a compact conformation of the polymer with only a small portion of amino groups exposed on the surface. Aromatic compounds such as AC and NNA bind Imaben-PEI at the apolar cluster of Imaben moieties. Stacking interactions between aromatic rings appear to be involved in the aggregation of Imaben groups of Imaben-PEI and complexation of AC and NNA to Imaben-PEI.

INTRODUCTION

Enzymatic catalysis is characterized by complex formation with substrates, various types of specificity, and a high degree of acceleration. By using a variety of synthetic compounds, attempts have been recently made to design artificial enzymes manifesting these features (*1–9*).

In order to develop effective artificial enzymes, it is necessary to incorporate several catalytic features into synthetic catalysts. Poly(ethylenimine) (PEI) derivatives have been considered to be suitable for accommodation of multiple catalytic elements (*8–11*). Each PEI (MW ca. 60,000) molecule contains ca. 1400 ethylamine moieties. About 25% of the amines are primary, 50% are secondary, and the remaining 25% are tertiary. The tertiary amine nitrogens represent branching points on the polymer backbone, and PEI is, therefore, a globular polymer.

Both recognition of substrates and catalytic turnover of the resultant supramolecular complexes are affected considerably by the nature of microenvironments on the polymer. In order to obtain effective artificial enzymes based on PEI, it is desirable to develop methods to create various kinds of microenvironments on

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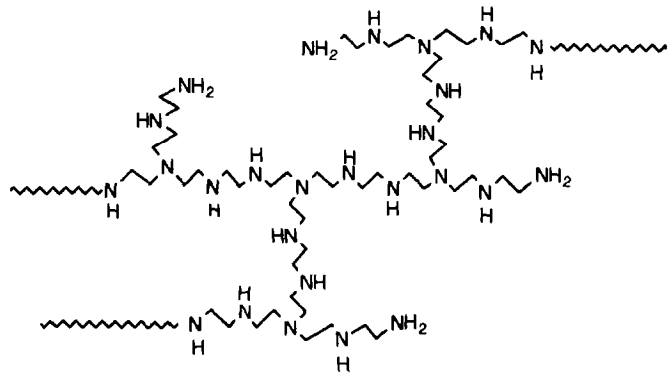
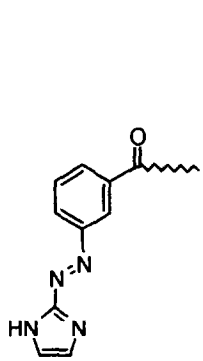
PEI and to have knowledge on the binding and the kinetic characteristics of the microenvironments.

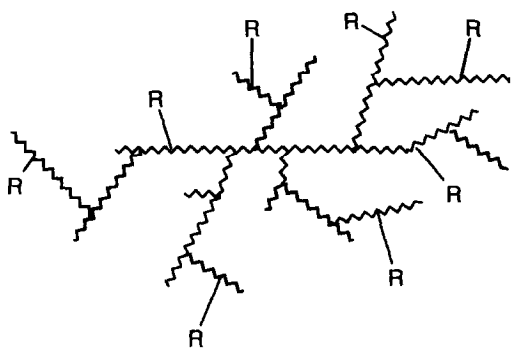


PEI

In the present study, we have attached 3-[2-imidazolylazo]benzoyl (Imaben) groups to PEI, leading to Imaben-PEI, to create hydrophobic microenvironments with metal binding capability. In this article, the nature of microenvironments of Imaben-PEI is described.



Imaben-



Imaben-PEI: R = Imaben

lauPEI: R = $C_{12}H_{25}$

EXPERIMENTAL PROCEDURES

Materials

3-[2-Imidazolylazo]benzoic acid. To an aqueous solution (0.1 N HCl, 30 ml) of 3-aminobenzoic acid (1.37 g) kept in an ice bath, $NaNO_2$ (1 g) dissolved in 5 ml water was added. This solution was added dropwise to an aqueous solution

(30 ml) of imidazole (2.4 g) whose pH was maintained at 9–11. The pH of the reaction mixture was adjusted to 6 and yellow precipitates were collected and recrystallized from acetone to obtain 3-[2-imidazolylazo]benzoic acid, mp 180–183°C (dec).

N-{3-[2-Imidazolylazo]benzoyloxy}-succinimide. To a solution (30 ml) of 3-[2-imidazolylazo]benzoic acid (0.80 g) dissolved in dimethyl formamide, dicyclohexyl carbodiimide (0.92 g) and *N*-hydroxysuccinimide (0.43 g) were added and the mixture was stirred at room temperature for 48 h. After precipitates were removed, the residue obtained by evaporation of the solvent was recrystallized from ethyl acetate to obtain *N*-{3-[2-imidazolylazo]benzoyloxy}-succinimide, mp 176–178°C (dec).

3-[2-Imidazolylazo]benzoyl-poly(ethylenimine) (Imaben-PEI). PEI purchased from Sigma (MW ca. 50,000) was ultrafiltered through a PM-30 membrane (Amicon) to remove fractions of small size (MW < 30000). From the amount of small fractions excluded, the average MW of PEI used in the present study is estimated as ca. 60,000. An acetonitrile solution (30 ml) of *N*-{3-[2-imidazolylazo]benzoyloxy}-succinimide (0.67 g) was added to an aqueous solution (40 ml) of PEI (0.90 g) dropwise over a period of 1 h at room temperature. After stirring for additional 5 h, the mixture was acidified to pH 4 with HCl and was purified by dialysis against 12 liters water three times. By comparing the visible spectrum of Imaben-PEI with that of *N*-{3-[2-Imidazolylazo]benzoyloxy}-succinimide, the content of Imaben group in Imaben-PEI was estimated as 6% of the monomer residues of PEI.

Lauryl-poly(ethylenimine) (LauPEI). This polymer was prepared by laurylation of PEI according to the literature (*12*), and the content of lauryl group was 10% of the monomer residues of PEI.

2-Nitro-1-naphthyl acetate (NNA). To a methylene chloride solution (20 ml) of acetyl chloride (0.20 g), 2-nitro-1-naphthol (0.47 g) and triethylamine (0.25 g) were added and the mixture was stirred for 1 h at room temperature. The mixture was washed with a 1% $NaHCO_3$ solution and water. After drying with $MgSO_4$, the residue obtained after evaporation of the solvent was recrystallized from ethyl acetate–ether to obtain NNA, mp 111–113°C.

Nickel chloride was prepared as reported previously (*13*). 9-Anthracenecarboxylic acid was converted into its sodium salt after recrystallization from ethanol. Distilled water was deionized prior to use.

Physicochemical Studies

Spectrophotometric measurements for Ni(II) binding, analysis of 9-anthracenecarboxylate (AC), and deacylation of NNA were performed with a Beckman DU-64 spectrophotometer at 25°C. Temperature was controlled to within ±0.1°C with a Haake E12 circulator, and pH was adjusted to 7.00 with 0.05 M *N*-(2-hydroxyethyl)-1-piperazineethanesulfonate.

For the binding study of AC to Imaben-PEI or lauPEI, a solution of both the polymer and AC contained in a dialysis tube was equilibrated against a solution containing only AC. Narrow and tall dialysis tubes were used to minimize concentration changes due to osmotic pressure. By measuring the decrease in the concentration of AC outside the dialysis tube after equilibrium was reached, the amount of AC bound to the polymer was calculated.

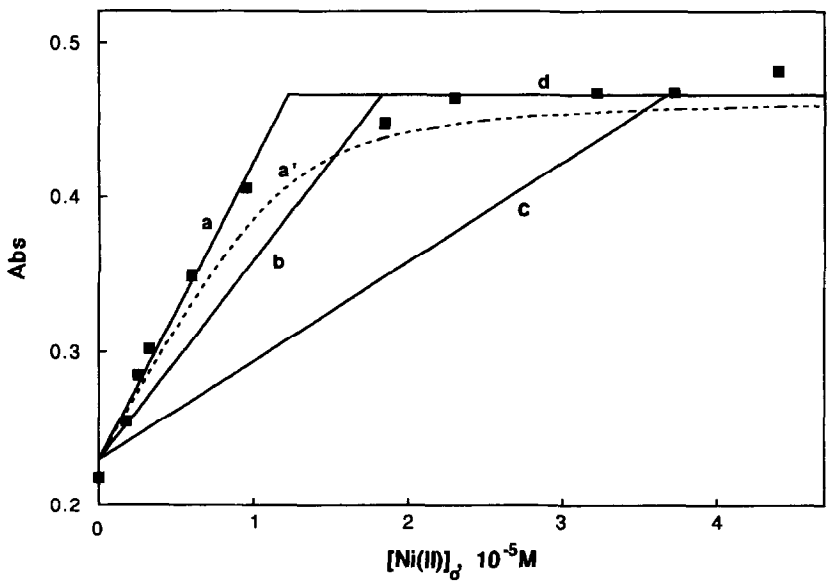


FIG. 1. Plot of absorbance of Imaben-PEI at 485 nm against $[Ni(II)]_0$ at 25°C and pH 7. The total concentration of the Imaben moiety of Imaben-PEI was 3.66×10^{-5} M. Curve a′ is drawn by assuming that one Ni(II) ion can be bound for three Imaben residues with dissociation constant of 10^{-6} M. Lines a–d are drawn as explained in the text.

Kinetics for deacylation of NNA was carried out in the presence of 1% (v/v) acetonitrile which was used as the solvent of the stock solution of NNA. The initially added concentration of NNA in the kinetic measurements was 1.2×10^{-5} M.

RESULTS

Binding of Ni(II) Ion to Imaben-PEI

In the present study, two derivatives of PEI are prepared. Imaben-PEI contains aromatic hydrophobic groups whereas lauPEI possesses alkyl hydrophobic groups. The content of the Imaben group in Imaben-PEI is 6% of the monomer residues and that of the lauryl group in lauPEI is 10% of the monomer residues.

The Imaben portion of Imaben-PEI contains a metal-chelating site, with the two imine nitrogens (an azo nitrogen and an imidazole nitrogen) being the donor atoms (*9, 13–16*). It is known that metal complexation of the Imaben portion results in a considerable change in visible spectrum. In Fig. 1, the absorbance change accompanying the addition of Ni(II) ion to Imaben-PEI is illustrated. Here, the total concentration of the Imaben portion of Imaben-PEI is kept constant at 3.66×10^{-5} M whereas the total concentration of Ni(II) ion ($[Ni(II)]_0$) is varied up to 5×10^{-5} M. The absorbance reaches a saturation value below 2×10^{-5} M of

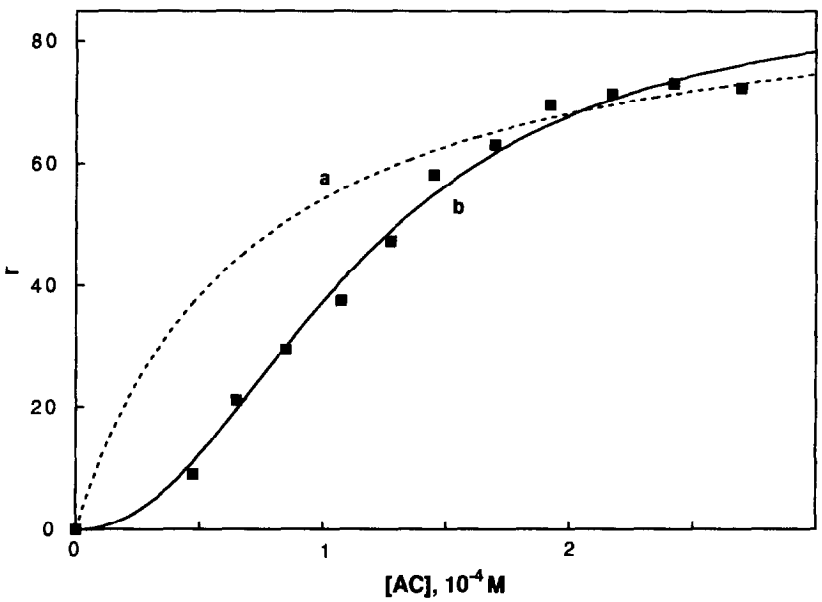


FIG. 2. Plot of r (number of AC bound per Imaben-PEI molecule) against [AC] (equilibrium concentration of uncomplexed AC) at 25°C and pH 7. Curve a is drawn according to Eq. [1] with $n = 92$ and $K_d = 7 \times 10^{-5}$ M. Curve b represents the best fit obtained with Eq. [2] ($n = 88$, $\alpha = 2.2$, and $K = 2.2 \times 10^{-9}$).

$[Ni(II)]_0$, which is unexpectedly low if one Ni(II) ion is assumed to be bound to one or two residues of Imaben.

If a 1 : 1-type complex is formed between Ni(II) and Imaben with a extremely small dissociation constant ($<10^{-8}$ M), the absorbance change would consist of two straight lines (e.g., lines c and d of Fig. 1) intersecting at $[Ni(II)]_0 = [Imaben\text{-}PEI]_0$. If each Ni(II) ion is bound to two Imaben moieties with sufficiently small dissociation constant, the absorbance change would be given by two straight lines (e.g., lines b and d of Fig. 1) intersecting at $[Ni(II)]_0 = \frac{1}{2}[Imaben\text{-}PEI]_0$.

The data illustrated in Fig. 3 is consistent with binding of each Ni(II) ion to three Imaben moieties, since lines c and d intersect at $[Ni(II)]_0 = \frac{1}{3}[Imaben\text{-}PEI]_0$. In case that the dissociation constant is not very small, the curve (e.g., curve a′ of Fig. 1) representing the absorbance change should lie below the two intersecting lines. The dissociation constant for the Ni(II) complex of Imaben-PEI is, therefore, estimated as $\ll 10^{-6}$ M.

Binding of 9-Anthracenecarboxylate to Imaben-PEI and lauPEI

Binding of AC to Imaben-PEI was studied by the dialysis method. In Fig. 2, the number of AC molecules bound to each molecule of Imaben-PEI is plotted against the equilibrium concentration of AC uncomplexed to the polymer.

When bindings of each small molecule are independent of one another, complexation of the small molecules to a polymer is described by Eq. [1] (*17*). Here, r is the

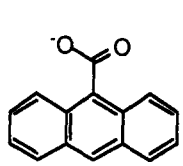
number of small molecules bound to each polymer molecule, [L] the equilibrium concentration of the small molecule uncomplexed to the polymer, n the maximum number of the small molecule that can be bound per polymer molecule, and K_d the dissociation constant for the resulting complex. Equation [1] has been successfully applied to binding of several types of small molecules to PEI derivatives (*10, 18*), and predicts a saturation curve such as curve a of Fig. 2 for the plot of r against [L].

$$r = n[L]/(K_d + [L]) \quad [1]$$

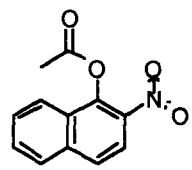
In Fig. 2, deviation of the data from Eq. [1] is obvious especially at low [AC] concentrations. The data are better analyzed by Eq. [2], a Hill-equation-type analogue of Eq. [1], suggesting cooperativity of initially bound AC for the subsequent binding of additional AC molecules.

$$r = n[L]^{\alpha}/(K + [L]^{\alpha}) \quad [2]$$

The binding of AC to lauPEI was also examined by the dialysis method, and no appreciable complexation of AC to lauPEI was observed.



AC



NNA

Deacylation of 2-Nitro-1-naphthyl Acetate in the Presence of Imaben-PEI, lauPEI, or PEI

Kinetics of the deacylation of NNA was measured in the presence of Imaben-PEI, lauPEI, or PEI under the conditions of $[Pol]_0$ (the initially added concentration of the polymer; expressed in monomer residue molar concentrations) $\gg$ $[NNA]_0$ (the initially added concentration of NNA) by following the release of 2-nitro-1-naphthol spectrophotometrically. For many reactions accelerated by several derivatives of PEI, saturation kinetics has been observed, which was analyzed according to the scheme of Eq. [3] (*8, 10*). The expression of pseudo-first-order rate constants (k_0) measured under the condition of $[Pol]_0 \gg S_0$ is given by Eq. [4].

$$C + S \underset{K_m}{\rightleftharpoons} CS \xrightarrow{k_{cat}} \text{products} \quad [3]$$

$$k_0 = k_{cat}C_0/(K_m + C_0) \quad [4]$$

The rate data obtained are illustrated in Fig. 3. For PEI and lauPEI, k_0 is proportional to $[Pol]_0$, indicating very weak complexation of NNA to PEI or

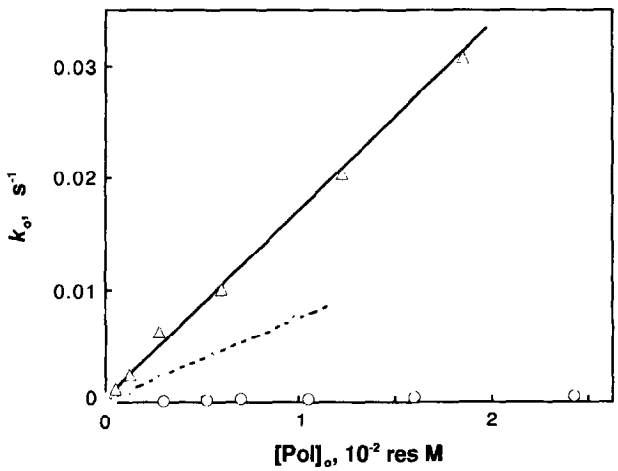


FIG. 3. Plot of k_0 against $[Pol]_0$ (expressed in monomer residue molar concentration, resM) for the deacylation of NNA by PEI (X), lauPEI (Δ), and Imaben-PEI (○). The slopes of the straight lines are 0.73 s^{-1} $resM^{-1}$ for PEI and 1.7 s^{-1} $resM^{-1}$ for lauPEI.

lauPEI ($K_m \gg$ the largest $[Pol]_0$ employed). For Imaben-PEI, the rate data manifest saturation kinetics (Fig. 4) although the deviation from linearity for the plot of k_0 against $[Pol]_0$ is not large.

DISCUSSION

For binding of Ni(II) ion by Imaben-PEI, two striking features are revealed: very tight complexation and binding of one Ni(II) ion for three Imaben residues. Binding of Ni(II) ion has been previously studied with Imaben derivatives which

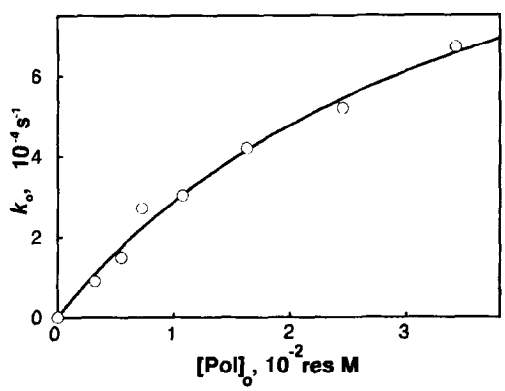
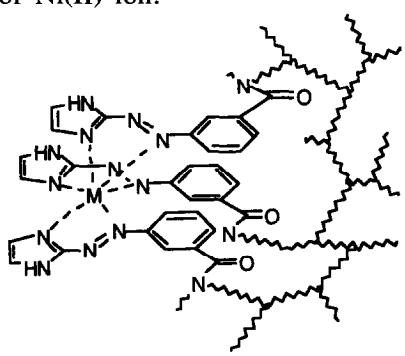


FIG. 4. Plot of k_0 against $[Pol]_0$ for the deacylation of NNA by Imaben-PEI. The curve is constructed according to Eq. [4] with k_{cat} = 0.0014 s^{-1} and K_m = 0.038 resM.

are not attached to polymers (*13, 19*). These studies indicated binding of one Imaben moiety to each Ni(II) with dissociation constants of about 10^{-3} M in water or 95% (v/v) DMSO. On the other hand, dissociation constant for Ni(II) ion bound to Imaben-PEI is $\ll 10^{-6}$ M.

The observed data are best explained by the complexation of Ni(II) ion to the aggregated Imaben moieties on the polymer surface (e.g., **I**). Binding of Ni(II) ion by more than one Imaben residues within the aggregates explains the very small dissociation constant. Ligation of six imine nitrogens to one Ni(II) as illustrated in **I** is consistent with the binding of one Ni(II) ion to three Imaben residues.[2] The binding data indicate that Imaben-PEI can be used as a scavenger of Ni(II) ion.[3]



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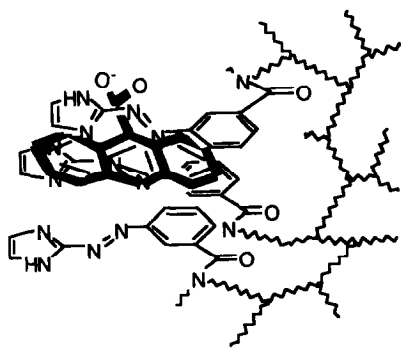


II

Binding of AC to Imaben-PEI is facilitated to a much greater extent compared with AC's binding to lauPEI. Each Imaben-PEI contains about 80–90 Imaben residues. For each molecule of Imaben-PEI, about 80–90 molecules of AC can be bound. Thus, about one AC can be bound per Imaben moiety.

Although the lauryl groups of lauPEI also form hydrophobic clusters (*20*), complexation of AC to lauPEI is negligible. Thus, hydrophobic interactions alone do not explain the efficient binding of AC to Imaben. Instead, the facilitated complexation of AC by Imaben may be taken to suggest that stacking interaction (e.g., **II**) is involved in the binding. This is also consistent with the complementary planar shapes of AC and Imaben.

The Hill-equation-type plot illustrated in Fig. 3 is frequently observed with allosteric proteins such as hemoglobin. The results of Fig. 3, therefore, can be taken to indicate that an initially bound AC facilitates subsequent binding of another AC. It is possible that the initially bound AC modifies the structure of the Imaben cluster so that the subsequent AC is more readily accommodated.

[2] It is possible that the Ni(II) ion is ligated by four nitrogens from two Imaben residues and electrostatic effects of the bound Ni(II) ion prevents binding of another Ni(II) ion in a proximal position resulting in the observed 1 : 3 stoichiometry. The possibility of ligation by four nitrogens from three Imaben residues is not excluded.

[3] When further Ni(II) is added in excess of the content of Imaben residues, additional Ni(II) ions may be bound to the ethylenediamine moieties of PEI (*10*).

Much slower rates were observed for the deacylation of NNA in the presence of Imaben-PEI compared with that of lauPEI or PEI. The scheme of Eq. [3] is kinetically equivalent to that of Eq. [5] (*10*). Failure to observe saturation kinetics indicates very large K_m ($K_m \gg C_0$; see Eqs. [4] and [6]) for lauPEI or PEI.

$$\begin{array}{ccc} S + C & \underset{K_m}{\rightleftharpoons} & CS \\ & \big\downarrow k_2 \qquad k_{com} \big\downarrow & \\ & \text{product} & \end{array} \qquad [5]$$

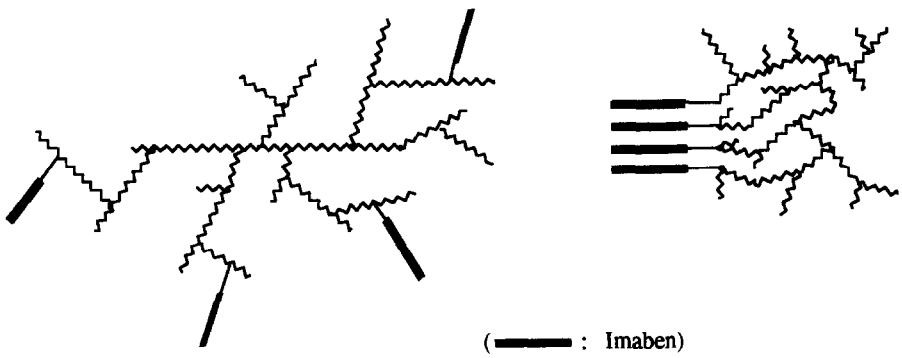
$$k_0 = (k_2K_m + k_{com})C_0/(K_m + C_0) \qquad [6]$$

Previous studies (*10, 11, 18, 21*) have indicated that PEI derivatives without any newly introduced nucleophilic groups deacylate activated esters through the attack of the amino groups of the polymers at the ester substrates. The proportionality between k_0 and C_0 observed for unmodified PEI is attributable to direct collision (k_2) between PEI and the ester ($k_2K_m \gg k_{com}$ in Eq. [6]) as revealed previously by comparing deacylation rates of PEI and propylamine (*21*).

The reactivity of lauPEI toward NNA is only about twice that of PEI. Laurylation of PEI occurs on primary amines (*21*), lowering the reactivity of the modified amines. Hydrophobic microenvironments obtained by laurylation, however, would retard protonation of the amines, raising the reactivity. Thus, the reactivity of lauPEI may be explained by the direct collision. It is, however, also possible that NNA is deacylated in the CS complex formed with lauPEI as implied previously (*22*). The hydrophobic microdomains of lauPEI can raise the effective concentrations of the hydrophobic ester and the amino groups of the polymer resulting in faster deacylation as in micellar catalysis.

The slow rates observed for Imaben-PEI indicate that the aminolysis (k_{com}) within the CS complex is not efficient. This may be taken to indicate the inaccessibility of the bound ester to the amino groups of the polymer. If the substrate is stacked (e.g., **II**) with the clustered Imaben moieties, the bound ester may be protected from the attack by the amino groups. Since the 2-nitronaphthyl and Imaben moieties have complementary shapes, it is likely that stacking interaction is involved in the complexation.

Under the experimental conditions, a significant fraction of NNA is uncomplexed to Imaben-PEI. Much slower deacylation of NNA by Imaben-PEI compared with that by PEI, therefore, indicates that the direct attack by the amino groups of the Imaben-PEI at NNA is much less efficient than that of PEI. If the Imaben portions of Imaben-PEI do not interact with one another, the polymer would be in an extended conformation (e.g., **III**). On the other hand, if the Imaben moieties forms tight aggregates as proposed by **I** and **II**, Imaben-PEI would have a compact conformation (e.g., **IV**). Since the compact conformation would allow lesser amino groups exposed on the exterior, direct attack by the amino groups at NNA would be slow.



III IV

Several kinds of microenvironments have been created on PEI. For example, hydrophobic environments have been obtained through attachment of alkyl chains by alkylation or acylation of amino groups of PEI. Laurylation is a typical example (*23*). When 4-imidazolylmethyl group is introduced, imidazole groups form hydrophobic domain (*24*). Recently, well-defined hydrophobic sites have been constructed on PEI by connecting β-cyclodextrin to PEI (*11*). In addition, highly cationic microdomains have been built by the formation of macrocyclic metal complexes on PEI (*10*).

In the present study, another unique apolar microdomain is obtained by aggregation of Imaben residues of Imaben-PEI. The aggregated Imaben moieties bind Ni(II) ion very strongly. The apolar domain of Imaben-PEI shows remarkable affinity for AC, a polycyclic aromatic compound, and stacking interaction appears to be important in this process of molecular recognition.

ACKNOWLEDGMENT

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