Transamination by Polymeric Enzyme Mimics

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This paper is dedicated to *Duilio Arigoni* in honor of his 75th birthday. He has been one of the great pioneers in bioorganic chemistry.

Pyridoxamine was linked to a series of polyethylenimines (PEIs) with $M_n = 600$, 1800, 10,000, and 60,000, both simply permethylated and with additional attached dodecyl chains. They were examined in the transamination of pyruvic acid and of phenylpyruvic acid, and showed *Michaelis–Menten* behavior. The values of k_2 and of K_M determined showed only small variations with polymer size. Thus, the previously reported strong advantage of pyridoxamines attached to the $M_n = 60{,}000$ PEI, relative to simple pyridoxamine alone, is seen to almost the same extent with the smaller PEIs.

Introduction. – Enzymes are proteins that are large in size relative to their active sites. One argument for this is necessity: biological information is linear in character (genetic sequences), while an effective catalyst must have a well-defined three-dimensional structure. For the spontaneous folding of a linear polypeptide into such a structure, significant size is needed. Since chemists can design in three dimensions, this idea suggests that the large size of natural enzymes might not be needed in an artificial synthesized enzyme mimic. However, there is at least one other feature of the large size of enzymes – they can be hydrophilic on the exterior, thus compatible with H_2O, while carrying out their catalyzed reaction in a nonaqueous interior.

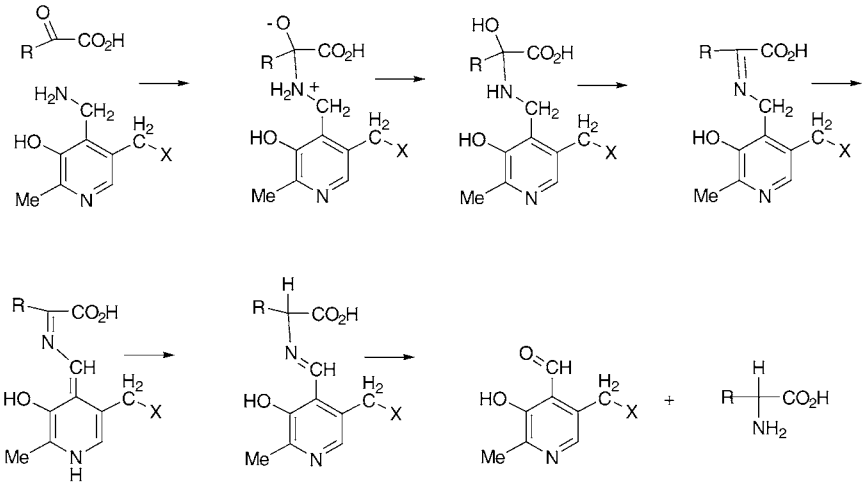
It is well-known in chemistry that a strongly H-bonding medium such as H_2O may slow reactions in which general acids and bases are involved when they must desolvate before reacting. For example, the much greater effectiveness of *t*-BuOK in dimethyl sulfoxide (DMSO) solution than in *t*-BuOH reflects the slowing effect of solvent H-bonding to the *t*-BuO^- ion. Thus, a protein can be thought of as a very large drop of DMF suspended in H_2O, using the H_2O for biological reasons and to promote hydrophobic binding of substrates into the enzyme while protecting the substrate and catalytic groups from deleterious H-bonds with H_2O. For this reason, there has been interest in examining synthetic polymers as enzyme mimics that might achieve this microscopic two-phase effect.

Irving Klotz pioneered the use of polyethylenimine (PEI) derivatives as enzyme mimics [1–7]. He showed that attachment of dodecyl groups and methyleneimidazole groups to PEI yielded a catalyst that was very effective in hydrolyzing 4-nitrophenyl acetate [1] and in the decarboxylation of isoxazole derivatives [3], and other derivatives were also effective in hydrolyzing activated esters and anilides [4–7]. He called these catalysts 'synzymes'. His reference [7] included *Junghun Suh* as a co-author, and *Suh* has pursued this area further.

Suh summarized his work in a recent account [8], and has used not only PEI [9] but also polystyrene [10] with appropriate attached basic groups. Recently, *Kirby* and co-workers have described studies on the 'synzymes' derived from PEI, catalyzing the ring opening of benzisoxazoles [11][12]. Although no one has attached pyridoxamine to PEI and other similar synthetic polymers previously, *Schultz* and co-workers attached it to an antibody [13], *Imperiali* and co-workers have attached it to peptides [14–17], and *Distefano* and co-workers have attached it to proteins [18–21]. Also, *Murakami* and co-workers have made an artificial transaminase by linking pyridoxamine to a membrane [22].

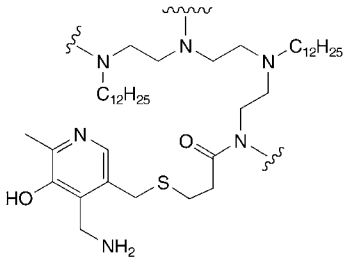
However, there were no previous examples in which a cofactor such as pyridoxamine was incorporated into such polymers, so we undertook such a study. The PEIs titrate between pH 3 and 13, and are half-protonated at pH 8. At such intermediate pH values, they have the strongest acid and base groups that can exist at equilibrium, and thus can be very potent general acids and general bases. In the transamination sequence (*Scheme 1*), there are many steps in which general acids and general bases function, so we expected and achieved some striking effects from the polymer.

Scheme 1

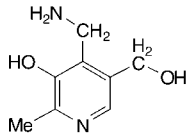


We have described the synthesis of a polymeric mimic of transaminase enzymes using commercial polyethylenimine (PEI) [23]. The polymer is highly branched and polydisperse, with a number-average molecular weight M_n of 60,000 and a weight-average molecular weight M_w of 750,000 (so the polydispersity index $M_w/M_n = 12.5$). (These molecular-weight values, supplied by the distributor, *Aldrich Chemical Company*, have been reconfirmed with the manufacturer. The M_n is obtained by gel-permeation chromatography, while the M_w is obtained by light scattering.) Based on the M_n value, the average polymer contains *ca.* 1400 monomer residues. About 25% of the amino groups of this PEI are primary, *ca.* 50% secondary, and the remaining tertiary.

We alkylated the polymer with lauryl iodide, acylated the product with the *N*-hydroxysuccinimide ester of 3,3′-dithiodipropionic acid, and reductively methylated the remaining primary and secondary amino groups with formaldehyde and sodium cyanoborohydride. The disulfide bonds of the polymer were reduced by $NaBH_4$ to liberate thiol groups. These were then *S*-alkylated with pyridoxamine carrying a $BrCH_2$ group in place of the $HOCH_2$ at C(5), to yield the final H_2O-soluble polymeric pyridoxamine reagent **1**. H-NMR Analysis indicated that 12% of the N-atoms of the polymer carried lauryl groups, and 29% carried propionylpyridoxamine groups. The elemental analysis (C/N ratios) indicated 11 and 22%, respectively, in reasonable agreement with the NMR results.



1



2

The polymeric compound **1** was used in the amination of keto acids and compared with the results using pyridoxamine **2** in H_2O. With the laurylated catalyst and pyruvic acid, the rate acceleration (per pyridoxamine unit) relative to pyridoxamine was 10,000 (at zero buffer concentration), and the polymer-bound pyridoxamine showed no buffer catalysis while pyridoxamine did. The N-atoms of the polymer – which titrate from pH 3 to 13 because of electrostatic repulsion, so at pH 8 there are both amine and ammonium groups acting as the strongest possible bases and acids – act as general acids and bases in the reaction. With simple pyridoxamine, the buffer plays this role. With chains shorter than lauryl, the polymeric pyridoxamine slowed, as much as by a factor of ten with Me groups replacing the lauryl groups.

The polymer system showed saturation effects when titrated with substrates, *Michaelis–Menten* kinetics; we have reported the k_2 and K_M values of different substrate-polymer reaction pairs [24]. The results are listed in *Tables 1* and *2*.

Table 1. Michaelis *Constants* ($k_2 \times 1000$ [min^{-1}], K_M [mM]) *for Transamination by the Polymeric Pyridoxamine Reagents Based on Polyethylenimine* (PEI) *with* $M_n = 60{,}000$ *with/without Added* C_{12} *Units*[a])

Reagent	Pyruvic acid		Phenylpyruvic acid	
	k_2	K_M	k_2	K_M
1, but lacking C_{12}	7.2 ± 0.7	12 ± 2	13 ± 1	27 ± 3
1 with C_{12}	290 ± 60	42 ± 6	370 ± 30	4.0 ± 0.6

[a]) Constants reported are measurements averaged from two trials and data analysis with *Eadie–Hofstree* and *Hanes–Wolff* kinetics approximations. Five or six distinct substrate concentrations were used to define the kinetics curve for each catalyst/substrate pair. Catalyst concentrations were determined by UV measurements (pyridoxamine, $\lambda = 324$ nm), ca 1.0×10^{-4} M, T = 40°, pH = 7.5 in H_2O with 0.25M HEPES buffer and 0.1M KCl.

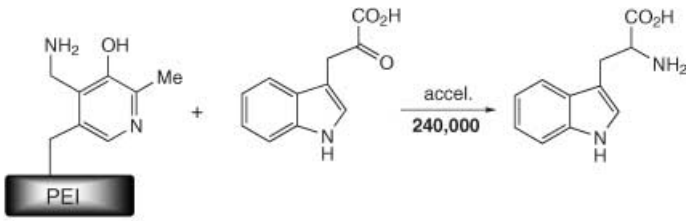
Table 2. Michaelis *Constants* ($k_2 \times 1000$ [min^{-1}], K_M [mM]) *for the PEI-Laurylated Transaminase Mimic* **1** *with Various Substrates*[a])

Substrate	k_2	K_M	k_2/K_M
Glyoxylic acid	92 ± 6	30 ± 5	3.1
Pyruvic acid	100 ± 0	28 ± 1	3.6
4-methyl-2-oxopentanoic acid	55 ± 4	13 ± 3	4.2
α-Ketoglutaric acid	120 ± 0	7.4 ± 0.9	16
Phenylpyruvic acid	200 ± 1	5.5 ± 0.6	36
Indole-3-pyruvic acid	110 ± 2	1.3 ± 0.2	85

[a]) Reported constants have been determined from UV measurement, $T = 30°$, pH = 7.5. All constants reported are measurements averaged from two trials and data analysis with *Eadie–Hofstree* and *Hanes–Wolff* kinetic approximations in H_2O with 0.25M HEPES buffer and 0.1M KCl. Five or six distinct substrate concentrations were used to define the kinetics curve for each catalyst/substrate pair. k_{cat}/K_M Values are in $min^{-1}\,M^{-1}$.

The added C_{12} chains in the catalyst modify both the k_2 constants and the substrate binding constants. The k_2 value increases with laurylation for all substrates, consistent with the idea that catalytic rates can increase in a nonpolar solvent. Substrate binding decreased with laurylation for rather polar pyruvate but increased for hydrophobic substrates such as phenylpyruvate. As a result, a pyridoxamine unit in a laurylated catalyst is 240,000-times faster than is pyridoxamine (*Scheme 2*) in reductively aminating indolepyruvate to tryptophan [24].

Scheme 2



The polyethylenimine we have used so far is quite large and quite polydisperse. Thus, we have investigated the properties of other available polymers in such artificial transaminating enzymes. For example, we have reported a transaminase mimic based on linear polyallylamine rather than branched PEI [23]. It was effective, but less so than the $M_n = 60{,}000$ branched PEI-based transaminase mimic. In this paper, we will describe the results of such mimics with smaller and more monodisperse PEIs. We have also examined new enzyme mimics based on some dendrimers, with defined sizes and shapes. These will be described elsewhere.

Results and Discussion. – Samples of three branched polyethylenimines were obtained from *Aldrich Chemical Company:* $M_n = 600$, $M_w = 800$; $M_n = 1800$, $M_w = 2000$; $M_n = 10{,}000$, $M_w = 25{,}000$. All of these are less polydisperse than is the previously examined $M_n = 60{,}000$ PEI. These were then converted to analogs of compound **1** by our standard procedure. As before and described above, we attached *ca.* 10% of lauryl groups using lauryl iodide, but, in this case, only *ca.* 5% of pyridoxamine units linked

through propionyl groups. We also prepared the same polymers without laurylation, which were, therefore, simply fully methylated. We prepared new samples of the polymers from the PEI of $M_n = 60{,}000$ and $M_w = 750{,}000$, again with and without laurylation. In a somewhat different purification procedure from that described previously (supporting information of [23][24]), we dialyzed the polymeric reagents at pH 9.0 and filtered the resulting solutions to remove precipitated polymers before lowering the pH to 7.5 and obtaining the polymeric reagents by freeze-drying the clear solutions. The polymers based on PEI $M_n = 600$ were not dialyzed, but purified by size-exclusion chromatography.

The analyses by NMR – examining the integrated ^{1}H ratios for the pyridoxamine and lauryl-group signals relative to the polymer-backbone signals – were as follows for the laurylated polymers: M_n 600: pyridoxamine $4.7 \pm 0.2\%$, lauryl groups $11.4 \pm 0.3\%$; M_n 1800: pyridoxamine $4.2 \pm 0.2\%$, lauryl groups $9.6 \pm 0.3\%$; M_n 10,000: pyridoxamine $4.1 \pm 0.2\%$, lauryl groups $9.1 \pm 0.3\%$; M_n 60,000: pyridoxamine $4.1 \pm 0.2\%$, lauryl groups $8.9 \pm 0.3\%$, for the polymers without lauryl groups: M_n 600: pyridoxamine $4.0 \pm 0.2\%$; M_n 1800: pyridoxamine $4.3 \pm 0.2\%$; M_n 10,000: pyridoxamine $4.0 \pm 0.2\%$; M_n 60,000: pyridoxamine $4.3 \pm 0.2\%$.

Then, the transamination of pyruvic acid was performed as follows: solutions of the polymer at 0.1 mM pyridoxamine were incubated with excess pyruvic acid at five different concentrations (2.5, 5.0, 7.5, 10.0, and 12.5 mM) and 0.25M HEPES buffer, 0.1M KCl, and 0.01M EDTA at pH 7.5 and 60°, and the UV spectrum for pyridoxamine (λ_{max} 324 nm) rapidly decreased and was replaced by the characteristic spectrum of pyridoxal (λ_{max} 385 nm). The pseudo-first-order rate constants from two independent runs at these five pyruvate concentrations were averaged and are listed in *Table 3*. Also in *Table 3* are the rate constants for the pyridoxamine polymers in which the laurylation step was omitted, and all the primary and secondary amino N-atoms of the polymer carry only Me groups. The reactions were performed at 60°, not at the lower temperature of the previous [23] study.

The data were analyzed by *Eadie–Hofstee* and *Hanes–Wolff* equations for *Michaelis–Menten* kinetics in which substrate binds to the polymeric reagent. These two data treatments gave slightly different values for k_2 and K_m, since they weight the points differently, so the values from the two treatments were averaged and are also listed in *Table 3*.

We also examined the reaction rates of our pyridoxamine polymers with phenylpyruvic acid, forming phenylalanine. In this case, high concentrations of substrate led to precipitation of some of the polymers carrying lauryl groups, so the phenylpyruvate concentrations were only 1.0, 2.0, 3.0, 4.0, and 5.0 mM. However, this change does not affect the values of k_2 and K_m, which are listed in *Table 3*. We also list the relative rate constants k_2 per pyridoxamine unit for the polymeric compounds relative to simple pyridoxamine itself in *Table 3*.

It is striking that the relative and absolute values of k_2 with pyruvic acid hardly increase as the unlaurylated polymers are larger, and that the increase with polymer size is also negligible in the laurylated cases. However, substrate binding reflected in K_M does increase in strength by a factor of three from the smallest to the largest polymer. With phenylpyruvic acid as substrate, there is almost no variation in binding constant, but a two- to three-fold increase in rate constant.

Table 3. *Pseudo-First-Order Rate Constants* [$\times 10^3 \text{min}^{-1}$] *for the Transamination Reaction between Various Polymeric Pyridoxamines and Pyruvic Acid or Phenylpyruvic Acid with Different Substrate Concentrations at 60°* [a])

Pyruvic acid							
Keto acid [mM]	2.5	5.0	7.5	10.0	12.5	K_M [mM]	k_2 [$\times 10^3$ min^{-1}]
PEI(600)	1.5 ± 0.1	3.7 ± 0.1	5.2 ± 0.1	6.5 ± 0.1	7.5 ± 0.1	$k_2/K_M = 0.6$ M^{-1}min^{-1}	
PEI(600)-C_{12}	3.4 ± 0.1	7.7 ± 0.2	11 ± 0.2	13 ± 0.2	16 ± 0.1	27 ± 0.5	50 ± 1
PEI(1800)	8.5 ± 0.5	16 ± 1	23 ± 1	30 ± 1	35 ± 1	47 ± 1	160 ± 5
PEI(1800)-C_{12}	12 ± 5	20 ± 1	28 ± 2	36 ± 2	40 ± 2	18 ± 0.5	100 ± 5
PEI(10,000)	11 ± 1	19 ± 2	27 ± 1	35 ± 1	38 ± 2	22 ± 1	110 ± 5
PEI(10,000)-C_{12}	22 ± 1	36 ± 2	46 ± 2	55 ± 2	63 ± 1	11 ± 0.5	120 ± 5
PEI(60,000)	12 ± 1	22 ± 1	13 ± 1	37 ± 2	41 ± 1	18 ± 1	100 ± 10
PEI(60,000)-C_{12}	26 ± 1	43 ± 2	52 ± 2	64 ± 2	71 ± 1	9.5 ± 0.3	120 ± 10
Phenylpyruvic acid							
Keto acid [mM]	1.0	2.0	3.0	4.0	5.0	K_M [mM]	k_2 [$\times 10^3$ min^{-1}]
PEI(600)	1.1 ± 0.1	2.7 ± 0.2	4.1 ± 0.2	5.0 ± 0.1	6.0 ± 0.1	$k_2/K_M = 0.6$ M^{-1}min^{-1}	
PEI(600)-C_{12}	9.5 ± 0.5	17 ± 1	22 ± 1	27 ± 1	31 ± 1	6.4 ± 0.1	70 ± 1
PEI(1800)	8.5 ± 0.5	18 ± 1	25 ± 1	30 ± 2	34 ± 1	11 ± 1.7	110 ± 10
PEI(1800)-C_{12}	18 ± 1	33 ± 2	50 ± 2	58 ± 1	63 ± 1	8.2 ± 0.5	170 ± 10
PEI(10,000)	12 ± 1	17 ± 1	29 ± 1	35 ± 2	38 ± 1	6.8 ± 1	110 ± 20
PEI(10,000)-C_{12}	30 ± 2	56 ± 2	78 ± 2	91 ± 2	100 ± 5	6.7 ± 0.5	240 ± 10
PEI(60,000)	12 ± 1	22 ± 1	32 ± 2	38 ± 1	43 ± 1	9.1 ± 0.1	120 ± 10
PEI(60,000)-C_{12}	39 ± 2	69 ± 2	89 ± 2	100 ± 2	110 ± 5	4.0 ± 0.1	200 ± 10

[a]) Reaction conditions: 0.1 mM pyridoxamine derivative with 0.25M HEPES buffer, 0.1M KCl, 0.01M EDTA, pH = 7.5.

In *Table 4*, we list the values of the ratio k_2/K_m, which corresponds to a second-order rate constant. It is clear that laurylation with all the polymers increases this value for phenylpyruvic acid more than for pyruvic acid, reflecting some hydrophobic binding of the phenylated substrate. However, again the variations in k_2/K_m values among the different polymers are not large, although they do reach maxima with the largest

Table 4. *The Second-Order Rate Constants* k_2/K_M *for the Transamination with Polymeric Pyridoxamines and with Simple Pyridoxamine at 60°* (rates relative to pyridoxamine in parentheses)

Reagent	Pyruvic acid	Phenylpyruvic acid
Pyridoxamine	0.004 ± 0.001 [a])	0.004 ± 0.001 [a])
PEI(600)	0.59 (150) [b])[c])	1.2 (300)
PEI(600)-C_{12}	1.8 (450)	10.9 (2,700)
PEI(1800)	3.3 (820)	10 (2,500)
PEI(1800)-C_{12}	5.6 (1,400)	20.7(5,700)
PEI(10,000)	5.0 (1,200)	16.2 (4,000)
PEI(10,000)-C_{12}	10.9 (2,700)	35.8 (9,000)
PEI(60,000)	5.6 (1,400)	13.2 (3,300)
PEI(60,000)-C_{12}	12.6 (3,200)	50 (12,000)

[a]) Extrapolated to zero buffer. [b]) These polymer values are not extrapolated to zero buffer, but the buffer effects are small. [c]) The values in parentheses are relative to the pyridoxamine value.

polymer. Thus, we conclude that there is not a major advantage to the use of the large polydisperse $M_n = 60{,}000$ PEI in our systems. The bulk of the polymer effect on transamination by pyridoxamine is seen with smaller more monodisperse polymers.

There is a cautionary note that needs to be sounded. The smaller polymers are indeed quite effective, but we have occasionally seen evidence of aggregation, and, indeed, that forced us to do the studies shown in *Table 3* at lower phenylpyruvate concentrations to prevent substrate-induced precipitation. Thus, the 'smaller' polymers might be operating as aggregates, and, thus, not be so small after all.

In our work with dendrimer-based pyridoxamines as enzyme mimics, being reported elsewhere, we see that there is an increase in rate as increased generations of the dendrimer are used. In the dendrimer case, computer simulations indicate that only with the higher generations is the pyridoxamine encased in a medium and not fully exposed to solvent H_2O. Something similar may be involved in the current study with PEIs of various sizes, but their structures are too poorly defined to be sure.

Conclusions. *1*) Conversion of pyruvic acid to alanine and of phenylpyruvic acid to phenylalanine by pyridoxamine is greatly accelerated, relative to simple pyridoxamine, when the pyridoxamines are attached to polyethylenimines (PEIs). *2*) Attachment of dodecyl chains to the pyridoxamine polymers increases their effectiveness, modifying both the binding constants and the catalytic rate constants. The binding is particularly increased with a hydrophobic phenylpyruvate substrate. *3*) The effects are seen with PEIs ranging from $M_n = 600$ up to 60,000, and the variation in polymer size does not strongly affect the polymer-induced acceleration. *4*) Thus, the smaller PEIs, with better defined sizes, are suitable for other explorations of the ability of synthetic polymers to mimic the role of the macromolecular character of natural enzymes in promoting enzyme reaction rates. *5*) With all the pyridoxamine-PEI reagents, the acceleration reflects the general acid/base contributions from the polymer and the rate advantage of a non-polar medium for the reactions.

We thank the *NIH* and *NSF* for support of this work. *Lei Liu* has been partially supported by a *Guthikonda* graduate fellowship.

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Received August 4, 2003