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Pyridoxal 5′-Phosphate Binding in Lysine-Modified PAMAM Dendrimers: A Biomimetic Approach

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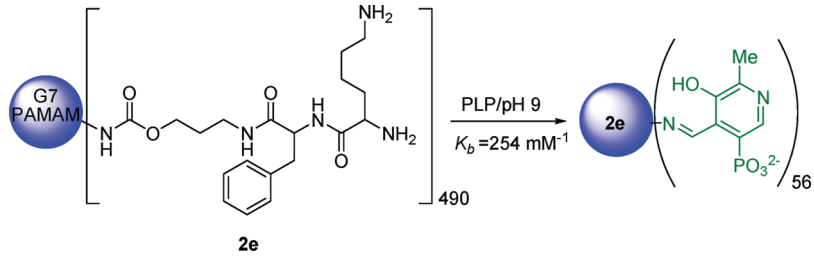
ABSTRACT



2e

(G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (2, APO = aminopropanol, Phe = phenylalanine, Lys = lysine) were prepared and used in a binding study with pyridoxal 5′-phosphate. The results revealed a positive dendritic effect, and binding ability was found to vary with the environment. (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (2) demonstrated better binding ability at higher pH, and protonation of lysine was considered to affect binding. The strongest binding affinity was K_b = 254.3 mM^{-1} at pH 9, which was shown by (G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$ (2e).

Pyridoxal 5′-phosphate (PLP) is the major active component in six vitamin B_6 metabolites and is crucial for the activities of a variety of enzymes.[1] PLP can regulate a number of biological functions, including metabolism of amino acids, replenishment of one carbon unit, and synthesis of amino sugars. Moreover, PLP is also involved in the metabolism of homocysteine[2] and polyunsaturated fatty acids.[3] In addition to its role in enzymatic reactions, levels of B_6 and/or PLP are associated with a variety of physiological responses, such as calcium ion flux.[4] In addition, PLP serves as either a biomarker or regulator of different syndromes and diseases;[5] PLP has been shown to prevent progression of diabetic nephropathy,[6] to inhibit apoptosis,[7] and to assist the recovery from the damage caused by reperfusion after

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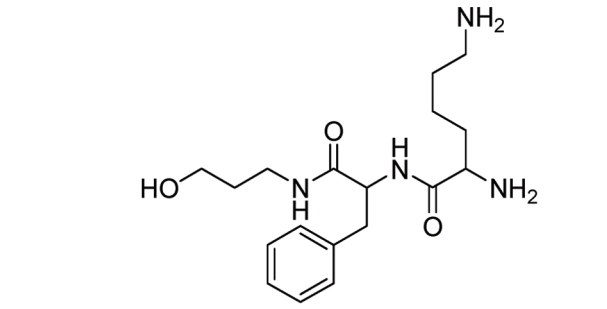
ischemia.[8] Although PLP is involved in a variety of enzymatic reactions, the reaction mechanisms share common features. In PLP-associated enzymes, PLP is usually bound to lysine residues in proteins, forming a Schiff base, and this species is known as an internal aldimine. Binding of the holoenzyme to inbound substrate causes the formation of an external aldimine, in which PLP forms a new imine bond with the substrate and carries out the certain enzymatic reactions. The exchange between internal and external aldimine is important for the progress of enzymatic reactions involving PLP. In addition, because of the aromaticity of PLP, phenylalanine, tyrosine, and tryptophan residues can improve the binding of PLP to protein.[9]

Dendrimers are globular and monospheric polymers that are produced by an iterative process.[10] A dendrimer thus possesses a well-defined architecture with a high number of end groups and tunable physical properties. These unique characteristics make dendrimers attractive platforms for execution of desired biological activities,[11] including roles as cell-membrane transporters[12] and as carriers of drugs[13] and imaging reagents.[14] In addition, because dendrimer sizes and shapes are similar to those of proteins, they are often referred to as "artificial proteins".[15] Several studies have examined dendrimers associated with pyridoxal or pyridoxamine,[16] and the studies revealed that noncovalent linkages between cofactors and dendrimers are better than nonexchangable covalent linkages in terms of biological activity.[17] To achieve reversible binding, hydrophobic interactions are widely used. Therefore, natural PLP has to be functionalized with a hydrophobic chain, and extra modifications must be made before PLP can be employed in the desired reactions.



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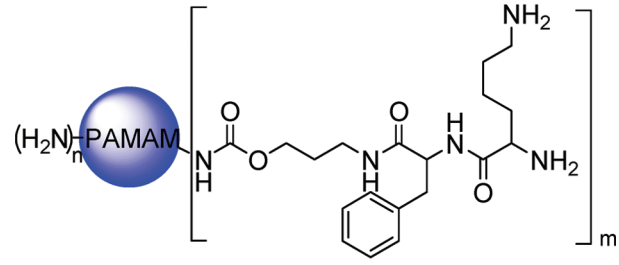

2a,(G:3)-*dendri*-PAMAM-(APO-Phe-Lys)$_{32}$
2b,(G:4)-*dendri*-PAMAM-(APO-Phe-Lys)$_{59}$
2c,(G:5)-*dendri*-PAMAM-(APO-Phe-Lys)$_{119}$
2d,(G:6)-*dendri*-PAMAM-(APO-Phe-Lys)$_{253}$
2e,(G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$

Figure 1. Designed peptide **1** and the synthetic dendrimers **2**.

In contrast, we suggest that a dendrimer which binds unmodified PLP in a more natural manner may shorten the effort of construction and improve catalytic activity.

To achieve reversible binding, physical entrapment of guest molecules by dendrimers might be desirable. Such a construction is known as a "dendritic box",[18] in which a variety of interaction forces are applied to prevent guest molecules from escaping. However, release of the box contents will be difficult if the box has a strong binding affinity for the guest molecules; this system is therefore not suitable for our purpose.[19] Thus, we considered the dendritic effect, which is another important feature of dendrimers.[20] The dendritic effect is a complex phenomenon usually demonstrated by dendrimers. Two major factors have been proposed to contribute to the dendritic effect. One is a clustering of reaction centers caused by the dense distribution of functional groups in dendrimers. The other is the creation of a nanoenvironment for preorganization of reaction sites; as a consequence, the desired reaction is improved. Reymond and colleagues have demonstrated the application of a positive dendritic effect in the construction of enzyme models.[21] We postulated that a suitably designed dendrimer,

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with sufficient numbers of surface functional groups, would be able to bind PLP to form natural aldimine moieties. The polyamidoamine (PAMAM) dendrimer is widely used, and in this molecule, both the tertiary amine and the secondary amide can be either hydrogen-bond donors or acceptors. This feature, along with the amide skeleton, makes the PAMAM dendrimer a good platform with which to demonstrate protein function.[22] Furthermore, a recent study indicated that the conformation and density of PAMAM dendrimers were flexible depending on their surroundings.[23] These characteristics make the PAMAM dendrimer similar to enzymes; therefore, the PAMAM dendrimer was used as a core structure in this study. Lysine-containing peptides (**1**, Figure 1) were introduced onto the surface of a PAMAM dendrimers in which lysine was anticipated to play the same role as in PLP-associated enzymes. In addition, we integrated a phenylalanine residue to increase the binding affinity of PLP using $\pi-\pi$ interaction.

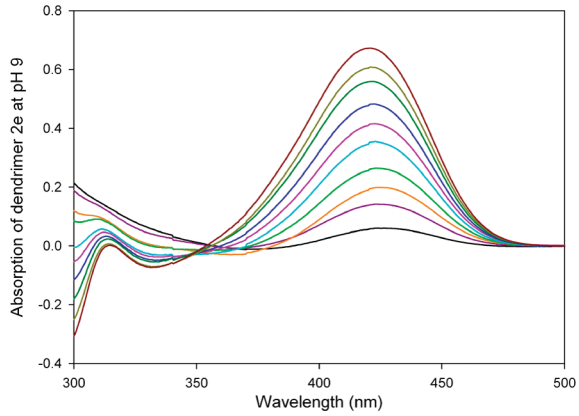

Figure 2. Difference spectrum of PLP and (G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$ **2e** with dendrimer vs PLP at pH 9. The concentration of dendrimer **2e** was 0.07 mM, and the concentration of PLP was varied from 0 to 0.014 mM.

The detailed procedure for synthesizing (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2a**−**e**, APO = aminopropanol, Phe = phenylalanine, Lys = lysine) is reported in the Supporting Information. The products were identified by NMR and MALDI-mass spectrometry. The degrees of substitution of the modified PAMAM dendrimer were 32, 59, 119, 253, and 490 for **2a**−**e**, respectively, and the polydispersities are narrow. The PDI values are 1.05, 1.12, 1.09, 1.07, and 1.14 for **2a**-**e**, respectively. To examine the UV spectra of free and bound PLP, free lysine was titrated with different concentrations of PLP, and UV spectra were recorded and individually analyzed at pH 5, 7, and 9. The UV spectrum of free PLP showed two absorption maxima at 325 and 388 nm. Upon mixing with lysine, a peak at 422 nm, which is correlated with the presence of aldimine, appeared, and the peaks at 325 and 388 nm disappeared.[24] Thereafter, (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**) were titrated with different concentrations of PLP at three different pH values. The difference spectra of PLP and (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**) versus PLP were used to determine binding affinities (Figure 2).

At pH 5, neither a growth nor decline in absorption was identified in difference spectra, except in those of (G:4)-*dendri*-PAMAM-(APO-Phe-Lys)$_{59}$ (**2b**). The difference spectrum of dendrimer **2b** at pH 5 clearly showed a reduction of intensity at 388 nm but enhancement of the peak at 325 nm, and no change in intensity was found between 420 and 450 nm. This implied that the addition of (G:4)-*dendri*-PAMAM-(APO-Phe-Lys)$_{59}$ did not cause the formation of bound PLP at pH 5; rather, it only contributed to the equivalent of two free PLP moieties. Presumably, the low pH environment led to protonation of lysine residues of (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**) and, consequently, prevented them from forming imine bonds with PLP.

At pH 7 and 9, an obvious decrease in absorption at 388 nm accompanied by a rise in absorption around 430 nm was observed. This finding was the first evidence of the existence of bound PLP. A Scatchard−Klotz plot was applied to determine the binding of PLP and (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**). The results are presented in Figure 3

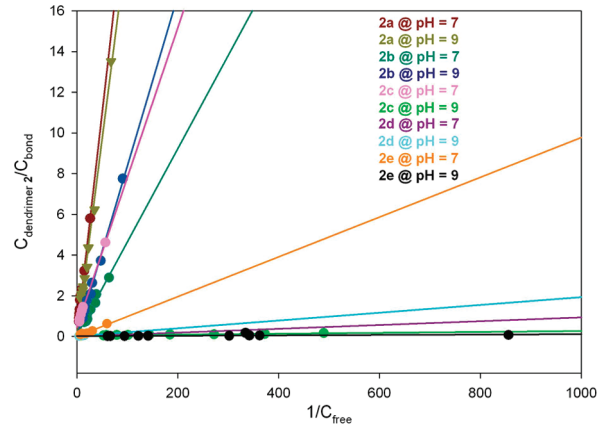

Figure 3. Scatchard−Klotz plots of **2a**−**e** at pH 7 and 9.

and Table 1. The linearity of the plot indicated that only one type of binding site existed. This fact is consistent with our original assumption that the PLPs were assumed to bind to lysine residues. In addition to (G:6)-*dendri*-PAMAM-(APO-Phe-Lys)$_{253}$ (**2d**), four other generations of dendrimers showed better binding ability in basic than in neutral solution, except for the binding of (G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$ (**2e**). This finding indicates that the binding behavior of dendrimer **2** was affected by the environment. Remarkably, (G:6)-*dendri*-PAMAM-(APO-Phe-Lys)$_{253}$ (**2d**) exhibited relatively low binding affinity and binding number. A

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(24) The extinction coefficient is 3052.5, 3083, and 3094.2 M^{-1} cm^{-1} at pH 5, 7, and 9.

Table 1. Binding Constant (K_b) and Binding Number (n) of **2** at pH 7 and 9

dendrimer	pH	K_b (mM^{-1})	n
2a	7	0.37	12.5
	9	1.34	16.3
2b	7	0.15	35.5
	9	0.31	39.1
2c	7	6.49	2.1
	9	242.01	20.7
2d	7	14.33	77.5
	9	7.79	67.6
2e	7	0.78	129.9
	9	254.30	56.2

detailed examination of the UV/vis spectra of this dendrimer indicated no obvious isosbestic point. Presumably, one or more species intervene between the exchange of the internal aldimine and external aldimine of **2d**. Therefore, the binding constant is likely to be underestimated, despite high correlation coefficients shown by Scatchard−Klotz analysis (R^2 = 0.98 at pH 7 and 0.97 at pH 9).

Moreover, the (G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$ (**2e**) demonstrated a higher binding number and a stronger binding constant than did any of the lower generation analogues. This result confirms our hypothesis that increases in the number of end groups and dendrimer size improves binding ability. The higher generation of dendrimer structure increases the area density of branching and improves preorganization, which results in stronger binding affinity. In comparison, when either free peptide **1** or (G:3−7)-*dendri*-PAMAM-$(NH_2)_x$ (X = 32, 64, 128, 256, or 512 for G2−7, respectively) was analyzed in the same manner, a peak between 420 and 440 was observed in the UV/vis spectra. However, Scatchard−Klotz analysis yielded no reliable statistical result because of a low correlation coefficient (Table S2, Supporting Information). Therefore, we conclude that no obvious binding exists between PLP and either peptide **1** or free PAMAM dendrimer at the tested concentrations. We thus believe that the binding behavior of (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**) results from cooperation of the PAMAM dendrimer and the anchoring ability of synthetic peptide **1**.

In summary, this study has demonstrated for the first time that peptide-conjugated PAMAM dendrimers can bind natural PLP via imines bonds to form a natural aldimine. Furthermore, we have shown that the pH environment is critical with respect to binding affinity. In acidic solution, no obvious binding was seen, and the binding affinity is higher at high pH. The binding ability showed a positive trend with increasing dendrimer complexity, except in the case of (G:6)-*dendri*-PAMAM-(APO-Phe-Lys)$_{253}$ (**2d**). The highest binding constant measured was 254.3 mM^{-1} with dendrimer (G:7)-*dendri*-PAMAM-(APO-Phe-Lys)$_{490}$ (**2e**) at pH 9. Because only one type of binding site existed, and as no obvious binding was seen with unmodified (G:3−7)-*dendri*-PAMAM-$(NH_2)_x$, the lysine residue in (G:3−7)-*dendri*-PAMAM-(APO-Phe-Lys)$_x$ (**2**) is suggested to serve as the binding site for PLP. The positive relationship between the size of the dendrimer and the stability of the imine bond may assist in the development of new carrier systems for medical applications. Moreover, this discovery sets the stage for a future investigation of the application of peptide-conjugated PAMAMs as artificial enzymes demonstrating useful biological activities.

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Supporting Information Available: Detailed synthetic procedure for dendrimer **2**, characterization spectra for synthetic compounds, and additional UV spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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