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Schiff bases formed between pyridoxal 5′-Phosphate and Amino-β-cyclodextrins: Intramolecular remote ion pair interactions of the phosphate with ammonium moieties

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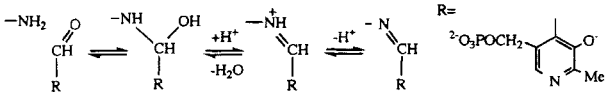
Keywords: Equilibrium binding constants, Pyridoxal 5′-phosphate, 6-Amino-β-cyclodextrins, Ion pair interactions, CD spectra.

Equilibria involved in the Schiff base formations of pyridoxal 5′-phosphate (PLP) and 5′-deoxypyridoxal with mono-, di-, and peraminocyclodextrins have been studied to determine equilibrium binding constants in aqueous media at several pH values. These results, as well as the circular dichroism study, show that remote electrostatic interactions between the negatively charged 5′-phosphate group and a second ammonium group on the cyclodextrins play a significant role in the complexation.

INTRODUCTION

Pyridoxal 5′-phosphate (PLP) is an obligatory cofactor for transaminase, aldolase, and α-decarboxylase, which involve formation of the Schiff base between an



enzyme-lysine residue and the aldehyde group of PLP in the initial stage of the reactions.[1] The tight binding of PLP by enzyme proteins results from the covalent C=N double bond as well as the summation of several substituent interactions which are not available with a simple alkylamine. In such PLP-dependent enzymes engaging in a covalent imine linkage formation, 20–40 times greater noncovalent contribution to the stability of the PLP-cofactor complex has been reported.[2] Thus, characteristic features of the imine double bond formation between PLP and amines[3] or amino acids[4] have been intensively investigated. Particularly, functions of the ε-amino group of the lysine residue in the enzyme catalytic site have been studied in detail by using model systems composed of poly(L-lysine) or poly(aminopropylene)[5,6] and PLP or the phosphate group-free 5′-deoxypyridoxal (DPL),[4] since several imine forming enzymes such as acetoacetate decarboxylase have been demonstrated to have a second lysine group close to the active site,[7] which acts to lower the pKa value of the active lysine forming the imine.[8,9]

As mentioned above, there are multipoint interactions between an apoenzyme and the coenzyme, i.e. hydrogen-bonding and/or electrostatic forces involving the phenolic OH group and the imine N-atom or other types of interactions that may include groups in the side chains or the peptide main chains. However, no interactions including the phosphate group have been explicitly implicated thus far. For example, García del Vado and his coworkers reported that the stabilities of the Schiff bases of poly(L-lysine) with PLP and pyridoxal are virtually identical to each other.[5] This means that there is no definite work demonstrating the importance of the phosphate group of PLP for electrostatic stabilization in PLP complex formation. Electrostatic interactions may add stability to the complexation, although they are of secondary importance. In this work special attention will be given to the influence of a second, remote amino group on the formation of the covalent imine linkage between PLP or its deoxy derivative and several types of amines. In order to carry out our study from such a viewpoint, cyclodextrin-based amines were selected. These model systems are more sophisticated than those employed in previous

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investigations, since the amino groups are held in the cis position to each other on the rim of a cyclodextrin (CD).

EXPERIMENTAL SECTION

Materials

Pyridine was refluxed over CaH for three hours and then distilled under nitrogen. p-Toluenesulfonyl chloride was recrystallized twice from hexane prior to use. 4,4′-Biphenyldisulfonyl chloride, commercially available from Aldrich, was recrystallized from benzene-hexane and dried over P_2O_5 for twenty four hours at 40°C under vacuum. MeCN was distilled over P_2O_5. β-Cyclodextrin (β-CD) purchased from Tokyo Kasei Co. (Japan) was vacuum-dried over P_2O_5 at 110°C. PLP was purchased from Wako Pure Chemical Ind. (Japan) and used as received.

Mono[6-deoxy-6-(p-toluenesulfonyl)]-β-CD (β-CD-OTs) was prepared according to the literature procedure with a minor modification[10]: β-CD (10.0 g, 8.8 mmol) was allowed to react with p-toluenesulfonyl chloride (2.0 g, 10.5 mmol) in 300 mL pyridine for twenty four hours at room temperature. Evaporation of the pyridine under reduced pressure at 30°C gave a white solid, which was washed with a copious amount of ethyl ether and then recrystallized twice from 300 mL of water (80°C). Filtration and vacuum-drying at 50°C of the resulting white solid afforded 2.19 g (19.3%) of β-CD-OTs: dec. 170–178°C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.48 (s, 3H, Me), 3.2–4.5 (m, 63H), 4.85 (s, 7H, CH), 5.71 (m, 14H, sec-OH), 7.45, 7.78 (all m, 2H each, arom CH), 7.78 (m, 2H, arom CH). TLC on silica gel (PrOH-AcOEt-H_2O-NH_3, 5:3:3:1 by volume) showed a single spot at R_f=0.5, anisaldehyde test positive. However, as HPLC on an octadecylsilyl silica gel (ODS) column (DMF-H_2O, 1:1 V/V) indicated that the product was contaminated with only traces of β-CD and pyridine, further purification was performed by preparative HPLC (LC-908 Recycling Preparative HPLC, Japan Analytical Inc.) with the same eluent as described above, giving the desired product of 100% purity.

Heptakis(6-deoxy-6-bromo)-β-CD was prepared by bromination with excess $MeSO_2Br$ in DMF according to the literature,[11] and characterized by ^{13}C NMR spectrometry.

Heptakis(6-deoxy-6-amino-2,3-di-O-methyl)-β-cyclodextrin (PACD: 1) was synthesized from the corresponding 6-perbromocyclodextrin by its iodonation with NaI/DMF, azidonation with NaN_3/DMF, methylation with NaH/MeI/DMF, and reduction with PPh_3/aq. NH_3 in this order according to the literature method.[12] The same procedures, excluding the methylation, were adopted to prepare heptakis(6-deoxy-6-amino)-β-cyclodextrin (HACD: 2). After complete removal of volatile materials, $CHCl_3$ was added to the residue, and the product was extracted with dilute hydrochloric acid. Lyophilization of the aqueous solution gave the hydrochloride of 2, which was further applied on a short Sephadex G-15 column to remove excess hydrogen chloride, affording the desired product as the hydrochloride adduct.

6,6′-Deoxy-6,6′-A,D-diamino-β-cyclodextrin (ADCD: 4), a new compound, was synthesized in a manner analogous to that adopted for preparation of 2 with only a minor modification, as follows: to a stirred solution of β-CD (18.8g; 16.6 mmol) in 500 mL pyridine under nitrogen atmosphere at 30°C was added 4,4′-biphenyldisulfonyl chloride (5.0 g; 14.2 mmol) in a portionwise fashion. The reaction mixture was then stirred at 30°C for three hours. The solvent (pyridine) was removed *in vacuo* at 40°C, and the oily residue was added dropwise to 300 mL of MeCN-H_2O (1:5.5 V/V) with vigorous stirring to give white precipitates. After the precipitates were removed by filtration, the solvent was distilled off from the filtrate. The resulting solid was thoroughly washed with a copious amount of ethyl ether to give 3.7 g of the crude product, which was subsequently purified by HPLC (LC-908 Recycling Preparative HPLC, Japan Analytical Inc.) on an ODS column with MeCN-H_2O (1:4 V/V) as eluent, giving 3.2 g (2.3 mmol) of the desired product, 4,4′-diphenyldisulfonyl-A,D-β–cyclodextrin (A,D-capped β-CD), upon removal of the solvents by lyophilization; a single spot on TLC, anisaldehyde test positive.[13] To a solution of 3.0 g of A,D-capped β-CD in 30 mL of DMF was added 3.6 g of pulverized KI, and the mixture was stirred for twenty hours at 80 °C under nitrogen. The solvent was removed at 30 °C under reduced pressure, the resulting solid was dissolved in 40 mL of H_2O, and 2 mL of tetracholoethylene was added to the aqueous solution with stirring at 0 °C to afford a white precipitate. The precipitate was removed by centrifugation and filtration followed by washing with H_2O and vacuum-drying of the resulting solid to give 2.55 g (88.2%) of A,D-diiodo-β-CD; no UV absorption at 270 nm. A,D-Diiodo-β-CD (2.4 g, 1.77 mmol) and NaN_3 (1.15 g, 17.7 mmol) were dissolved in 30 mL of DMF, and the mixture was heated at 65 °C for twenty four hours with stirring under nitrogen. Then, the solvent was removed, and the residue in H_2O was reprecipitated upon addition of tetrachloroethylene at 0 °C. The precipitate was recovered by filtration and vacuum-dried over P_2O_5 to give diazido-β-CD; 1.98 g (94.4 %), a single IR peak at 2,100 cm^{-1} due to the N_3 group. The diazido derivative (800 mg; 0.68 mmol) in 50 mL DMF was treated with triphenylphosphine (178 mg; 6.8 mmol) for two hours and then 3 mL of 30 % aq. NH_3 for twenty hours at room temperature. After removal of volatile materials

and acidification with dilute hydrochloric acid, the unreacted triphenylphosphine and phosphine oxide produced were completely extracted with chloroform. Lyophilization of the aqueous solution gave the hydrochloride of A,D-diamino-β-CD, which was subsequently applied on a short Sephadex G-15 column for purification. The hydrochloride adduct was further applied on an ion exchange resin column gave the HCl-free AD-amino derivative (4); mp. 148–151°C; a single spot at R_f=0.21 by TLC (BuOH-EtOH-H_2O, 5:4:3 by volume); no IR peak at 2,100cm^{-1}; MS (pos. FAB) m/e 1133.1 (calcd. for $C_{42}H_{72}N_2O_{33}$: 1133); Anal Calcd. for $C_{42}H_{72}N_2O_{33}$•$5H_2O$: C, 41.24;H, 6.75; N, 2.29. Found: C, 41.22; H, 6.50; N, 2.08.

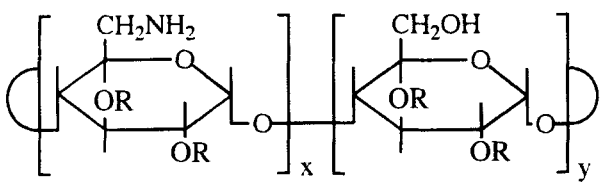
6,6′-Deoxy-6,6′-A,B-diamino-β-cyclodextrin (ABCD: 3) as the hydrochloride was prepared from 1,4-dimethoxyphenyl-2,5-disulfonyl-A,B-β-cyclodextrin according to the same method as applied to purification of the AD isomer[13]; MS (pos. FAB) m/e 1133.2 (calcd. for $C_{42}H_{72}O_{33}N_2$: 1133); Anal Calcd. for $C_{42}H_{72}N_2O_{33}$ •2HCl•$5H_2O$: C, 38.39; H, 6.59; N, 2.13. Found: C, 38.43; H, 6.48; N, 1.87.

Mono(6-deoxy-6-amino)-β-cyclodextrin (MACD: 5) in the HCl-free form was prepared from mono[6-deoxy-(p-toluenesulfonyl)]-β–cyclodextrin by iodonation, azidonation and then PPh_3 reduction according to a previously reported procedure[14]; MS (pos. FAB) m/e 1134.3 (calcd. for $C_{42}H_{71}NO_{34}$: 1134); Anal Calcd. for $C_{42}H_{71}NO_{34}$•$3H_2O$: C, 42.46;H, 6.53; N, 1.17. Found: C, 42.65; H, 6.60; N, 0.93.

5′-Deoxypyridoxal (DPL: 6) was synthesized from pyridoxine hydrochloride according to a previous method.[15]

Physical measurements A stock solution (0.1 M) of PLP was prepared in distilled water. Aliquot amounts of the stock PLP solution, 0.1 M acetate or phosphate buffer, and aqueous KCl solution were mixed, and an ionic strength of the resulting solution was maintained at 0.1 with KCl. To this solution was added an appropriate amount of an amine stock solution, and the solution was mixed at 25°C to establish an equilibrium quickly.

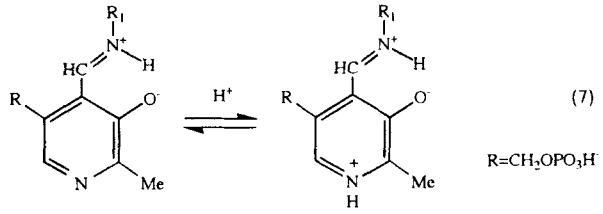
PACD (1) : x=7; y=0; R=Me
HACD (2) : x=7; y=0; R=H
ABCD (3) : x=2; y=5; R=H (HCl form)
ADCD (4) : x=2; y=5; R=H
MACD (5) : x=1; y=6; R=H



Electronic absorption spectra were run on a Hitachi the 220A spectrophotometer. The circular dichroism spectra were recorded on a JASCO J-720 spectropolarimeter under the same conditions as adopted for the UV-vis spectral measurements.

RESULTS AND DISCUSSION

As a typical experiment, 10^{-4} M PLP in an 0.1 M acetate, phosphate, or carbonate buffer was titrated with an amine dissolved in the same buffer. The equilibrium was attained rapidly, and caused an increase in concentration of the Schiff base immediately after an amine solution was added to the PLP solution. The Schiff base species of PLP, irrespective of protonation status of the pyridine nitrogen [i.e. protonated or unprotonated (7)], showed an absorption maximum (λ max) at 410–415 nm over the pH range 2.0–9.5 with an isosbestic point at 400–410 nm, depending on the properties of the amine used.[3] PLP itself gave a band maximum at 388 nm in the pH range



4.5–9.5 and the maximum shifted to 290 nm at a solution pH less than 4.0.

On progressive addition of an amine in excess of PLP, the absorbance at 450 nm (A_{450}) increased, at which no other absorption except for the Schiff bases was overlapping. An increase in the absorbance was monophasic and reached a saturation level along with progressive in-

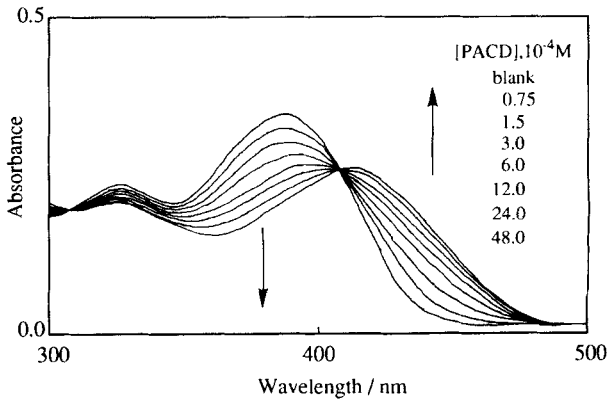


Figure 1 Electronic spectral change of PLP as titrated with PACD at pH 5.; PLP concentration, 1.0x10^{-4} M.

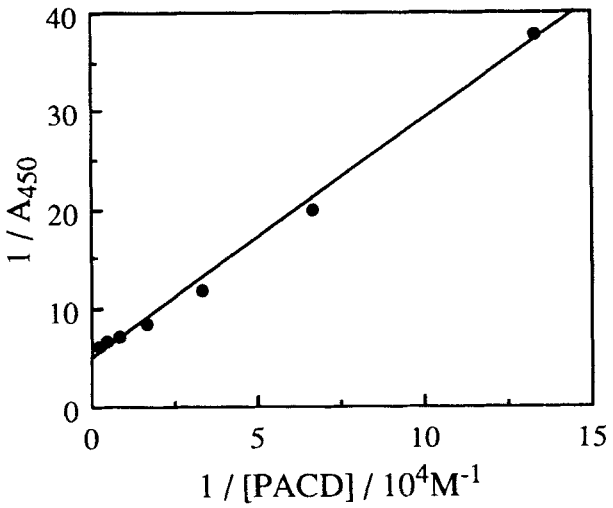


Figure 2 Double-reciprocal plot of the absorbance at 450 nm vs. PACD concentration.

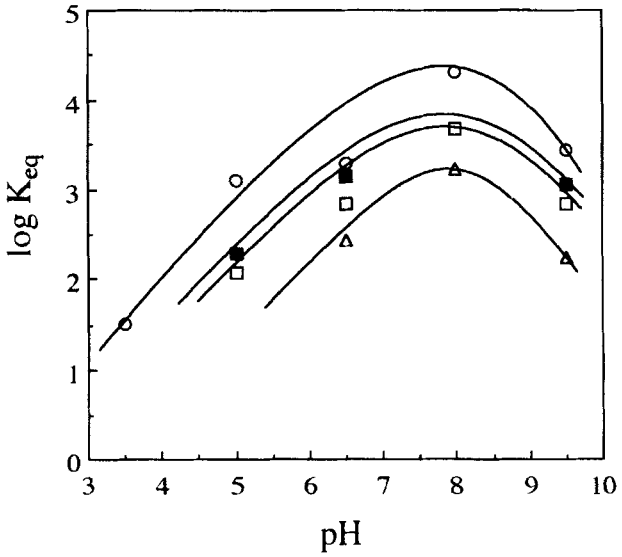


Figure 4 Correlations between log K_{eq} and pH for PLP systems: ○, PACD (1); □, ADCD (4); ■, ABCD (3); △, MACD (5).

crease of an amine concentration over the pH range of 2.0–9.5 in most, if not all, cases examined, and was accompanied by a decrease in the PLP absorbance. An equilibrium between PLP and a simple amine, such as n-butylamine ($BuNH_2$) or 1,4-diaminobutane, was also examined for comparison. By utilizing such amines as having low equilibrium constants (K_{eq}) with PLP, the absorbance was not saturated even when an amine concentration was greater then 0.1 M. Therefore, we were not able to determine exactly absorption maxima (λ max) and intensities (ε max) due to the corresponding Schiff bases for such cases. Figure 1 displays a typical spectral change for titration of PLP with PACD at pH 4.93. In general, an equilibrium concentration of the carbinolamine intermediate was very low under the experimental conditions and could be neglected in calculation of the apparent equilibrium binding constant (K_{eq}). The K_{eq} value was determined by a double-reciprocal plot of absorbance against amine concentration; plotting $1/A_{450}$ vs. 1/[amine] gives an intercept of $1/A_{max}$ on the y axis, and the slope of the line is $1/K_{eq}A_{max}$. A typical example of the plotting is shown in Figure 2. The data thus obtained are displayed in Figure 3. The results with DPL are given in Figure 4.

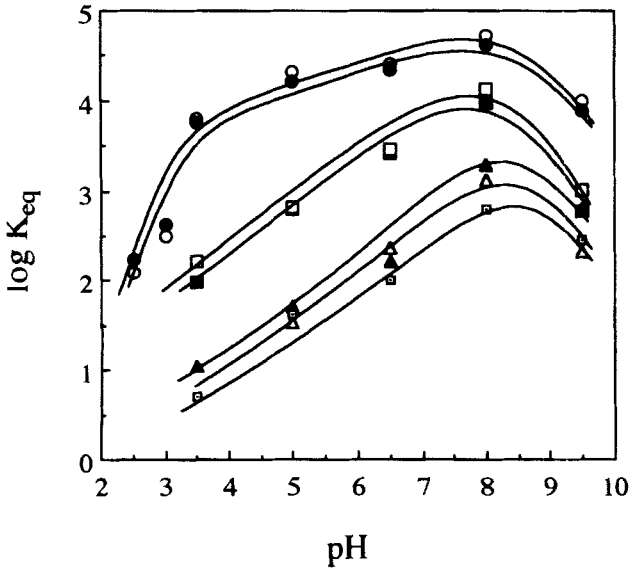


Figure 3 Correlations between log K_{eq} and pH for PLP systems: ○, PACD (1); ●, HACD (2); □, ADCD (4); ■, ABCD (3); △, MACD (5); ▲, 1, 4-diaminobutane; □, $BuNH_2$.

The K_{eq} values for PLP were found in the decreasing order: PACD > ADCD > ABCD > 1,4-diaminobutane > MACD > n-butylamine over the pH range examined, and the situation was essentially the same for DPL; in the series of the cyclodextrin derivatives the K_{eq} values increased with an increasing number of the amino substituent.

Unfortunately, owing to a limited number of available data it is rather difficult to evaluate individual thermodynamic parameters which comprise the overall stabilization increments more quantitatively. Nevertheless, the relative contribution of hydrophobic and electrostatic forces for the Schiff base formation can be estimated on the basis of careful comparisons of the obtained data in order to understand the origin of an extra stabilization energy.

It has been generally accepted that more basic amines tend to yield more stable imine bonds.[5] However, the PLP-Schiff base of less basic MACD (pKa 8.23) is more stable than that of more basic $BuNH_2$ (pKa 10.5). This stability trend is against an anticipated direction; namely,

the ability of MACD to form the Schiff base is not fully reconciled with its basicity. The hydrophobic effect caused by the cyclodextrin skeleton seems to prevail over the basicity effect. In fact, it was reported that an imine linkage is stabilized by the presence of a bulky non-polar group surrounding the imine double bond and by use of non-polar solvents.[6] Furthermore, as indicated by comparison of MACD/PLP with $BuNH_2$/PLP (an equilibrium system between, for example, MACD and PLP is abbreviated as MACD/PLP), the difference in Gibbs free energy of the stabilization coming from the cyclodextrin moiety is 0.46 kcal/mol at pH 8, and the corresponding difference between MACD/DPL and $BuNH_2$/DPL is 0.39 kcal/mol. These two values are quite close, and they could probably be identical within an estimated experimental uncertainty of ±0.1, indicating also that their identity is ascribed to nearly equal hydrophobic interactions in the MACD/PLP and MACD/DPL complexation.

On the other hand, as revealed from Figure 5, ADCD involves two amino substituents on the cyclodextrin skeleton, and adds stability to the complexation with DPL by no less than 0.62 kcal/mol, suggesting the possibility of intramolecular electrostatic interactions between the phenolate and the ammonium ions. In addition, it may be evident also from Figure 5 that a binding free energy for ADCD/PLP is lower than that for MACD/PLP by 1.4 kcal/mol. This means the phosphate group bound to a remote ammonium group liberates a large fraction of this free energy, as opposed to the conclusion of García del Vado and his coworkers.[5] They stated that any potential interactions of the phosphate group could be ruled out since stability assays on the poly(L-lysine) Schiff bases of pyridoxals, with and without a phosphate group, were identical to each other.

The K_{eq} value for PACD is, in particular, the largest among all the amines employed and is less pH-dependent over the pH range of 3.5–9.5; namely, the differences in stability between PACD/PLP and, for example, ADCD/PLP; 0.83 kcal/mol at pH 8, 1.32 at pH 6.5, 2.07 at pH 4.5, 2.13 at pH 3.5, and 1.1 at pH 3.0. On the other hand, such a tendency was not seen between any other couples of complexes; for example, the stability of the PACD/DPL complex relative to the ADCD/DPL complex is approximately constant over the pH range examined. These facts would reflect the unique character of the PACD molecule, in which the seven amino groups, each protonated or unprotonated, are forced to be cis oriented to each other; generally, when two charged acid or base molecules with the same electrical sign come close to each other, mutual repulsion will broaden their pKa values. Actually, unlike MACD (pKa 8.23) and diethanolamine (pKa 8.96) which gave a slope (n) of nearly unity for the Henderson-Hasselbalch plot, the n value for PACD (average pKa of the amino groups: 7.82)[16,17] was found to be 1.9, revealing the prominent importance of mutual interactions between the adjacent amino groups. All these observations lead us to conclude that a characteristic feature of the PACD/PLP comes predominantly from a congested arrangement of the amino groups interacting with the phosphate group.

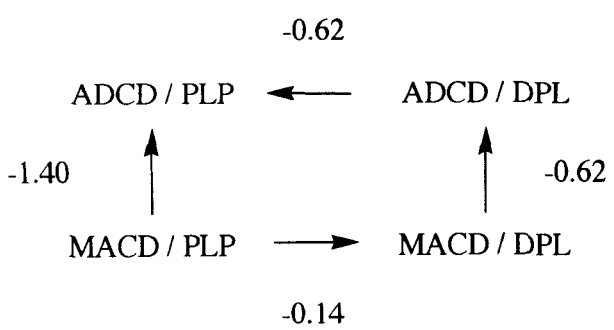


Figure 5 Free energy differences (Δ G kcal/mol) between relevant equilibria at pH 8 at 25°C: data from Figure 3.

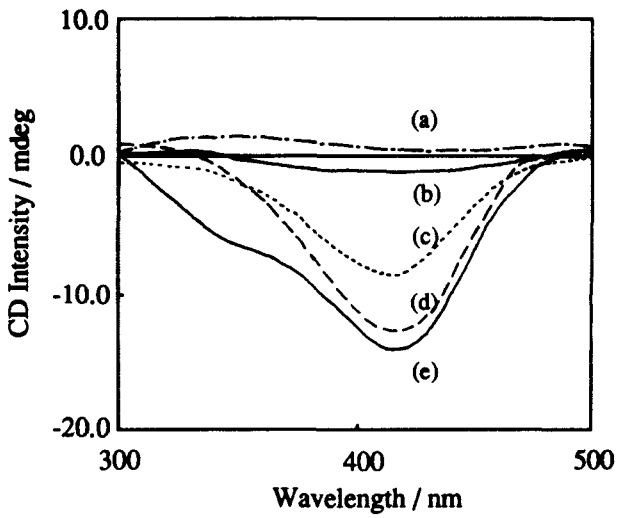


Figure 6 CD spectra at pH 5.0 for (a) PACD/DPL, (b) MACD/PLP, (c) ABCD/PLP, (d) ADCD/PLP, and (e) PACD/PLP. Concentrations in M: pyridoxals, 1.0×10^{-4} M; amines, 1.0×10^{-3} M.

Circular dichroism spectroscopy was also employed to study these interactions. The PLP complexes of cyclodextrin-based amines display a negative induced circular dichroism (ICD) band centered at 414–418 nm (refer to Figure 6). The intensity was subjected to change depending on the number and positions of the amino groups, and increases as the binding constant becomes greater. Namely, the ICD peak of the PACD/PLP is most intense, and that for the ADCD/PLP complex is slightly less intense, while the MACD/PLP complex shows no significant ICD band in the same region. The observations seem to demonstrate that cation-anion electrostatic interactions, probably involving charged hydrogen-

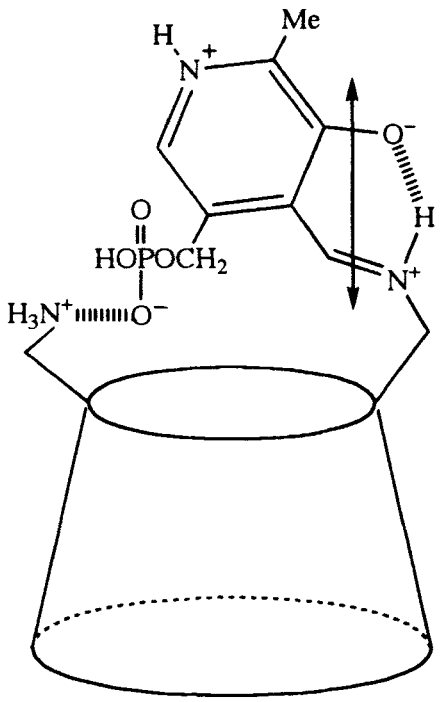


Figure 7 Plausible schematic illustration of PACD/PLP complex.

bonding between the ammonium and 5′-phosphate moieties (pH= ~ 7), are operating to stabilize the complexes.[18] Under neutral and weakly acidic conditions, the phosphate group exists as the mono or dianion species and the amino group predominantly in the quaternary form, so that they are allowed to interact with each other. Recently, the ε-amino group of the lysin residue was suggested to play a critical role for enzymic substrate binding and catalysis via ion pair interactions with the β- and γ-phosphate of GTP in mammalin protein.[19] Incidentally, the PACD/PLP Schiff base formation was not influenced by altering buffer concentrations. This seems to indicate that such intramolecular cation-anion interactions are significantly strong. Consistent with this conclusion, no ICD band was observed for combinations of DPL with cyclodextrin-based amines, because DPL lacks a phosphate group capable of capping the narrower face of the cyclodextrin cavity. Furthermore, the present ICD study allows us to conclude that the narrower rim of the cyclodextrin cavity is appreciably capped by a PLP chromophore part that has an electronic transition moment (shown below by an arrow) approximately parallel to the molecular rotation axis of the cyclodextrin cavity, as schematically illustrated in Figure 7.[20]

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