# Thiamin Diphosphate–Rhodium(III) and 2-(1-Hydroxyethyl)thiamin Diphosphate–Rhodium(III). Models for Metal Ion Activation of Enzyme-Bound Thiamin Diphosphate

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Rh(III) complexes of thiamin diphosphate (TDP) and 2-(1-hydroxyethyl)thiamin diphosphate (HETDP) were prepared (as models for enzymic metal–coenzyme interactions) by the reaction of rhodium(III) hexahydrate with the diphosphates. The complexes were characterized by $^{1}H$, $^{13}C$, and $^{31}P$ NMR. Doublets in $^{31}P$ NMR spectra at $\delta \sim 8$ and $\delta \sim 3$ ppm indicate bidentate coordination to Rh(III) at the pyrophosphate moiety. The $^{1}H$ and $^{13}C$ NMR signals for the pyrimidine group of TDP in the Rh(III) complexes are essentially the same as those of the same group in uncomplexed TDP, establishing that the pyrimidine N1′ position is not coordinated to the metal ion. The $^{13}C$ NMR signals for C2$\alpha$ and C2$\beta$ of the two pairs of diastereomers arising from coordination of HETDP to Rh(III) are completely resolved. The signals for phosphorus in these diastereomers are partially resolved in the $^{31}P$ NMR spectrum. Monodentate $\beta$-Rh(III)–TDP and $\beta$-Rh(III)–HETDP complexes, as well as bidentate $\alpha,\beta$-Rh(III)–TDP and $\alpha,\beta$-Rh(III)–2-HETDP complexes, were characterized by $^{31}P$ NMR and separated by gel filtration. The complexes do not bind to the apoenzyme of pyruvate decarboxylase although the bidentate complexes are structurally similar to the Ca–TDP complex determined by X-ray crystallographic analysis of the holoenzyme form of transketolase. The inaccessibility of the binding sites to the Rh(III) complexes suggests an obligatory ordered binding of a free metal ion precedes association of the coenzyme.

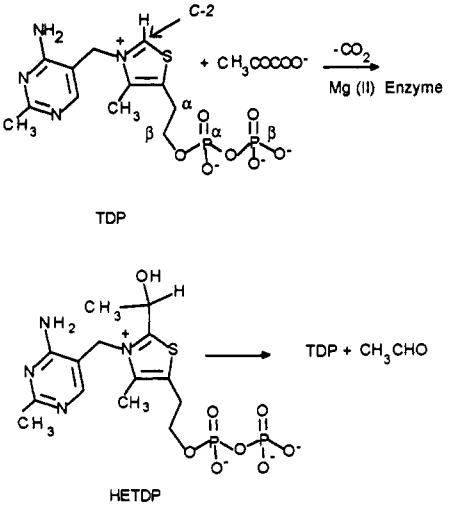
## Introduction

The coenzyme thiamin diphosphate (TDP) functions as a covalent catalyst in enzymic reactions involving the apparent formation of acyl carbanions.[1] The conversion of pyruvate to carbon dioxide and acetaldehyde is such a process (Scheme I) in which the intermediate formed from the addition of pyruvate to TDP is decarboxylated to give the adduct of acetaldehyde, 2-(1-hydroxyethyl)thiamin diphosphate (HETDP).[2,3]

In order to establish the interactions of metal ions and thiamin diphosphate in the enzyme, Jordan and co-workers have studied the activation of the apoenzyme of pyruvate decarboxylase from yeast by combinations of thiamin diphosphate and metal ions as well as inhibition by thiamin thiazolone diphosphate and metal ions.[4] While divalent metal ions are normally required for the binding of TDP to the apoenzyme, Jordan finds that lanthanides in the +3 state can activate the apoenzyme to almost the same extent as magnesium ion.

Fluorescence enhancement studies show that the metal ion binds in a hydrophobic region of the protein.[4] Since previous studies have shown that TDP binds in a hydrophobic region of the protein,[5,6] these results indicate that the metal and coenzyme bind in similar environments, implicating metal–coenzyme complex formation. The first X-ray crystal structure of a thiamin diphosphate-dependent enzyme, transketolase, has recently been reported by Lindqvist, Schneider, and co-workers.[7] The structure of the holoenzyme with calcium ion shows that the metal ion coordinates to the pyrophosphate group of the coenzyme and does not interact with other parts of the coenzyme. While such a structure is stable in the enzyme, complexes of metal ions known to activate TDP are labile and thus cannot be studied as isolated species.

**Scheme I**




Stable (substitution-inert) complexes of metal ions, such as Cr(III), Co(III), and Rh(III), with phosphate substrates are models of labile divalent metal complexes of nucleoside diphosphates and triphosphates.[8–10] The complexes are competent substrates in some enzymic reactions.[8,10] Structural analysis of the stable complexes provides information about the likely mode of coordination in the unstable complexes.[11] Rh(III) species are diamagnetic and can be analyzed by NMR methods[10,12,13] while Cr(III) and Co(III) complexes are paramagnetic. Earlier studies from our laboratory of paramagnetic Cr(III)TDP complexes were thus not analyzed by NMR.[14] Rh(III) complexes of TDP and HETDP permit NMR characterization.

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**Table I. Comparison of $^1H$,[a] $^{13}C$,[b] and $^{31}P$[c] NMR Data for Free Ligands and Complexes. Differences in Other $^{13}C$ NMR Shifts Were <0.5 ppm; Differences in Other $^1H$ NMR Shifts Were <0.1 ppm**

| | TDP δ (J, Hz) | RhTDP δ (J, Hz) | HETDP δ (J, Hz) | RhHETDP δ (J, Hz) |
|---|---|---|---|---|
| C2-*H* | 9.50 | 9.50 | | |
| C2-*CH* | | | 5.32 q (6.5) | 5.31–5.27 m |
| C2-CH-*CH*$_3$ | | | 1.56 d (6.5) | 1.55 d (6.2) |
| α-*CH*$_2$ | 3.16 m | 3.22 m | 3.05 t (5.6) | 4.16 m |
| β-*CH*$_2$ | 4.03 q (5.8) | 4.13 q (6.3) | 3.78 t (5.7) | 4.79–4.57 m |
| *C*2 | 155.7 | 155.7 | 179.6 | 179.89, 179.83 |
| C2-*CH* | | | 65.2 | 65.3 |
| C2-CH*CH*$_3$ | | | 22.7 | 22.7 |
| β-*CH*$_2$ | 65.7 d (4.8) | 66.5 d (5.8) | 65.5 d (5.1) | 66.90 d (5.7), 66.80 d (5.7) |
| α-*P* | −10.7 dd | 3.5 d (20.8) | | 3.56 d (20.4) |
| β-*P* | | 8.4 d (20.9) | | 8.10 d (20.4), 8.03 d (20.8) |

[a] 200 MHz, $D_2O$, pH 2, ref to HOD. [b] {$^1H$} 100 MHz, $D_2O$, pH 2, ref to dioxane (external standard). [c] {$^1H$} 81 MHz, 20% $D_2O$/80% $H_2O$, pH 2, ref to 85% $H_3PO_4$ (external standard).

## Experimental Section

**Materials and Methods.** NMR parameters are summarized in Table I. UV–vis spectra and kinetics data were obtained on a computer-interfaced spectrophotometer. The cell holder temperature was maintained with a circulating water bath. Dowex 50W-X2-200 resin was bleached prior to use by the method of Dunnaway–Mariano and Cleland.[15] $RhCl_3{\cdot}3H_2O$ was obtained from the Aldrich Chemical Co. or Lancaster Synthesis. Thiamin diphosphate was obtained from the Sigma Chemical Co. $Rh(H_2O)_6{\cdot}3ClO_4$ was prepared by standard methods.[16,17]

**Synthesis of Complexes.** *Caution*: Organic perchlorates are known to explode violently, particularly when concentrated. Small quantities should be used and handled with care behind safety shields.

**$Rh(H_2O)_4TDP^{3+}$.** $Rh(H_2O)_6{\cdot}3ClO_4$ was prepared by reducing the volume of a solution of $RhCl_3{\cdot}3H_2O$ (1 mmol, Aldrich) in 25 mL of $H_2O$ and 20 mL of 70% $HClO_4$ to 5 mL by boiling of water.[16] The solution was diluted to 50 mL and adjusted to a pH meter reading of 2 with $KHCO_3$ (satd) and water, as needed. The solution was filtered, TDP (0.463 g, 1 mmol) was added, and the solution was adjusted with $KHCO_3$ (satd) to pH 3.0. The yellow solution was stirred at 80 °C for 30 min. After cooling, the acidity of the solution was adjusted to give a pH meter reading of 2 with 1 M $HClO_4$. The sample was placed directly onto a prepared 2.5 × 35 cm Dowex 50W-X2-200 column[15] at 4 °C, which had previously been washed with 0.05 M $HClO_4$. The column was eluted with a gradient of 250 mL each of 0.05 M $HClO_4$ and 2.0 M $HClO_4$. The desired complex eluted at 1.0–1.2 M $HClO_4$, usually the third pale orange band isolated from the column. The 5.0-mL fractions were adjusted to pH 2.0 using $KHCO_3$ (satd) or (s), concentrated as needed by rotary evaporation at 35 °C, and monitored for product purity by $^{31}P$ NMR. The orange complex was best used and stored as the solution; degradation sometimes occurred upon evaporation of the solution to dryness. The product is stable for more than a month at pH 2.0, 4 °C. It was separated from residual $KClO_4$ by repeated concentration of the solution to dryness by rotary evaporation at 35 °C, dissolving the yellow-orange solid in cold water, methanol, or ethanol, and filtering. The yield (based on integration of free and coordinated TDP by $^{31}P$ NMR) was 20–30%. Gel filtration (Bio-Rad P2 resin in 0.05 M $HClO_4$, 1.5 × 40 cm column, 2-mL fractions) was used to remove residual unreacted $Rh(H_2O)_6^{3+}$ and to separate β-monodentate and bidentate RhTDP. NMR data ($^1H$, $^{13}C$, $^{31}P$) are summarized in Table I. Additional spectral data for bidentate-RhTDP at pH 2: UV–vis: $\lambda_{max}$ 327 (ε = 67) and 414 (ε = 75); $\lambda_{min}$ 308 (ε = 57) and 368 (ε = 37) based on ε = 13 400 $cm^{-1}$ $M^{-1}$ at 245 nm for TDP.[18] These extinction coefficients show that the complex is 1:1 between TDP and Rh(III). Spectral data for β-monodentate RhTDP, pH 2: $^{31}P\{^1H\}$ NMR (81 MHz): δ 6.4 (d, $J_{PP}$ = 19.0, 1P); δ −9.7 to −10.2 (m, 1P).

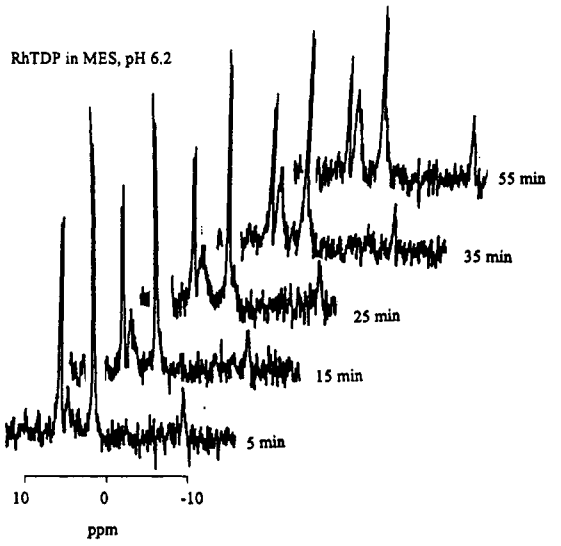


**Figure 1.** Stability of RhTDP (22mM) in 0.5 M MES with 0.1 M $Mg^{2+}$ at pH 6.2, followed by $^{31}P\{^1H\}$NMR. Time elapsed is since combination of complex and buffer solutions, immediately followed by adjustment of solution pH to 6.2. Acquisition time = 1.0 s, 200 transients, at 30 °C.

**$Rh(H_2O)_4HETDP^{3+}$.** HETDP was prepared by addition of acetaldehyde to TDP.[19] The complex was prepared and purified as described above for RhTDP. Based on integration of the $^{31}P$ NMR spectrum of the crude material, the yield of complex was 30%. Both β-monodentate and α,β-bidentate isomers were isolated. NMR data ($^1H$, $^{13}C$, $^{31}P$) are summarized in Table I. Additional spectral data for α,β-bidentate RhHETDP at pH 2: UV–vis: $\lambda_{max}$ 327 (ε = 89) and 413 (ε = 89); $\lambda_{min}$ 311 (ε = 78) and 369 (ε = 49) based on ε = 14 050 $cm^{-1}$ $M^{-1}$ at 260 nm and ε = 14 360 $cm^{-1}$ $M^{-1}$ at 246 nm for HETDP.[19] Spectral data for β-monodentate RhHETDP, pH 2: $^{31}P\{^1H\}$ NMR (81 MHz): δ 7.4 (d, $J_{PP}$ = 19.1), δ −9.2 to −9.6 (m) ppm.

**Stability of Rh(III) Complexes.** $^{31}P\{^1H\}$ NMR was used to monitor the stability of α,β-RhTDP in 0.05 M MES, pH 6.2, containing 0.1 M $MgSO_4$. The sample consisted originally of 86% α,β-RhTDP and 14% β-RhTDP, with no free TDP. Figure 1 shows the spectral change as a function of time at pH 6.2. The peak at δ −10 increased by 8% over the entire time course, indicating a slow increase in the amount of either β-RhTDP or TDP itself. UV–vis experiments were not useful in measuring complex stability because of the very minor differences in the spectra of α,β-RhTDP and β-RhTDP and the tendency of $Rh(H_2O)_6^{3+}$ to polymerize at pH 6.2.[20]

**Enzyme Isolation and Cofactor Extraction.** The holoenzyme of pyruvate decarboxylase was isolated from brewer's yeast[21] with a modification of the cell lysing procedure. Frozen brewer's yeast (300 g) was broken into pieces (at 4 °C) and ground in a blender as a 2:1 mixture with dry ice until a fine powder was obtained. The powder was thawed at room temperature in the first buffer described in the literature preparation. The CM-Sephadex purified holoenzyme was routinely obtained with specific activity of 35–45. It was stored as the ammonium sulfate precipitated pellet at −20 °C.

A modification of Hübner's procedure[22] was used to remove TDP and Mg(II) from the holoenzyme. Purified holoenzyme paste (10–50 mg) was dissolved in sufficient 0.2 M glycine phosphate buffer (pH 9.3, containing 0.01 M EDTA and 0.02 M dithio-

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threitol), usually 2–3 mL, to adjust the solution to pH 8.4–8.5. After being stirred on ice for 20 min, the mixture was concentrated to about 0.2 mL by centrifugation using Amicon Centriprep-10 and Centricon-10 filters at 2600*g* and 4300*g*, respectively. The retained solution was diluted with the glycine phosphate buffer (adjusted first to pH 8.5) and concentrated again. The retentate was then diluted with 0.05 M MES buffer (pH 6.2, containing 0.3 M ammonium sulfate and $10^{-2}$ M dithiothreitol) and concentrated twice. A final dilution with the MES buffer (and centrifugation at 15000*g* to remove insoluble materials) yields apoenzyme which retains 1% of activity in the presence of magnesium ion alone and about 40% activity in the presence of TDP alone. The apoenzyme was reconstituted to about 60% of the holoenzyme activity by incubation in 0.05 M MES, pH 6.2, with 10 mM magnesium chloride and 10 mM TDP for 10 min at room temperature.

**Enzyme Kinetics.** Pyruvate decarboxylase activity was measured by following the conversion of NADH to $NAD^+$ at 340 nm in a coupled assay with alcohol dehydrogenase (ADH) at 30 °C.[6] Assay solutions contained 2.75 mL of 0.3 M citrate buffer, pH 6.0, 50 μL of yeast alcohol dehydrogenase in buffer (200 units/mL), 50 μL of NADH (5.0 mg/mL), and 100 μL of 1 M pyruvate solution. These were mixed in the cell and incubated at 30 °C for 10 min; 50 μL of PDC in solution was added to initiate the reaction. The PDC solution consisted either of holoenzyme or apoenzyme preincubated at room temperature (21 °C) with one or more cofactors or $Rh(H_2O)_4TDP^{3+}$; all component solutions contained 0.05 M MES and were adjusted to pH 6.2. For the stock $Rh(H_2O)_4TDP^{3+}$ solutions, MES buffer (to give 0.05 M final concentration) was added and the pH adjusted to 6.2 with 0.5 M NaOH immediately prior to use. Before adding the enzyme solution to each cell, a background run was taken over 5 min which was substracted from the data acquired with the enzyme present. For each assay, the maximum slope was calculated. The data for each set of experiments were then fit directly to the Michaelis–Menten equation using GraFit (from Erithacus Software Ltd., Microsoft Windows 3.0) to obtain $k_{cat}$ (deduced from the maximal velocity of the native enzyme).

## Results

**Synthesis of Complexes.** $Rh(H_2O)_4TDP^{3+}$ (RhTDP) and $Rh(H_2O)_4HETDP^{3+}$ (RhHETDP) were prepared by analogy to the procedure for Rh(III)ADP[13] from $Rh(H_2O)_6{\cdot}3ClO_4$. TDP or HETDP[19] was combined with $Rh(H_2O)_6{\cdot}3ClO_4$[16,17] at pH 3.0 and heated at 80 °C for 30 min. Cation-exchange chromatography of the crude reaction mixtures (perchloric acid gradient) separated product from unreacted ligand and byproducts. Saturated potassium bicarbonate was used to adjust to pH 2 and to precipitate potassium perchlorate. Residual amounts of $Rh(H_2O)_6^{3+}$ could be subsequently removed by gel filtration. Attempts to substitute less destructive conditions (e.g., 1% formic acid or dilute HCl with other resins) in the cation-exchange chromatography were unsuccessful because of side reactions between the eluant and the desired product.

Bidentate and monodentate rhodium complexes were isolated from both RhTDP and RhHETDP. $^{31}P$ NMR spectra indicated that the monodentate complex forms within the first 5 min, with almost complete conversion to the bidentate complex within 30 min. In the course of purification of the bidentate product, the amount of monodentate complex present increases as a function of the duration of contact of the complex with concentrated acid, suggesting that bidentate complex slowly hydrolyzes to the monodentate form. Fractions of pure, stable bidentate complex were isolated in the final gel filtration step. Residual monodentate complex eluted first off the column in a mixture of the monodentate and bidentate complexes.

**Structure Determination.** The modes of TDP and HETDP coordination to Rh(III) were determined by the $^{31}P$ NMR spectra of the complexes,[23] Table I. In each case,

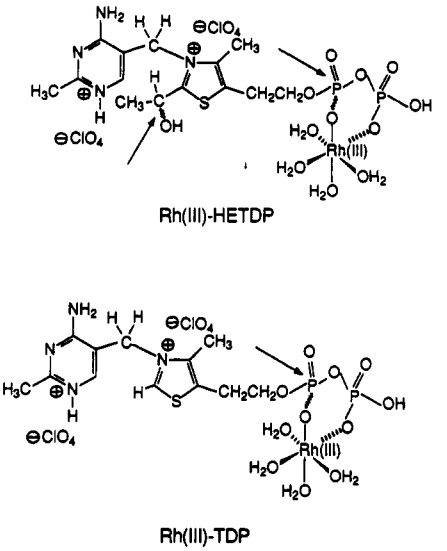


**Figure 2.** Structures of monodentate and bidentate RhTDP and RhHETDP complexes based upon this work. Stereocenters indicated by arrows.

both α,β-bidentate and β-monodentate complexes are formed in which coordination of the ligands to Rh(III) was through the diphosphate moiety, Figure 2. Bidentate coordination of the diphosphate to the metal ion is indicated by doublets in the $^{31}P$ NMR spectra for RhTDP and RhHETDP at δ ~8 and ~3 ppm; doublets at δ ~7 and ~−10 ppm indicate β-monodentate coordination of the phosphate. The report of bidentate coordination by the pyrophosphate group of TDP to Cu(II) in the X-ray structure of Cu(phen)(TDP)$(H_2O)$[24] supports this assignment.

$^1H$ NMR and $^{13}C$ NMR data for each of the bidentate complexes and the corresponding free ligands are summarized in Table I. Data are shown only for those signals where a significant shift was observed upon complexation. The lack of perturbation in the pyrimidine ring signals establishes that N1′ is *not* coordinated to the metal ion. The mode of metal ion coordination to thiamin and HET, which have no pyrophosphate moiety and cannot serve as coenzymes, is exclusively through N1′.[25,26] In contrast, our results support coordination of the coenzyme, TDP, and the enzymic intermediate, HETDP, to metal ions through the diphosphate moiety alone. This is also consistent with the fact that the pyrimidine N1′ is protonated at the reaction pH.

Two or more stereoisomers arise from coordination of a bidentate organic phosphate to a metal ion.[27] In the $^{13}C$ NMR spectrum of bidentate RhHETDP, the two pairs of diastereomers resulting from coordination of racemic HETDP to Rh(III) are well-resolved for the C2 and β-$CH_2$ signals (Table I). They are also partially resolved in the $^{31}P$ NMR spectrum.

**Complex Stability.** The stability of the bidentate complex, α,β-RhTDP, in 0.05 M MES (pH 6.2, containing 0.1 M $Mg^{2+}$) was monitored by $^{31}P$ NMR, Figure 1. Although the peaks at δ 7.5 and 3 ppm broadened and shifted upfield slightly over 50 min, very little increase in the peak at δ −10 ppm was observed, indicating that hydrolysis to

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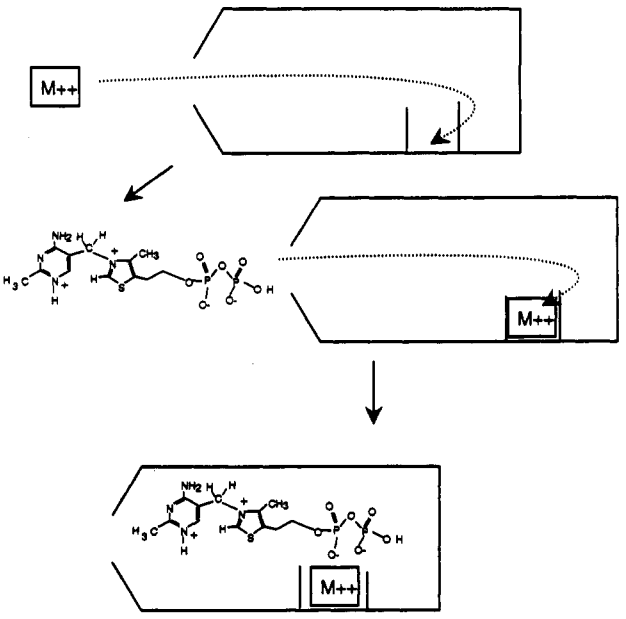


**Figure 3.** Proposed mode of enzyme–metal–coenzyme binding.

form free TDP or β-RhTDP was limited. The broadened and shifted signals probably indicate increased lability of the bidentate complex, since the chemical shifts remain at the values characteristic of bidentate complexes. Stability studies with β-RhTDP or with the RhHETDP complexes were not undertaken.

**Enzyme Studies.** The $K_m$ of TDP is 6.4 μM,[28] and thus low levels will cause a significant background activity if the complex with rhodium is not highly active. The coenzyme activities of α,β-RhTDP and α,β-RhHETDP were tested with brewer's yeast pyruvate decarboxylase apoenzyme in the presence and absence of Mg(II). For α,β-RhHETDP, no catalytic activity for pyruvate decarboxylation was observed under either set of conditions. For α,β-RhTDP, in the absence of Mg(II), no activity was observed. In the presence of saturating quantities of Mg(II), a small increase in activity as a function of the concentration of α,β-RhTDP was observed, consistent with Michaelis–Menten kinetics. However, $V_{max}$ was sufficiently small that <1% contamination of the complex solution by free TDP would provide this activity. Such contamination cannot be detected by the $^{31}P$ NMR methods used in this study, and such a low level of activity indicates that the complex is inactive in enzymic catalysis.

Since α,β-RhTDP is not active as a coenzyme, an alternative measure of the extent to which it binds to PDC is its ability to inhibit activation of PDC by TDP in the presence of Mg(II). High concentrations of α,β-RhTDP provide neither significant inhibition nor activation. The binding is sufficiently weak that we can be sure that α,β-RhTDP is neither an active coenzyme nor an inhibitor.

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α,β-RhTDP has also been found not to bind to transketolase.[29]

## Discussion

The complexes of Rh(III) with TDP and HETDP involve exclusive coordination of the Rh(II) to the pyrophosphate moiety of each. Uncomplexed versions of both TDP and HETDP bind to apoenzymes in the presence of Mg(II) while the Rh(III) complexes apparently do not. This lack of binding to an enzyme is not an inherent property of Rh(III) complexes of pyrophosphates: Rh(III) complexes of pyrophosphate itself and of nucleoside di- and triphosphates are substrates for a number of enzymes.[10,13] Furthermore, PDC is activated by metal ions which have +3 as well as +2 ionization states[4] so the lack of binding is not due to the central charge on the metal.

The crystal structure of transketolase[7] shows the coenzyme is bound with calcium ion coordinated to the pyrophosphate moiety. Thus, the mode of coordination of the metal in the bidentate Rh(III) complexes is similar to that of the divalent metal ion with TDP in the enzyme. The crystal structure shows the coenzyme bound within a deep hydrophobic cleft, consistent with Gubler's observations of fluorescence quenching of thiochrome derivatives.[30] Access to the coenzyme binding site is strictly limited, and it appears that a pre-formed complex, such as RhTDP, would not be able to enter if the site remains unchanged.

The binding site of the cofactor in PDC has also been shown to be hydrophobic.[4-6,30] Ordered binding of the metal ion followed by the cofactor can overcome steric problems as well as take advantage of electrostatic direction of association. The highly ionic pyrophosphate group of TDP and HETDP will respond to electrostatic attraction in the binding pocket.[31] Therefore, if the metal ion first associates with the binding region, then the pyrophosphate residue will permit the entire cofactor to be attracted into the hydrophobic region (Figure 3). On the other hand, if the cofactor associates with the metal ion prior to association with the apoenzyme, as for RhTDP, the electrostatic attraction will be eliminated or reduced while steric problems are increased. In addition, for RhTDP, the slow rate of exchange of ligands on rhodium may prevent additional coordination of groups from the protein to coordinate to the metal at the coenzyme binding site.

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