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Competence of Thiamin Diphosphate-Dependent Enzymes with 2'-Methoxythiamin Diphosphate Derived from Bacimethrin, a Naturally Occurring Thiamin Anti-vitamin

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Abstract

Bacimethrin (4-amino-5-hydroxymethyl-2-methoxypyrimidine), a natural product isolated from some bacteria, has been implicated as an inhibitor of bacterial and yeast growth, as well as in inhibition of thiamin biosynthesis. Given that thiamin biosynthetic enzymes could convert bacimethrin to 2'-methoxythiamin diphosphate (MeOThDP), it is important to evaluate the effect of this coenzyme analogue on thiamin diphosphate (ThDP)-dependent enzymes. The potential functions of MeOThDP were explored on five ThDP-dependent enzymes: the human and *Escherichia coli* pyruvate dehydrogenase complexes (PDHc-h and PDHc-ec, respectively), the *E. coli* 1-deoxy-D-xylulose 5-phosphate synthase (DXPS), and the human and *E. coli* 2-oxoglutarate dehydrogenase complexes (OGDHc-h and OGDHc-ec, respectively). Using several mechanistic tools (fluorescence, circular dichroism, kinetics, and mass spectrometry), it was demonstrated that MeOThDP binds in the active centers of ThDP-dependent enzymes, however, with a binding mode different from that of ThDP. While modest activities resulted from addition of MeOThDP to *E. coli* PDHc (6–11%) and DXPS (9–14%), suggesting that MeOThDP-derived covalent intermediates are converted to the corresponding products (albeit with rates slower than that with ThDP), remarkably strong activity (up to 75%) resulted upon addition of the coenzyme analogue to PDHc-h. With PDHc-ec and PDHc-h, the coenzyme analogue could support all reactions, including communication between components in the complex. No functional substitution of MeOThDP for ThDP was in evidence with either OGDH-h or OGDH-ec, shown to be due to tight binding of ThDP.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01300. Experimental procedures, references, Scheme S1, and Figures S1–S7 (PDF)

Notes

The authors declare no competing financial interest.

Bacimethrin (4-amino-5-hydroxymethyl-2-methoxypyrimidine), an antibacterial agent and thiamin (Th) antagonist, is toxic for bacteria, and the toxicity can be reversed by thiamin.^{1–4} Biosynthetic enzymes producing thiamin diphosphate (ThDP) could convert bacimethrin to 2'-methoxythiamin diphosphate (MeOThDP), one of the simplest known chemically similar analogues of ThDP, in which the C2'-methyl group of the 4'-aminopyrimidine ring is replaced by a 2'-methoxyl group.³ The toxicity of the bacimethrin on expression of thiamin biosynthetic genes was reported to be weak in comparison with the effect of thiamin itself.^{2,5} An alternative explanation of bacimethrin toxicity was that upon conversion to MeOThDP, it could inhibit some or all of the ThDP-dependent enzymes in the cell. In experiments with *Escherichia coli* cells growing on minimal medium with bacimethrin and nutritional supplements, the OGDHc-ec, 1-deoxy-D-xylulose 5-phosphate synthase (DXPS), and transketolase were identified as major targets for inhibition by MeOThDP.¹ Given the antibacterial and thiamin antagonist properties of bacimethrin, it is important to establish its potential effects on ThDP-dependent enzymes. These studies could also contribute to development of novel inhibitors against DXPS, a potential antimicrobial drug target.^{6–10}

We here explore the potential functions of MeOThDP on a selection of ThDP enzymes available to our groups, PDHc-h and PDHc-ec, OGDHc-h and OGDHc-ec, and DXPS, all five of which commence their reaction sequences by a ThDP-dependent decarboxylation of a 2-oxo acid. While all five enzymes appear to bind MeOThDP according to quenching of the intrinsic fluorescence of each enzyme, only PDHc-h, PDHc-ec, and DXPS appear to be capable of utilizing MeOThDP as a coenzyme, as evidenced by their activities. To reach this conclusion, steady-state kinetics, circular dichroism (CD) spectroscopy, and in the case of PDHc-ec and PDHc-h reductive acetylation of the E2p component (more precisely, its lipoyl domain) via mass spectrometry were employed.

EXPERIMENTAL PROCEDURES

Synthesis of 2'-Methoxythiamin Diphosphate

The MeOThDP (**11**) was synthesized following the convergent synthetic strategy previously described in the literature with major modifications (see Scheme 1).¹¹ For pyrimidine precursor **4**, *O*-methylisourea bisulfate and ethoxymethylene malononitrile were reacted in the presence of sodium methoxide to give **3**. Nitrile intermediate **3** was then reduced to the corresponding amine by Raney nickel-catalyzed hydrogenation in the presence of ammonia to give **4**.^{12,13} The other precursor, compound **7**, was synthesized with little modification of the method described in the literature.¹⁴ Acetyl butyrolactone **5** was chlorinated at room temperature using sulfuryl chloride followed by acid-catalyzed decarboxylation to afford **6**, which was further acetylated to give **7**. Intermediate compound **9** was prepared by reacting **4**

and **7** with carbon disulfide followed by acid-catalyzed dehydration. Oxidation of compound **9** with acidic hydrogen peroxide affords 2'-methoxythiamin (**10**), which was converted to the corresponding diphosphate using recombinant mouse thiamin pyrophosphokinase and ATP.¹⁵ The resulting MeOThDP (**11**) was purified by high-performance liquid chromatography (see the Supporting Information for the experimental procedure). For the synthesis of MeOThDP precursors **3**, **4**, **6**, **7**, **9**, and **10**, see the Supporting Information.

Protein Expression and Purification

Expression and purification of E1p-ec, 3-lip E2p-ec, and E3-ec followed reported protocols.^{16,17}

E1p-h was overexpressed in *E. coli* BL21(DE3) cells harboring pET28-*b*-PDH α /PDH β (a co-expression vector with coding sequences of both E1 α and E1 β subunits) and was purified using a Ni Sepharose column as described previously.¹⁸ The recombinant E2p-h-E3BP subcomplex and E3-h were overexpressed in *E. coli* cells and were purified as described previously.¹⁹ Details for expression and purification of E1o-ec, E2o-ec, and E3-ec²⁰ are provided in the Supporting Information. Expression and purification of E1o-h, E2o-h, and E3h were conducted as reported recently.²¹

For purification of DXPS, *E. coli* BL21(DE3) cells harboring the *dxs*-pET37b plasmid were grown in LB medium supplemented with 50 μ g/mL kanamycin. Protein expression was induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside. Cells were grown for 15 h at 25 °C. Cells were resuspended in 50 mM Tris-HCl (pH 8.0) containing 20 mM MgCl₂, 1 mM ThDP, 1 mM TCEP, 1 mM PMSF, 1% (v/v) protease inhibitor cocktail, 10 units of Turbo DNase, and 10% glycerol (lysis buffer). Cells were lysed with a French press, and the clarified supernatant was incubated with 4 mL of Ni²⁺-NTA resin dissolved in lysis buffer containing 5 mM imidazole for 1 h at 4 °C with rotation. DXPS was then eluted with a stepwise gradient of imidazole (5 to 500 mM) in 3 mL fractions. Fractions containing DXPS were combined and diluted by 8-fold with 50 mM Tris-HCl (pH 8.0) containing 10% glycerol. The DXPS was further purified using a HiTrap Q column (5 mL, GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 1 mM TCEP and 10% glycerol. DXPS was eluted with a stepwise gradient of NaCl (0–1.0 M) over 10 column volumes. Fractions containing DXPS (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were combined and were dialyzed against 1000 mL of 50 mM Tris-HCl (pH 8.0) containing 10 mM MgCl₂, 1 mM ThDP, 0.1 M NaCl, and 10% glycerol for 15 h at 4 °C with the following dialysis against 2000 mL of 50 mM Tris-HCl (pH 8.0) containing 10 mM MgCl₂, 1 mM ThDP, 1 mM β -mercaptoethanol, 0.1 M NaCl, and 10% glycerol for an additional 4 h at 4 °C. DXPS was flash frozen in liquid nitrogen and stored at –80 °C. The Arg478Ala substituted DXPS was purified as reported previously.⁹

Preparation of the Apoenzymes

Apo-E1p-ec (no ThDP bound in the active centers) was prepared by using a PD-10 column (GE Healthcare) equilibrated with 50 mM KH₂PO₄ (pH 7.0) containing 0.15 M NaCl with no addition of ThDP and MgCl₂. The percent of apo-E1p-ec was determined by measuring of the E1p-ec specific activity and the overall PDHc activity in the absence and presence of

saturation concentrations of ThDP (0.20 mM) in the reaction assay. On the basis of these measurements, the apo-E1p-ec content could be calculated. Apo-E1p-h was prepared by using a column equilibrated with 50 mM KH₂PO₄ (pH 7.5) containing 0.15 M NaCl, 0.50 mM DTT, and 1.0 mM benzamidine-HCl, followed by dialysis for 15 h at 4 °C against the same buffer. For apo-DXPS preparation, the column was equilibrated with 50 mM HEPES (pH 8.0) containing 0.15 M NaCl, 1 mM DTT, and 1% glycerol. The apo-DXPS was further dialyzed against the same buffer for 15 h at 4 °C. The apo-DXPS content was analyzed by CD by recording the progress curves of DXP formation in the presence and absence of ThDP in the reaction assay as described for E1p-ec above.

Enzyme Activity Measurements in the Complexes

Overall PDHc and E1p Specific Activities—The overall activity of PDHc-ec and PDHc-h was measured by the formation of NADH at 340 nm as reported previously.^{19,22} PDHc-ec was reconstituted by premixing the recombinant E1p-ec, E2p-ec, and E3-ec components at a microgram mass ratio of 1:1:1. PDHc-h was reconstituted by premixing E1p-h, E2p-h-E3BP, and E3-h at a microgram mass ratio of 1:3:3. Both were reconstituted in 50 mM KH₂PO₄ (pH 7.0) containing 0.15 M NaCl for 60 min at 25 °C. The E1 specific activity was measured by the rate of reduction of DCPIP as reported previously.^{19,22}

Overall OGDHc and E1o Specific Activities—The overall OGDHc-ec, OGDHc-h, and E1o specific activities were determined as reported previously^{20,21} (see also the Supporting Information).

DXPS Activity Measurement—The assay was conducted by direct detection of DXP formation by CD₂₉₀ and 37 °C, as reported previously with some modifications.⁹ The assay mixture (2.4 mL) contained 50 mM KH₂PO₄, 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 0.10 mM ThDP (0.10 mM MeOThDP), 2.0 mM MgCl₂, 1.0 mM pyruvate, 1.0 mM DTT, 1 mM GAP, and 1% glycerol. The reaction was started by addition of 0.02 mg of DXPS, and the spectrum was recorded for 400 s at 37 °C. To calculate the DXPS activity and the corresponding rate constants, the recently reported molar ellipticity of DXP of 13200 deg cm³ dmol⁻¹ was used.²³

Fluorescence Spectroscopy—For the fluorescence titration of apo-DXPS with ThDP or with MeOThDP, apo-DXPS (0.058 mg/mL, concentration of active centers of 0.858 μM) in a mixture of 50 mM KH₂PO₄ and 50 mM Tris (pH 8.0) containing 0.1 M NaCl, 2 mM MgCl₂, and 1% glycerol was titrated with ThDP (0.60–60 μM) or MeOThDP (0.60–70 μM). Fluorescence spectra were recorded at 25 °C using a Cary Eclipse spectrometer using the method reported previously.²⁴ The excitation wavelength was 295 nm, and the emission spectra were recorded in the range of 300–450 nm in 3 mL quartz cuvettes. The inner filter effect was corrected with the absorption of ThDP and MeOThDP at the excitation wavelength (295 nm) and the emission maximum (334 nm) with a dilution factor according to eq 1

$$F_{i,\text{corr}} = F_{i,\text{obs}} \times (V_i/V_o) \times \text{antilog} [0.5(A_{\text{ex}} + A_{\text{em}})] \quad (1)$$

where $F_{i,corr}$ is the corrected value of the fluorescence intensity at a given point of titration, $F_{i,obs}$ is the experimentally measured fluorescence intensity at the emission maximum, V_0 is the initial volume of the sample, V_i is the volume at a given point of titration (V_i/V_0 is the dilution factor), A_{ex} is the absorbance of the sample at the excitation wavelength, and A_{em} is the absorbance of the sample at the emission maximum. The K_d values for ThDP and MeOThDP were calculated using eq 2

$$(F_0 - F_i)/F_0 = (\Delta F_{max}/F_0 [ThDP]^n) / (S_{0.5}^n + [ThDP]^n) \quad (2)$$

where $(F_0 - F_i)/F_0$ is the relative fluorescence quenching following the addition of ThDP or MeOThDP, n is the Hill coefficient, and for $n = 1.0$, the value of $S_{0.5}$ is equal to K_d .

For the fluorescence titration of apo-E1p-ec by MeOThDP, E1p-ec (0.057 mg/mL, concentration of active centers of 0.57 μ M) in 20 mM KH_2PO_4 (pH 7.0) containing 2.0 mM $MgCl_2$ was titrated with MeOThDP (0.30–70 μ M). The inner filter effect was corrected with the absorption of MeOThDP at the excitation wavelength (295 nm) and emission maximum (334 nm) and with a dilution factor similar to that to DXPS presented above.

Circular Dichroism Spectroscopy

pH Titration of the AP Form of MeOThDP Bound on E1p-h—For the pH titration experiment, a reaction mixture in 2.4 mL of 50 mM KH_2PO_4 and 50 mM Tris (pH range of 7.2–8.54 was used) contained E1p-h (3.0 mg/mL, concentration of active centers of 39.4 μ M), 2.0 mM $MgCl_2$, 0.25 mM MeOThDP, 0.15 M NaCl, and 1.0 mM DTT. The pH was adjusted using a symphony pH electrode (VWR), and CD spectra were recorded after each adjustment. The pK_a was determined from the fit of the intensity of the CD band at 309 nm versus pH according to eq 3, for a single ionizing group

$$(CD_{309})_H = (CD_{309,max} \times [H^+]) / (K_a + [H^+]) \quad (3)$$

where $(CD_{309})_H$ is the observed value of CD_{309} at a particular pH.²⁶

Titration of E1p-ec by Acetylphosphinate—For CD titration of E1p-ec by acetylphosphinate (AcPhi), E1p-ec (2.0 mg/mL, concentration of active centers of 20 μ M) in 20 mM KH_2PO_4 (pH 7.0) containing 2.0 mM $MgCl_2$ and 0.20 mM ThDP (or 0.20 mM MeOThDP) was titrated with AcPhi (0.50–160 μ M). Difference CD spectra were recorded via subtraction of the CD spectrum of E1p-ec in the presence of ThDP (or MeOThDP). The intensity of the CD band at 319 nm (E1p-ec·ThDP) and 303 nm (E1p-ec·MeOThDP) was recorded and was plotted versus the concentration of AcPhi used. CD spectra were recorded using a Chirascan CD instrument (Applied Photophysics, Leatherhead, U.K.) at 30 °C. Data were fitted to a quadratic equation (eq 4)

$$CD = \left\{ E_t + L + K_d - \left[(E_t + L + K_d)^2 - 4E_tL \right]^{1/2} \right\} / (2E_t/CD_{max}) \quad (4)$$

where CD is the ellipticity at 319 nm (E1p-ec·ThDP) or 303 nm (E1p-ec·MeOThDP) at a given concentration of AcPhi, E_t and L are the total concentrations of E1p-ec and AcPhi

used for titration, respectively, K_d is the dissociation constant, and CD_{max} is the maximal ellipticity at 319 or 303 nm.^{27, 28}

Titration of Apo-DXPS by ThDP (MeOThDP) and Pyruvate—For CD titration of apo-DXPS with ThDP and pyruvate, the CD spectra of apo-DXPS (1.88 mg/mL, concentration of active centers of 27.8 μ M, 1.2% holoenzyme remained) in a mixture of 50 mM KH_2PO_4 and 50 mM Tris-HCl (pH 8.0) containing 0.10 M NaCl, 2.0 mM $MgCl_2$, and 1% glycerol were recorded: (i) in the absence of ThDP, (ii) in the presence of 0.15 mM ThDP, and (iii) in the presence of 0.15 mM ThDP and 100 μ M pyruvate.

For CD titration of apo-DXPS with MeOThDP and pyruvate, CD spectra of apo-DXPS (1.61 mg/mL, concentration of active centers of 23.81 μ M, 0.4% holoenzyme remained) were recorded: (i) in the presence of 0.15 mM MeOThDP under conditions similar to those with ThDP above and (ii) upon addition of 25–200 μ M pyruvate.

Titration of DXPS with Thiamin 2-Thiothiazolone Diphosphate (ThTTDP)—Apo-DXPS (2.0 mg/mL, concentration of active centers of 29.5 μ M) in a mixture of 50 mM KH_2PO_4 and 50 mM Tris-HCl (pH 8.0) containing 0.10 M NaCl, 2.0 mM $MgCl_2$, and 1% glycerol was titrated by 0.5–50 μ M ThTTDP. The concentrations of ThTTDP used for the titration were comparable to the concentration of active centers of DXPS. A quadratic equation (eq 4) was used to calculate $K_{d,ThTTDP}$. In the presence of 10 μ M MeOThDP, apo-DXPS (0.85 mg/mL, concentration of active centers of 12.5 μ M) was first complexed with MeOThDP in a mixture of 50 mM KH_2PO_4 and 50 mM Tris-HCl (pH 8.0) containing 0.10 M NaCl, 2.0 mM $MgCl_2$, and 1% glycerol and then titrated with 0.5–70 μ M ThTTDP. The ellipticity at 331 nm was plotted versus ThTTDP concentration, and data were fitted to a quadratic equation (eq 4).²⁴

FT-MS To Monitor Reductive Acetylation of the E2p-ec Lipoyl Domain by E1p-ec and Pyruvate—Isolation and purification of the lipoyl domain (LD_{h-ec}), its *in vitro* lipoylation, and its reductive acetylation by pyruvate and E1p-ec complexed with MeOThDP were performed as reported previously.²⁹ The reaction assay contained in 50 mM NH_4HCO_3 (pH 7.5) E1p-ec (0.10 μ M), MeOThDP (0.20 mM), 1 mM $MgCl_2$, and 100 μ M LD_{h-ec} . The reaction was started by addition of 2 mM pyruvate. Aliquots were withdrawn at different times of incubation and were quenched into 50% methanol and 0.1% formic acid. Samples were analyzed for the presence of unacetylated LD_{h-ec} and reductively acetylated LD_{h-ec} by FT-MS. The fraction of acetylated LD_{h-ec} at different times was determined by taking a ratio of the relative intensity of the acetylated LD_{h-ec} form to the total relative intensity (sum of unacetylated and acetylated LD_{h-ec}). The time dependence of this fraction was plotted, and data were fitted to a single-exponential equation (eq 5)

$$f = f_o + f_1 [1 - \exp(-k_1 t)] \quad (5)$$

where f is a ratio of the relative intensity of the acetylated form to the total relative intensity (acetylated and unacetylated forms of LD_{h-ec}) and k_1 is a rate constant. Under steady-state conditions, the value of k_1 was determined from a linear fit of the initial rate conditions.

RESULTS

Kinetic Studies of the PDH Complexes with MeOThDP

PDHc is a member of the superfamily of enzymes that catalyze oxidative decarboxylation of pyruvate generating the corresponding acyl-CoA and NADH (H⁺) according to the overall reaction in eq 6

The PDH complexes in prokaryotes and eukaryotes are composed of multiple copies of three catalytic enzymes: (1) pyruvate dehydrogenase (E1p), dihydrolipoamide acetyltransferase (E2p), and dihydrolipoamide dehydrogenase (E3; the E3 component is common to all such complexes in a particular cell).^{30–32} E1p is a ThDP-dependent enzyme and catalyzes two consecutive steps: (1) decarboxylation of pyruvate to CO₂ with the formation of a C2 α -hydroxyethylidene-ThDP (enamine) intermediate and (2) the reductive acetylation of the lipoyl groups covalently attached to E2p (Scheme 2). E2p catalyzes transfer of the acetyl group from S-acetyldihydrolipoyl-E2p to CoA to form acetyl-CoA. The transfer of electrons from dihydrolipoyl-E2p to FAD and then to NAD⁺ is performed by E3, which recycles dihydrolipoyl-E2p to lipoyl-E2p.

First, results with E1p-ec and E1p-h are presented, where most of the ThDP could be removed and could be replaced by MeOThDP, making interpretation of kinetic and spectroscopic data less ambiguous.

Functional Competence of MeOThDP in the Active Centers of E1p-ec and E1p-h

ThDP could be virtually completely removed from E1p-ec active centers with no overall PDHc-ec activity detected in the absence of ThDP (Table 1, top). Approximately 0.6–5% of the overall PDHc-h activity and 3.2–3.5% of the E1p-h specific activity could be detected for E1p-h under similar conditions, indicating the presence of some tightly bound ThDP still remaining (Table 1, bottom). In the presence of MeOThDP (0.2 mM) in the reaction assay, but with no ThDP present, approximately 5.9–11% of the overall PDHc-ec activity and 19% of the E1p-ec specific activity were detected with no significant difference between these two activities. However, for E1p-h, approximately 48–75% of the overall PDHc-h activity and 49% of E1p-h specific activity were detected in the presence of 0.2 or 0.5 mM MeOThDP in the reaction assay, suggesting the functional competence of MeOThDP on both E1p's (Table 1).

Activation of E1p-ec Follows a Similar Mechanism with ThDP and MeOThDP

Detailed analysis of the progress curves for NADH production by PDHc-ec complexed with MeOThDP revealed the presence of a lag phase that shortened with increasing concentration but was still in evidence even at the highest (150 μ M) concentration of MeOThDP used (Figure 1, top). A similar lag phase had been detected for binding of ThDP to PDHc-ec and was attributed to activation of E1p-ec by ThDP.³³ Thus, in this respect, ThDP and MeOThDP behave similarly. Kinetic analysis of the duration of the lag phase (represented as a plot of 1/lag phase vs [MeOThDP] in the bottom panel of Figure 1) revealed a linear part at

concentrations above 20 μM but deviated from linearity at concentrations below 20 μM (Figure 1, bottom, inset), suggesting that interaction of MeOThDP with E1p-ec involves more than a single step to reach a catalytically active E1p-ec, which is similar to that observed and reported earlier with ThDP.³³ According to the mechanism of E1p-ec activation by ThDP proposed earlier (eq 7), rapid equilibrium binding of ThDP is likely followed by a slow isomerization at ThDP concentrations below 1 μM .³³ At ThDP concentrations above 1 μM , a linear plot of 1/lag phase versus [ThDP] suggests a single-step binding mechanism (eq 7).³³

Using a data treatment similar to that used with ThDP,³³ kinetic constants could be calculated for binding of MeOThDP to E1p-ec from the lag phase (Table 2). The data revealed a similar mechanism of activation of E1p-ec by ThDP and MeOThDP at the first equilibration step (K_1) and at the subsequent isomerization step (k_2 and k_{-2}). However, the value of the forward rate constant for binding the second MeOThDP (k_3 of $0.49 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$) was ~14 times lower than that for ThDP ($0.70 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), producing E1p-ec with a lower catalytic efficiency.

Fluorescence Studies of Binding of MeOThDP to E1p-ec and E1p-h Suggest Similar Binding Constants for ThDP and MeOThDP but Different Binding Modes

Additional evidence of the similarity of binding of ThDP and MeOThDP to E1p-ec and E1p-h was obtained from fluorescence studies. Titration of E1p-ec with [MeOThDP] led to quenching of the intrinsic E1p-ec fluorescence, yielding a $K_{\text{d,MeOThDP}}$ of $2.68 \pm 0.56 \mu\text{M}$, comparable with that for ThDP ($K_{\text{d,ThDP}} = 7.0 \pm 0.9 \mu\text{M}$) (Figure S1, left) and with a $K_{\text{d,MeOThDP}}$ of $3.45 \pm 0.74 \mu\text{M}$ (calculated as $1/K_{\text{I,MeOThDP}}$ in Table 2). The major difference was in the percent of the E1p-ec fluorescence quenched by ThDP (9.9%) and MeOThDP (5.8%), suggesting a difference in their binding mode. Similar evidence was obtained using E1p-h (Figure S1, right). While ThDP quenched 19% of E1p-h fluorescence ($K_{\text{d,ThDP}} = 7.2 \pm 0.5 \mu\text{M}$), only 4% E1p-h fluorescence was quenched by MeOThDP ($K_{\text{d,MeOThDP}} = 16.2 \pm 4.5 \mu\text{M}$) (Figure S1, right). The kinetic data in combination with fluorescence studies suggest that while $K_{\text{d,MeOThDP}}$ and $K_{\text{d,ThDP}}$ for binding are similar, the MeOThDP has a lower catalytic efficiency and, likely, a different binding mode.

CD Studies of E1p-h Also Support a Different Binding Mode for MeOThDP

There is evidence of the presence of three tautomeric and ionization states of enzyme-bound ThDP: two neutral ones, the 4'-aminopyrimidineThDP (AP) and the 1'/4'-iminopyrimidineThDP (IP) tautomers, and the protonated 4'-aminopyrimidiniumThDP (APH⁺). While CD signatures were identified at Rutgers for the AP and IP forms, the APH⁺

form has so far only been detected by solid-state NMR (Scheme 1, left-handed).^{34–40} Neither the AP nor the IP forms were detected on the near-UV CD spectra of E1p-ec (α_2 homodimer with two active centers). However, E1p-h complexed with ThDP ($\alpha_2\beta_2$ heterotetramer with two active centers) revealed the simultaneous presence of both tautomeric forms, AP (negative CD₃₃₀) and IP (positive CD₃₀₅), that pertain to ThDP bound to E1p-h, suggesting that two active centers are occupied by different tautomeric forms of ThDP (Figure 2).³⁷ These two CD bands were missing on E1p-h complexed with MeOThDP, suggesting that the pK_a for the $([AP] + [IP])/[APH^+]$ equilibrium could be shifted (Figure 2).

The pH dependence of the CD spectrum of E1p-h with MeOThDP revealed the presence of a negative CD₃₀₉ at high pH that could be assigned to the AP form of bound MeOThDP, but no CD band corresponding to the IP form could be detected (Figure 3A). The pH dependence of the amplitude of the negative CD₃₀₉ could be fitted to a single proton titrating with a pK_a of 8.02 ± 0.03 for the $([AP] + [IP])/[APH^+]$ equilibrium (Figure 3B). This value is shifted to the alkaline side compared to that reported for ThDP (pK_a of 7.07 ± 0.1) on E1p-h.³⁶ According to these findings, the APH^+ form would be the predominant form at lower pH (7.2–7.6), an ionization state with no previously identified CD signature (Figure 3B). At higher pH, the APH^+ form is replaced by the AP form that becomes the predominant form at pH 8.45, revealing a negative CD₃₀₉ band (Figure 3B).

Next, the pH dependence of k_{cat} was determined in the overall PDHc-h activity, where E1p-h was complexed with MeOThDP, revealing a maximal k_{cat} at pH 7.25 that correlates well with the pH of optimal activity (pH 7.0–7.5) for E1p-h complexed with ThDP (Figure S2).³⁶ These data raised the following question: Is the alkaline shift in pK_a (APH^+) due to replacement of the C2'-methyl group of the 4'-aminopyrimidine ring with the 2'-methoxyl group, or are its origins due to a different binding mode? An experiment was conducted to determine the pK_a (APH^+) of bacimethrin in aqueous solution on the basis of the pH dependence of its UV spectrum (Figure 4). A plot of the ratio of the absorbances at 273 nm and at 265 nm versus pH fits to a single proton titrating with a pK_a of 5.32 ± 0.03 (Figure 4), actually lower than the pK_a of 5.9 reported for N1 protonation in 4-amino-5-(methoxymethyl)-2-methylpyrimidine, the closest analogue of bacimethrin.⁴¹ This comparison indicates that replacement of the 2'-CH₃ group with the 2'-OCH₃ group shifts the pK_a in the opposite direction in solution (less basic by 0.6 unit) than on the enzyme (more basic by 1.0 unit).

CD Observation of a Stable Predecarboxylation Intermediate Analogue Derived from MeOThDP and Acetylphosphate

It was demonstrated earlier that the pyruvate analogue AcPhi is a reversible tight-binding inhibitor of E1p-ec ($K_{i,AcPhi} = 0.76 \mu M$) and E1p-h ($K_{i,AcPhi} = 0.014 \mu M$).²⁸ Addition of AcPhi to E1p-ec also manifested itself in the appearance of a positive CD₃₁₉ on the difference spectra of E1p-ec, with the maximal intensity reached at an $[AcPhi]/[E1p-ec]$ molar ratio equal to 1/1, and indicating stoichiometric binding of AcPhi ($K_{d,AcPhi} = 0.062 \mu M$).²⁸ This CD band was assigned to 1',4'-iminophosphinolactylThDP, a reporter of the tetrahedral predecarboxylation intermediate on ThDP enzymes (LThDP in Scheme 2).⁴⁰

Here we found that a broad positive CD band developed upon titration of E1p-ec with AcPhi in the presence of MeOThDP, again suggesting formation of C2 α -phosphinolactylMeOThDP (CD_{max} at 303 and 340 nm), similar to but not identical in shape to the curve obtained upon addition of AcPhi to ThDP (CD_{max} at 319 nm) (Figure 5A,C). Also, at similar concentrations of E1p-ec active centers used for complexation with ThDP (20 μ M) or MeOThDP (14.4 μ M), and similar concentrations of AcPhi used in the titrations (0.5–130 μ M), the intensity of CD_{max} for formation of C2 α -phosphinolactyl-MeOThDP was 2-fold lower than that for C2 α -phosphinolactylThDP (compare panels B and D of Figure 5). The maximal CD intensity was reached at an [AcPhi]/[E1p-ec] molar ratio equal to 1/1 (ThDP; $K_{d,AcPhi}$ = 0.036 μ M) and 1/ 2.5 (MeOThDP; $K_{d,AcPhi}$ = 1.88 μ M), indicating that AcPhi binding was also affected (Figure 5B,D and Figure S3). The data provided above allowed us to determine for the first time a molar ellipticity ($[\Theta_{319}]$) of 25970 deg cm² dmol⁻¹ for the phosphinolactylThDP intermediate on E1p-ec.

The following results were obtained upon titration of E1p-h complexed with MeOThDP by AcPhi. Full saturation of E1p-h active centers was achieved with stoichiometric concentrations of AcPhi (Figure S4), which is similar to that observed for E1p-h complexed with ThDP.²⁸ A $K_{d,AcPhi}$ of 1.36 μ M (MeOThDP) was calculated as compared with a $K_{d,AcPhi}$ of 0.043 μ M (ThDP) (unpublished data).

Reductive Acetylation of the E2p-ec Lipoyl Domain by Pyruvate with E1p-ec Complexed with MeOThDP Was Adversely Affected

Reductive acetylation of the lipoyl moiety covalently bound to LD_h-ec by E1p-ec and pyruvate is the final step involving ThDP-bound covalent intermediates (Scheme 2). E1p-ec had been shown to reductively acetylate independently expressed LD_h-ec in the presence of pyruvate, providing a model reaction for studying the rate of the final reaction catalyzed by E1p-ec.²⁹ As seen from Figure 6, E1p-ec complexed with MeOThDP gave rise to time-dependent reductive acetylation of LD_h-ec in the presence of pyruvate. While 100% acetylation of LD_h-ec was reached over time, the rate constant of 4.4 s⁻¹ calculated for this reaction was at least 10 times slower than the rate constant of 52 s⁻¹ for E1p-ec complexed with ThDP as reported previously by Rutgers.²⁹ Because this reaction represents communication between the E1p-ec and E2p-ec components, the reduced rate constant signals that this communication is also affected by MeOThDP substitution.

In a similar experiment with E1p-h, two forms of the E2p-h lipoyl domain (LD1-h) were detected even after incubation with pyruvate for 900 s: unacetylated form with a mass of 11971.3 Da and acetylated form with a mass of 12015.3 Da. A plot of the ratio of the intensity of the acetylated to total intensity (acetylated and unacetylated LD1-h) versus time of incubation allowed us to calculate rates for this reaction with ThDP (10.25 s⁻¹) and MeOThDP (6.38 s⁻¹) and revealed that substitution of ThDP with MeOThDP only slightly affects the rate of acetyl transfer between E1p-h and LD1-h (Figure S5).

Interaction of OGDHc-ec and OGDHc-h with MeOThDP

According to measurement of the OGDHc activity presented in Table 3, it is clear that ThDP is tightly bound in the active centers of both E1o-h and E1o-ec. Upon reconstitution of E1o

with recombinant E2o and E3 components, approximately 72–76% (OGDHc-h) and 53–93% (OGDHc-ec) of the overall OGDHc activity could be detected in the absence of ThDP in the reaction assay. Similarly, in the E1o-specific assay, approximately 63–100% (E1o-h) and 92–100% (E1o-ec) activity could be detected in the absence of ThDP for E1o alone, which complicated studies with MeOThDP. In the presence of 0.20 mM MeOThDP, some inhibition of the overall OGDHc activity was observed for both OGDHc-h and OGDHc-ec as compared with that in the presence (and in the absence) of ThDP in the reaction assay (Table 3).

Interaction of the *E. coli* 1-Deoxy-D-xylulose 5-Phosphate Synthase with MeOThDP

DXPS is a ThDP-dependent enzyme that catalyzes formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and D-glyceraldehyde 3-phosphate (D-GAP) (Scheme 3).⁶ This reaction is the first step on the non-mevalonate pathway to isoprenoid biosynthesis in human pathogens that is absent in mammals. DXP is also a precursor in vitamin B1 and B6 biosynthesis.^{42,43} The reaction commences with the typical ThDP-catalyzed pyruvate decarboxylation, and LThDP was found to be remarkably stable in the absence of D-GAP.²³ The principal results on DXPS are the following.

Evidence of the Functional Competence of MeOThDP Bound to DXPS—In

kinetic experiments, in the absence of ThDP and MgCl₂ in the reaction assay, in three separate experiments maximal activities of 1.2, 0.34, and 0.38% of that in the presence of ThDP were detected, indicating that most of the ThDP could be removed from DXPS, thus forming apo-DXPS (Table 4 and Figure 7). The apo-DXPS could then be complexed with ThDP to fully active DXPS. When apo-DXPS with MeOThDP were mixed, activities of 0.26 ± 0.04 and 0.3 ± 0.01 $\mu\text{mol min}^{-1}$ (mg of DXPS)⁻¹ were detected in two independent experiments representing 8.8 and 14% of that in control experiments in the presence of ThDP, respectively, and indicating functional competence of MeOThDP bound to DXPS (Table 4 and Figure 7).

Fluorescence Evidence of MeOThDP Binding in the Active Centers of DXPS—

According to quenching of the intrinsic fluorescence of apo-DXPS by MeOThDP, MeOThDP binds in the active centers of DXPS with a $K_{d,\text{MeOThDP}}$ of 6.24 μM , a value similar to the $K_{d,\text{ThDP}}$ of 3.5 μM (Table 4 and Figure 8). Also, the percent quenching of DXPS fluorescence was similar for MeOThDP (27%) and ThDP (35%), suggesting similar activation of apo-DXPS by both (Figure 8).

CD Studies with ThTTDP Confirm Competition with MeOThDP at the Active Center of DXPS—

Thiamin 2-thiothiazolone diphosphate (ThTTDP) is a potent inhibitor of ThDP-dependent enzymes, where the C2=S bond replaces the C2-H bond in the thiazolium ring of ThDP.²⁸ ThTTDP bears some structural similarity to the C2 α -hydroxyethylidene-neThDP (the enamine or C2 α carbanion in Scheme 2) intermediate, the first postdecarboxylation intermediate. Upon titration of DXPS with ThTTDP, a broad positive CD band with a maximum at 331 nm was generated (Figure S6) and exhibited saturation at a [ThTTDP]/[DXPS active centers] molar ratio of 1.26, indicating stoichiometric binding of ThTTDP to DXPS ($K_{d,\text{ThTTDP}}$ of 0.39 ± 0.097 μM). A molar

ellipticity [Θ_{331}] of $66610 \text{ deg cm}^2 \text{ dmol}^{-1}$ was determined for ThTTDP bound to DXPS. At a MeOThDP concentration of $10 \text{ }\mu\text{M}$ (similar to the $K_{d,\text{MeOThDP}}$ of $6.24 \pm 0.23 \text{ }\mu\text{M}$ calculated from the fluorescence studies described above), a $K_{d,\text{ThTTDP}}$ of $0.60 \pm 0.14 \text{ }\mu\text{M}$ was calculated, a value slightly higher than the $K_{d,\text{ThTTDP}}$ of $0.39 \pm 0.097 \text{ }\mu\text{M}$ calculated in its absence (Figure 9). Also, saturation of apo-DXPS with ThTTDP was reached at a [ThTTDP]/[DXPS active centers] molar ratio of 2.6 compared to the ratio of 1.26 in its absence (Figure S6), indicating that ThTTDP and MeOThDP compete for the same active centers on DXPS.

Attempted Observation of the Predecarboxylation LThDP Intermediate by CD

—The near-UV CD spectrum of apo-DXPS complexed with ThDP displays a negative CD band at 320 nm , suggesting that ThDP is bound in its AP tautomeric form to DXPS, similar to that reported for E1p-h above, and for DXPS reported earlier (Figure 10A).²³ DXPS complexed with ThDP titrated with pyruvate ($100\text{--}1000 \text{ }\mu\text{M}$) at $5 \text{ }^\circ\text{C}$ displayed a positive CD band at 312 nm , suggesting formation of the $1',4'$ -iminopyrimidinylLThDP intermediate (Figure 10A).²³ These two CD bands were not apparent when apo-DXPS was complexed with MeOThDP (Figure 10B), indicating that MeOThDP has a different binding mode that does not give rise to the CD signatures for different forms of ThDP. Similar observations were made on the Arg478Ala variant of DXPS [for which an activity of $0.059 \pm 0.007 \text{ }\mu\text{mol min}^{-1} (\text{mg of Arg478Ala DXPS})^{-1}$ was determined] (Figure S7). Earlier, it was reported that Arg478 is important for GAP binding and its recognition, but not crucial for catalysis through LThDP formation.⁹ Again, the characteristic negative CD band at 320 nm was not apparent on MeOThDP binding (Figure S7). Upon titration with donor substrate pyruvate, the positive CD band at 313 nm assigned earlier to LThDP was also evident for the Arg478Ala DXPS complexed with ThDP, but not with MeOThDP (Figure S7).

We found no CD evidence of formation of putative predecarboxylation intermediate MeOLThDP (corresponding to LThDP in Scheme 3) on DXPS with MeOThDP (it is to be noted that LThDP was exceptionally stable on DXPS with ThDP). Therefore, we sought evidence of reactivity once more from carboligase reactions: formation of acetoin (CD band with a maximum at 275 nm) or acetolactate (CD band with a maximum at 303 nm).^{23,44} Indeed, we obtained evidence of formation of (*R*)-acetolactate by DXPS (with ThDP) upon addition of pyruvate, confirming decarboxylation of pyruvate to the enamine. The carboligation of the enamine with pyruvate as an acceptor led to (*R*)-acetolactate (Figure 11). This apparently is not the case with DXPS with MeOThDP, leading to the conclusion that substitution of ThDP with MeOThDP affects both DXP synthesis and carboligase activities of the DXPS.

DISCUSSION

The data presented in this paper clearly indicate that MeOThDP is a functionally competent analogue of ThDP. Approximately 5.9–11% of NADH production could be detected for PDHc-ec when ThDP in E1p-ec was replaced with MeOThDP. Also, upon addition of MeOThDP to apo-DXPS, ~8.8 and ~14% of DXP formation could be detected; however, a low catalytic efficiency was demonstrated for both enzymes. Among the five enzymes tested, PDHc-h in the presence of MeOThDP revealed the highest level of product formation

(~48–75% NADH produced as compared with 0.6–5% NADH produced in the absence of ThDP). With OGDHc-ec and OGDHc-h, only a modest inhibition was observed in the presence of MeOThDP, but again a high percent of tightly bound ThDP was detected for both enzymes [72 and 76% for OGDHc-h and 53 and 93% for OGDHc-ec in duplicate preparations (Table 3)]. Because of these results, our major focus was on E1p-ec, E1p-h, and *E. coli* DXPS, from which most of the ThDP could be removed, and their activity could be completely restored upon complexation with ThDP. The results led to the following conclusions.

- i. MeOThDP binds in the active centers of ThDP-dependent enzymes with binding constants that were not very different from those of ThDP. This was evident from fluorescence, CD, and kinetic experiments with E1p-ec, E1p-h, and DXPS. However, the binding mode of MeOThDP could be different from that of ThDP judging by the amplitudes and appearance of CD signals and the percent of fluorescence quenching.
- ii. CD experiments revealed that upon addition of MeOThDP to apo-E1p-h, the pK_a for the $([AP] + [IP])/[APH^+]$ equilibrium is shifted from 7.07 (with ThDP) to 8.02 (with MeOThDP), which is a property of MeOThDP bound on E1p-h. However, it was also shown that in a model 4-aminopyrimidine, replacement of 2-CH₃- by 2-CH₃O- leads to a 0.6 unit pK_a reduction in solution (see ref 41 for the pK_a of an appropriate model with 2-CH₃, rather than with 2-OCH₃, shown in Figure 4). We believe that this comparison is valid and parallels what would be expected from a comparison of the pK_a 's at the N1' atom of ThDP and MeOThDP, as this substitution at the C2' position is the only difference in structure between ThDP and MeOThDP. Therefore, the observed elevation in the pK_a on the enzyme must pertain to the interaction of MeOThDP with the protein environment, plausibly because of the incorrect alignment of the N1' atom with the conserved Glu, typically within hydrogen bonding distance in the complex with ThDP. The fact that the APH⁺ form is stabilized on ThDP enzymes was especially striking and evident from solid-state NMR results for three enzymes;³⁹ here with the ThDP analogue, we observe even further stabilization leading to an even higher pK_a . This pK_a shift to more alkaline values is likely also reflected in the bell-shaped k_{cat} -pH profile with deduced pK_a 's of 6.4 and 7.8. These values suggest that a group on the enzyme with a pK_a of 7.8 should be in its conjugate acid form in the rate-limiting step, and we suggest that this observation pertains to the APH⁺ form of MeOThDP, with a pK_a of 8.02 according to direct CD determination.
- iii. Kinetic analysis of PDHc-ec revealed a similar mechanism of activation by MeOThDP as was found with ThDP. However, the rate constant of binding of the second MeOThDP was approximately 14 times slower as compared with ThDP, a decrease that could be attributed to a slower association rate constant of the second MeOThDP to be bound, possibly to the second active center in this homodimer.
- iv. Both E1p components revealed formation of a stable predecarboxylation intermediate derived from MeOThDP and acetylphosphinate; however, the binding mode was different for E1p-ec according to the appearance of the CD spectra.

Perhaps reflecting the “poorer” fit of the MeOThDP to the coenzyme binding site, the stoichiometry of binding is 2.6, compared to 1.0 for ThDP, the latter displaying essentially “quantitative” binding.

- v. Evidence was obtained for formation of (R)-acetolactate by apo-DXPS complexed with ThDP, confirming decarboxylation of pyruvate to the enamine. This apparently is not the case with apo-DXPS complexed with MeOThDP, leading to the conclusion that substitution of ThDP with MeOThDP affects both DXP synthase and carboligase activities of the DXPS.
- vi. The MeOThDP-bound covalent intermediates are competent in reductive acetylation of the lipoyl moiety of the lipoyl domain covalently bound to E2p-ec and E2p-h.
- vii. MeOThDP was shown to effectively compete with ThTTDP on DXPS, and hence with ThDP. This is a useful experiment, because the UV-CD property of bound TTThDP permits studies also of the reversibility of binding, or lack thereof, of ThDP analogues.

These results are important as far as potential inhibition by MeOThDP is concerned and point to difficulties to be anticipated in the design of inhibitors for ThDP enzymes that would depend on rapid/reversible dissociation of ThDP to be effective.

The results are also important in demonstrating the tolerance of the enzymes for the subtle substitution at position 2' of ThDP, and its clear and surprisingly dramatic consequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Th	thiamin
MeOThDP	2'-methoxythiamin diphosphate
ThDP	thiamin diphosphate
ThTTDP	thiamin 2-thiothiazolone diphosphate
AP	4'-aminopyrimidine tautomeric form of ThDP

AcPhi	acetylphosphinic acid sodium salt
D-GAP	D-glyceraldehyde 3-phosphate
DXP	1-deoxy-D-xylulose 5-phosphate
LThDP	C2 α -lactyl-ThDP
TCEP	tris(2-carboxyethyl)phosphine
OGDHc	2-oxoglutarate dehydrogenase complex
PDHc	pyruvate dehydrogease complex
DXPS	1-deoxy-D-xylulose 5-phosphate synthase
E1p-ec	<i>E. coli</i> pyruvate dehydrogenase first component
E2p	dihydrolipoamide acetyl-transferase
E3	dihydrolipoamide dehydrogenase
LD_h-ec	E2p-ec hybrid lipoyl domain, where residues 1–33 are from the N-terminal end of the first lipoyl domain and residues 34–85 are from the C-terminal end of the third lipoyl domain of wild-type three-lipoyl domain E2p-ec
E1p-h	E2p-h, and E3-h, components of human pyruvate dehydrogenase complex
LD1-h	outer lipoyl domain of E2p-h
E3BP	E3h-binding protein
CD	circular dichroism electronic spectroscopy
FT-MS	Fourier transform mass spectrometry with the electrospray ionization sampling method
ec	<i>E. coli</i> origin
h	human origin

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Figure 1.

Binding of MeOThDP to E1p-ec by kinetic analysis of the overall PDHc-ec activity. The top panel shows the Progress curves for NADH production in the overall PDHc-ec reaction where MeOThDP was complexed with E1p-ec at the indicated MeOThDP concentrations. Its inset shows the progress curve for NADH production in the presence of 200 μM ThDP. The bottom panel shows the [MeOThDP] dependence of the lag phase determined from the progress curves for NADH production. Its inset shows the dependence of the reciprocal of the lag phase on [MeOThDP]. See Experimental Procedures for details.

Figure 2.

CD spectra of E1p-h complexed with ThDP and MeOThDP. E1p-h (1.5 mg/mL, concentration of active centers of 19.6 μ M) in 50 mM KH_2PO_4 (pH 7.0) containing 2.0 mM MgCl_2 was complexed with 0.20 mM ThDP (spectrum 2, colored blue) or 0.20 mM MeOThDP (spectrum 3, colored red). The positive CD_{305} (IP form of ThDP) and the negative CD_{330} (AP form of ThDP) bands relative to the baseline were detected on E1p-h complexed with ThDP.

Figure 3. pH dependence of the CD band for the AP form of MeOThDP bound on E1p-h. (A) Near-UV CD spectrum of E1p-h complexed with MeOThDP at pH 8.54. (B) pH dependence of the AP form of MeOThDP. The trace is a fit to an equation with a single proton titrating, $(CD_{309})_H = (CD_{309,max} \times [H^+]) / (K_a + [H^+])$, where $(CD_{309})_H$ is the observed value of CD_{309} at a particular pH.²⁶ See Experimental Procedures for details.

Figure 4.

UV spectroscopic determination of the pK_a for N1-protonated bacimethrin. The top panel shows the pH dependence of the absorption spectra of bacimethrin. Spectra were recorded in a triple buffer containing 50 mM MES, 0.1 M Tris-HCl, 50 mM acetic acid, and 5% methanol in the pH range of 4.65–7.43. The bottom panel shows the pH dependence of the Abs₂₇₃/Abs₂₆₅ ratio. The trace is a fit to an equation with a single proton titrating, $(\text{Abs}_{273}/\text{Abs}_{265})_H = [(\text{Abs}_{273}/\text{Abs}_{265})_{\text{max}} \times [\text{H}^+]] / (K_a + [\text{H}^+])$.

Figure 5.

CD titration of E1p-ec with acetylphosphinate. (A) Difference CD spectra of E1p-ec (2.0 mg/mL, 20 μ M of active centers) complexed with 0.20 mM ThDP and titrated with AcPhi (0.5–130 μ M). (B) Dependence of CD₃₁₉ on AcPhi concentration. (C) Difference spectra of E1p-ec (1.45 mg/mL, concentration of active centers of 14.4 μ M) complexed with 0.20 mM MeOThDP and titrated with AcPhi (0.5–160 μ M). (D) Dependence of CD₃₀₃ on AcPhi concentration. Data in panels B and D were fitted using a quadratic equation (see Experimental Procedures for details), and the trace is the regression fit line.

Figure 6.

Time dependence of the fraction of reductively acetylated LD_h-ec during a steady-state experiment using MS. The trace is a fit to a single exponential. In the inset, the solid line represents a linear fit to initial rate conditions. See Experimental Procedures for details.

Figure 7.

Progress of the DXPS reaction monitored by CD at 290 nm reflecting DXP formation: (A) apo-DXPS (1.2% holoenzyme), (B) apo-DXPS complexed with 0.10 mM ThDP, and (C) apo-DXPS complexed with 0.1 mM MeOThDP. See conditions in Experimental Procedures.

Figure 8.

Quenching of the intrinsic fluorescence of DXPS by ThDP and MeOThDP. The top panel shows the dependence of the relative fluorescence quenching on the concentration of ThDP. DXPS (0.058 mg/mL, concentration of active centers of 0.858 μM) in a mixture of 50 mM KH_2PO_4 and 50 mM Tris (pH 8.0) containing 0.1 M NaCl, 2 mM MgCl_2 , and 1% glycerol was titrated with ThDP (0.3–70 μM). The bottom panels shows the dependence of the relative fluorescence quenching on the concentration of MeOThDP (0.3–70 μM) under

conditions similar to those used for ThDP. Data points were fit using eq 2, and the line is the regression fit trace. For details, see Experimental Procedures.

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Figure 9.

CD titration of DXPS with ThTTDP in the presence of 10 μM MeOThDP. (A) Near-UV CD spectra of DXPS upon titration by ThTTDP. (B) Dependence of CD₃₃₁ on the concentration of ThTTDP. Data points were fitted using quadratic eq 4, and the line is the regression fit trace. (C) Dependence of CD₃₃₁ on the [ThTTDP]/[DXPS active centers] molar ratio. For details, see Experimental Procedures.

Figure 10.

Near-UV CD spectra of DXPS with ThDP and MeOThDP present. (A) Near-UV CD spectra of apo-DXPS with ThDP: (1) apo-DXPS, (2) apo-DXPS with 0.15 mM ThDP with evidence of 4'-aminopyrimidinylThDP (AP tautomer with negative CD_{320}), and (3) apo-DXPS with 0.15 mM ThDP and 100 μ M pyruvate with evidence of the formation of the C2 α -lactylThDP (LThDP) predecarboxylation intermediate (positive CD_{312}). (B) Near-UV CD spectra of apo-DXPS with MeOThDP: (1) apo-DXPS with 0.15 mM MeOThDP and (2–9) apo-DXPS

with 0.15 mM MeOThDP upon addition of 25–2000 μ M pyruvate. See conditions in Experimental Procedures.

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Figure 11.

CD evidence of formation of acetolactate from pyruvate by DXPS complexed with ThDP but not with MeOThDP. CD spectra of the remaining reaction mixture were recorded for (1) apo-DXPS (1.88 mg/mL, concentration of active centers of 27.8 μ M) incubated with 0.15 mM ThDP and 1 mM pyruvate at 5 °C for 15 h before protein was removed and (2) apo-DXPS (1.61 mg/mL, concentration of active centers of 23.81 μ M, 0.4% holoenzyme) incubated with 0.15 mM MeOThDP and 2 mM pyruvate at 5 °C for 15 h before protein was removed.

Scheme 1.
Synthesis of the 2'-Methoxythiamin Diphosphate

Scheme 2.
 Mechanism of PDHc-ec with the Role of ThDP on E1p-ec²⁹

Scheme 3.

Individual Steps in the DXP Synthase Catalytic Cycle with Pyruvate and GAP Present in LThDP Formation²³

Table 1

E1p Specific Activities and PDHc Activities of E1p-ec (top) and E1p-h (bottom)

overall PDHc-ec activity ^a [$\mu\text{mol min}^{-1} (\text{mg of E1p-ec})^{-1}$]			E1p-ec specific assay ^b [$\mu\text{mol min}^{-1} (\text{mg of E1p-ec})^{-1}$]		
with ThDP ^c	without ThDP ^c	with MeOThDP	with ThDP ^c	without ThDP ^c	with MeOThDP
36.9 $\pm$ 0.69 (100%)	0	3.18 $\pm$ 0.88 (8.6%)	0.36 $\pm$ 0.01 (100%)	0	0.069 $\pm$ 0.003 (19%)
24.8 $\pm$ 1.41 (100%)	0	2.76 $\pm$ 0.14 (11%)	0.29 $\pm$ 0.02 (100%)	1.2%	n/a ^d
32.7 $\pm$ 1.03 (100%)	0	1.93 $\pm$ 0.29 (5.9%)	n/a ^d	n/a ^d	n/a ^d
overall PDHc-h activity ^a [$\mu\text{mol min}^{-1} (\text{mg of E1p-h})^{-1}$]			E1p-h specific assay ^b [$\mu\text{mol min}^{-1} (\text{mg of E1p-h})^{-1}$]		
with ThDP ^c	without ThDP ^c	with MeOThDP	with ThDP ^c	without ThDP ^c	with MeOThDP
12.5 $\pm$ 0.4 (100%)	0.08 $\pm$ 0.03 (0.6%)	9.43 $\pm$ 0.46 (75%)	n/a ^d	n/a ^d	n/a ^d
16.2 $\pm$ 1.2 (100%)	0.83 $\pm$ 0.07 (5%)	9.08 $\pm$ 0.18 (56%)	0.09 $\pm$ 0.01 (100%)	0.0029 (3.2%)	n/a ^d
22.2 $\pm$ 2.2 (100%)	0.31 $\pm$ 0.06 (1.4%)	10.7 $\pm$ 0.36 (48%)	0.04 $\pm$ 0.01 (100%)	0.0012 (3.5%)	0.017 $\pm$ 0.01 (49%)

^aData for three independent preparations of the respective enzymes are presented. The overall PDHc activity was measured by the formation of NADH after reconstitution of E1p-ec with recombinant E2p-ec and E3-ec components and E1p-h with the recombinant E2-E3BP-h subcomplex and recombinant E3-h in the reaction assays as reported earlier (ref 22 for PDHc-ec and ref 19 for PDHc-h). The concentrations of ThDP and MeOThDP were similar: 0.2 mM (E1p-ec) and 0.2 and 0.5 mM (E1p-h).

^bThe E1p-ec specific activity was measured for E1p components alone in a model reaction (refs 19 and 22).

^cWith or without ThDP indicates the presence of ThDP (MeOThDP) or its absence, respectively, in the reaction assays for activity measurement. In both cases (with and without ThDP), the ThDP was first removed from the active centers of the respective enzymes before their activities were analyzed in different assay systems.

^dNo measurement of the E1p specific activity was conducted for a particular enzyme preparation.

Table 2

Rate Constants for Binding of ThDP and MeOThDP to PDHc-ec (see eq 7)

enzyme	K_1^a (M ⁻¹)	k_2^b (s ⁻¹)	k_{-2}^c (s ⁻¹)	k_3^d (M ⁻¹ s ⁻¹)	k_{-3}^e (s ⁻¹)
PDHc-ec ^f (ThDP)	1.2×10^6	1.12×10^{-2}	0.38×10^{-2}	0.71×10^3	0.76×10^{-2}
PDHc-ec ^{g,h} (MeOThDP)	0.3×10^6	0.87×10^{-2}	0.33×10^{-2}	0.49×10^2	0.69×10^{-2}

^a Binding constant.^b Forward rate constant of ThDP or MeOThDP binding.^c Dissociation rate constant of ThDP or MeOThDP.^d Forward rate constants for second ThDP or MeOThDP binding.^e Reverse rate constant for dissociation of second ThDP or MeOThDP.^f Data from ref 33.^g PDHc-ec was reconstituted from apo-E1p-ec, E2p-ec, and E3-ec.^h Rate constants were calculated using equations from ref 33.

Table 3

Overall OGDHc and E1o Specific Activities

enzyme source ^a	overall OGDHc activity ^b [$\mu\text{mol min}^{-1}$ (mg of E1o) ⁻¹]		E1o specific activity ^c [$\mu\text{mol min}^{-1}$ (mg of E1o) ⁻¹]	
	with ThDP ^d	without ThDP ^d	with ThDP ^d	without ThDP ^d
OGDHc-h	15.6 $\pm$ 1.3 (100%)	11.3 $\pm$ 0.3 (72%)	10.0 $\pm$ 0.35 (64%)	0.51 $\pm$ 0.02 (100%)
OGDHc-h	14.5 $\pm$ 0.5 (100%)	11.0 $\pm$ 0.6 (76%)	<i>e</i>	0.44 $\pm$ 0.08 (100%)
OGDHc-ec	31.7 $\pm$ 1.4 (100%)	16.8 $\pm$ 0.3 (53%)	13.2 $\pm$ 1.0 (42%)	0.54 $\pm$ 0.03 (100%)
OGDHc-ec	26.2 $\pm$ 1.7 (100%)	24.3 $\pm$ 0.3 (93%)	18.1 $\pm$ 0.1 (69%)	0.57 $\pm$ 0.02 (100%)

^aTwo different preparations of E1o-h and E1o-ec were used these studies.

^bThe overall OGDHc activity was measured by the formation of NADH after reconstitution of E1o-h or E1o-ec with the corresponding recombinant E2o and E3 components in the reaction assays reported previously.^{20,21}

^cE1o specific activities were measured as reported previously.^{20,21}

^dWith and without ThDP (MeOTHP) indicate the presence of ThDP (MeOTHP) and its absence, respectively, in the reaction assay. Apo-E1o-h and apo-E1o-ec were prepared by using a PD-10 column equilibrated with 50 mM HEPES (pH 7.5) containing 0.15 M NaCl and 2 mM MgCl₂ followed by dialysis for 15 h at 4 °C against the same buffer but with no MgCl₂ added. See the experimental procedures in the Supporting Information for details.

^eNo measurement of the E1p specific activity was conducted for a particular enzyme preparation.

Table 4

Kinetic Parameters for DXPS in the Presence of ThDP or Its Analogues

DXPS activity ^a [$\mu\text{mol min}^{-1}$ (mg of DXPS) ⁻¹]		[I]			
with ThDP	without ThDP	with MeOThDP	$K_{\text{d,ThDP}}^b$ (μM)	$K_{\text{d,MeOThDP}}^b$ (μM)	$K_{\text{d,ThTTDP}}^c$ (μM)
1.84 ± 0.09 (100%)	0.022 ± 0.01 (1.2%)	0.26 ± 0.04 (14%)	3.5 ± 0.1 (35%) ^d	6.24 ± 0.23 (27%) ^d	0.39 ± 0.10
1.58 ± 0.10 (100%)	0.005 ± 0.001 (0.34%)	^e			0.60 ± 0.14 (with MeOThDP)
3.39 ± 0.08 (100%)	0.013 ± 0.003 (0.38%)	0.30 ± 0.01 (8.8%)			

^aData are presented for three independent preparations of DXPS. Each activity is an average from three or four independent measurements.^b K_{d} values for ThDP and MeOThDP were calculated from quenching of the intrinsic fluorescence of DXPS.^cThe $K_{\text{d,ThTTDP}}$ was calculated from a CD titration experiment.^dPercent of quenching of the fluorescence of apo-DXPS. See Experimental Procedures for details.^eNo measurement of the E1p specific activity was conducted for a particular enzyme preparation.