Communication between Thiamin Cofactors in the Escherichia coli Pyruvate Dehydrogenase Complex E1 Component Active Centers

EVIDENCE FOR A "DIRECT PATHWAY" BETWEEN THE 4'-AMINOPYRIMIDINE N1' ATOMS*

Received for publication, September 22, 2009, and in revised form, December 29, 2009. Published, JBC Papers in Press, January 27, 2010, DOI 10.1074/jbc.M109.069179

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Kinetic, spectroscopic, and structural analysis tested the hypothesis that a chain of residues connecting the 4'-aminopyrimidine N1' atoms of thiamin diphosphates (ThDPs) in the two active centers of the Escherichia coli pyruvate dehydrogenase complex E1 component provides a signal transduction pathway. Substitution of the three acidic residues (Glu571, Glu235, and Glu237) and Arg606 resulted in impaired binding of the second ThDP, once the first active center was filled, suggesting a pathway for communication between the two ThDPs. 1) Steady-state kinetic and fluorescence quenching studies revealed that upon E571A, E235A, E237A, and R606A substitutions, ThDP binding in the second active center was affected. 2) Analysis of the kinetics of thiazolium C2 hydrogen/deuterium exchange of enzyme-bound ThDP suggests half-of-the-sites reactivity for the E1 component, with fast (activated site) and slow exchanging sites (dormant site). The E235A and E571A variants gave no evidence for the slow exchanging site, indicating that only one of two active sites is filled with ThDP. 3) Titration of the E235A and E237A variants with methyl acetylphosphonate monitored by circular dichroism suggested that only half of the active sites were filled with a covalent predecarboxylation intermediate analog. 4) Crystal structures of E235A and E571A in complex with ThDP revealed the structural basis for the spectroscopic and kinetic observations and showed that either substitution affects cofactor binding, despite the fact that Glu235 makes no direct contact with the cofactor. The role of the conserved Glu571 residue in both catalysis and cofactor orientation is revealed by the combined results for the first time.

* This work was supported, in whole or in part, by National Institutes of Health Grants GM-050380 (to F. J.) and GM-061791 (to W. F.). This work was also supported by the Veterans Affairs Merit Review Program (to W. F.). The atomic coordinates and structure factors (codes 3LPL, 3LQ2, and 3LQ4) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Communication between the thiamin diphosphate cofactors (ThDPs) at the active centers of ThDP enzymes has been reported for several years (1–6). An intriguing pathway for such interaction had been suggested by Perham’s group (2) on the basis of crystal structure studies on E1bs (the E1 component of the pyruvate dehydrogenase complex [PDHc] from Bacillus steatorrhophilus). According to this suggestion, there is a hydrated tunnel of acidic residues involved in proton shuttling from the N1' atom of the ThDP in one active site (activated ThDP, active site is closed) to the corresponding atom in the second active site (non-activated ThDP, active site is open) over a distance of 20 Å, dubbed a “proton wire” (2). This mechanism explains how two active sites communicate with each other, with no significant conformational changes in the structure of the subunits with the exception of the active site loops (2). At the same time, for E1h (human PDHc E1), a so-called “flip-flop” mechanism was proposed for active site communication through the shuttle-like motion of the heterotetramers, although the x-ray structure did not reveal directly a structural nonequivalence of the two active centers (4). Direct kinetic evidence of the chemical non-equivalence of the E1h active sites with respect to ThDP and substrate analog methyl acetylphosphonate binding was reported in support of the flip-flop mechanism. It was demonstrated that only one of the two bound ThDPs is in the activated state (5).

It was pointed out in a note accompanying the proton wire suggestion that not all ThDP enzymes have such an acidic proton wire arrangement of amino acids apparent in their x-ray structures (3), and, in fact, a study on the E1 component of a human branched-chain α-keto acid dehydrogenase demonstrated that a substitution of acidic residues on the path does not affect the overall activity of that complex. It was suggested that the two active sites in this enzyme operate independently during catalysis (6). This enzyme, however, does not have an

4 The abbreviations used are: ThDP, thiamin diphosphate; PDHc, pyruvate dehydrogenase complex; E235A-ThDP, the complex formed with the E235A E1 Ec variant and ThDP; E571A-ThDP, the complex formed with the E571A E1 Ec variant and ThDP; MAP, methyl acetylphosphonate; LThDP, C2α-lactyl-ThDP; PLThDP, 1’-4’-imino phosphonoacetyl thiamin diphosphate; ThTDP, thiamin 2-thiothiazolone diphosphate; N1'-MeThDP, N1'-methylated ThDP.
uninterrupted array of acidic residues between ThDPs. Our groups have studied the kinetic behavior and x-ray structure of E1ec (the PDHc E1 component from Escherichia coli), which, as seen in Fig. 1, indeed has such an apparent series of acidic and hydrogen bonding-capable residues, all able to participate in active center communication in an uninterrupted array of residues and water molecules between the two ThDPs (3). We therefore deemed this enzyme a satisfactory test case for the pathway suggested by the proton wire hypothesis. We report biochemical and biophysical experiments, including steady-state kinetics, spectroscopic observation of the rates of formation of both the first step of the reaction sequence (formation of the ylide) and of the first predecarboxylation intermediate, fluorescence determination of ThDP binding, and x-ray structural analyses of the E235A and E571A variants on the putative pathway. The biochemical results clearly indicate that the interaction between active centers is greatly impaired upon substitution of any of the three acidic residues (E571, E235, and E237) or of Arg$^{606}$ along the communication pathway, whereas the x-ray results reveal the structural basis for the diminished functionality in the E235A and E571A variants examined by crystallographic methods.

EXPERIMENTAL PROCEDURES

Construction of the E235A, E237A, E571A, and R606A Variants of E1ec—The pGS878 plasmid encoding E1ec, two synthetic oligonucleotide primers (Integrated DNA Technologies) complementary to the opposite strands of the DNA, and the QuikChange site-directed mutagenesis kit (Stratagene) were used for mutagenesis reactions. The following synthetic oligonucleotides (and their complements) were used as mutagenic primers (mismatched bases are underlined, and mutated nucleotides (and their complements) were used as mutagenic primers (Integrated DNA Technologies)).

**TABLE 1**

| Cofactor occupancy refinement for two active centers of E235A E1ec |
|----------------|-----------------|-----------------|-----------------|----------------|
|                | E235A-ThDP      | E235A-ThDP      | E235A-ThDP      | E235A-ThDP      |
|                | (0.5 mM)        | (5 mM)          | (0.5 mM)        | (5 mM)          |
| Pyrimidine ring| 1.00            | 0.63            | 1.00            | 0.93            |
| Thiazolium ring| 0.51            | 0.36            | 0.65            | 0.53            |
| Diphosphate group| 1.00          | 0.51            | 1.00            | 0.75            |

Activity and Related Measurements—The activity of E1ec and its variants was measured in the overall PDHc reaction after reconstitution with E2ec and E3ec components as reported previously (8). The reaction medium contained the following in 1.0 ml: 0.1 M Tris-HCl (pH 8.0), 1 mM MgCl$_2$, 0.20 mM ThDP, 2 mM sodium pyruvate, 2,5 mM NAD$^+$, 0.1–0.20 mM CoA, 2.6 mM dithiothreitol at 30 °C. The reaction was initiated by the addition of PDHc and CoA. Steady-state velocities were taken from the linear portion of the progress curve. One unit of activity is defined as the amount of NADH$^+$ produced (μmol/min/mg E1). The E1-specific activity was measured in the model reaction monitoring the reduction of 2,6-dichlorophenol-indophenol at 600 nm (8). The lag phases ($\tau$) in the progress curves for NADH$^+$ production were determined as described previously (9).

**TABLE 2**

| Crystallographic data and refinement statistics | Values |
|-----------------------------------------------|--------|
|                                               | E571A-ThDP | E235A-ThDP (0.5 mM) | E235A-ThDP (5 mM) |
| Parameters                                    |         |                   |                  |
| Space group                                   | P$_2$   | P$_2$             | P$_2$            |
| R factor (last shell)                         | 0.197(0.229) | 0.194(0.283)    | 0.199(0.253)     |
| R$_{pro}$ (last shell)                       | 0.238(0.286) | 0.223(0.317)    | 0.236(0.290)     |
| No. of residues                               | 1602    | 1602              | 1602             |
| No. of waters                                 | 725     | 632               | 677              |
| Average $\beta$ factor ($\beta^\text{a}$)     |         |                   |                  |
| Main chain                                    | 26.4    | 42.1              | 35.7             |
| Side chain                                    | 26.7    | 45.4              | 38.9             |
| Solvent atoms                                 | 29.4    | 47.1              | 41.1             |
| r.m.s. deviations                             |         |                   |                  |
| Bond lengths ($\AA$)                          | 0.010   | 0.006             | 0.006            |
| Bond angles (degrees)                         | 1.5     | 1.2               | 1.2              |

Hydrogen/Deuterium Exchange Kinetics at C2-H of the Enzyme-bound ThDP by Rapid Quench $^1$H NMR Spectroscopy—The deprotonation rate constants at the C2 carbon of E1ec-bound and free ThDP were determined using a hydrogen/deuterium exchange technique as described earlier (5, 10). The apo-E1ec enzymes were reconstituted with an equimolar amount of ThDP and 1 mM Mg$^{2+}$ in 20 mM KH$_2$PO$_4$ (pH 7.0) and mixed with D$_2$O (99.9%) in a 1:1 mixing ratio at 30 °C, by using a rapid quench flow device (QRF-3, Kintek Corp. (Austin, TX)) for reaction times up to 2000 ms and by manual mixing for longer reaction times. After acid quench of the exchange reaction, the isolated cofactor was analyzed by $^1$H NMR spectroscopy. The singlet signal of the C6'-H proton (8.01 ppm) was used as an internal non-exchanging standard. To obtain the exchange rates, the relative decay of the signal intensity of C2-H (9.68 ppm) was fitted to a monoeponential or double exponential function as detailed (5).

Fluorescence Spectroscopy—The fluorescence spectra of E1ec and its variants were recorded using a Cary Eclipse fluorescence spectrometer from Varian Inc. (8). The protein was diluted to a concentration of 0.034 mg/ml (concentration of active centers = 0.342 μM) in 10 mM KH$_2$PO$_4$ (pH 7.0), containing MgCl$_2$ (5 mM) and pyruvate (1.0 mM).
Circular Dichroism—CD spectra were recorded on an Aviv model 202 CD spectrometer and on a Chirascan CD spectrometer from Applied Photophysics (Leatherhead, UK) in a 1-cm path cell at 30 °C. The E1ec or its variants were diluted to a concentration of 2.0 mg/ml (concentration of active centers = 20 μM) in 20 mM KH2PO4 (pH 7.0) containing MgCl2 (2 mM) and 0.20 mM ThDP for E1ec and 0.50 mM ThDP for the E235A, E237A, E571A, and R606A variants, the maximum concentration of ThDP that could be used for CD titration. For titration experiments with methyl acetylphosphonate (MAP; a substrate analog), the E1ec variants were preincubated with ThDP for about 10 min before the first aliquot of MAP was added, and the steady-state level of the CD band was reached after ~2–2.5 h upon the addition of the last aliquot of MAP, eliminating the effect of the ThDP-dependent lag phase (lasting for about 40–100 s) on CD measurements.

Time-resolved CD—Stopped-flow CD experiments were carried out at 302 nm on a Pi Star –180 CDF instrument from Applied Photophysics with a path length of 10 mm and bandwidth of 2 nm at 30 °C. In a typical experiment, protein was diluted to a concentration of 5–10 mg/ml in 20 mM KH2PO4 (pH 7.0) containing MgCl2 (2 mM) and 0.20 mM ThDP for E1ec and 0.50 mM ThDP for the E235A, E237A, E571A, and R606A variants, the maximum concentration of ThDP that could be used for CD titration. For titration experiments with methyl acetylphosphonate (MAP; a substrate analog), the E1ec variants were preincubated with ThDP for about 10 min before the first aliquot of MAP was added, and the steady-state level of the CD band was reached after ~2–2.5 h upon the addition of the last aliquot of MAP, eliminating the effect of the ThDP-dependent lag phase (lasting for about 40–100 s) on CD measurements.

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\[
CD = a_1 \times \exp(-k_1 \times t) + a_2 \times \exp(-k_2 \times t) \quad (\text{Eq. 1})
\]

where \(k_1\) and \(k_2\) are the rate constants for intermediate formation, and \(t\) is time (s).

Crystallization and Data Collection—Crystallization of the E571A and E235A variants of the E1ec complexed with ThDP in the presence of Mg2+ was done separately by the sitting drop vapor diffusion method. The best crystals were obtained at a reservoir solution with 15–20% polyethylene glycol 2000 monomethyl ether, 10% propanol, 0.2% NaN3, and 60 mM HEPES (pH 7.0) at 22 °C. Drops were 6–10 μl, consisting of equal parts of reservoir and protein solution. For crystallization of the E571A-ThDP, the protein concentration was 25.6 mg/ml, whereas for the E235A-ThDP, it was 28 mg/ml with the ThDP concentrations at both 0.5 and 5 mM in the protein solution. In all cases, the crystals grew within 4–6 weeks and were isomorphous to E1ec-ThDP crystals (12). The crystals typically have dimensions of 0.15 × 0.20 × 0.30 mm. Low temperature (~180 °C) data sets were collected on the SERCAT (sector-22ID) beamline at the APS (Advanced Photon Source, Argonne National Laboratory). All data sets were processed with the HKL2000 package (13). The E571A-ThDP data set was truncated at 2.1 Å resolution, whereas the E235A-ThDP data sets were truncated at 1.96 and 1.98 Å resolution for the complexes of E235A crystallized with 0.5 and 5 mM ThDP, respectively.

Structure Determination and Refinement—Because the E1ec-ThDP crystals are isomorphous with the E571A-ThDP and E235A-ThDP crystals, the atomic coordinates of the E1ec-ThDP structure, refined to 1.85 Å resolution (12), were used as the starting model. The initial model included 1602 amino acids, with the cofactors ThDP and Mg2+ along with neighboring active site residues omitted. Following rigid body refinement, simulated annealing refinement, and molecular replacement, the model was refined to 1.65 Å resolution.

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![ThDP complexes of E1ec](image)

**FIGURE 1.** Shown are the amino acids of E1ec that connect one ThDP cofactor to the other. Residue Glu571 (E571) is the highly conserved glutamate present in ThDP-dependent enzymes and forms a hydrogen bond directly with the ThDP cofactor.

### Table 3: Kinetic parameters for E1ec and its variants

| Variant | Overall activitya | Overall assay, 1.0 mM ThDP | DCPiP assay, 0.2 mM ThDPb | K_{m,THDP}c | K_{m,pyruvate}d |
|---------|-------------------|-----------------------------|--------------------------|------------|---------------|
| E1ec    | 52.2 ± 1.90 (%)   | 52.2 ± 1.90 (%)             | 0.52 (100%)              | 1.6 ± 0.10 | 0.29 ± 0.01   |
| E235A   | 5.92 ± 0.84 (11.4%) | 14.1 ± 2.75 (27%)          | 0.43 (83%)               | 133 ± 8.6  | 0.99 ± 0.11   |
| E237A   | 3.73 ± 0.42 (7.2%) | 6.3 ± 0.75 (12%)           | 0.057 (11%)              | 633 ± 104  | 1.36 ± 0.13   |
| E571A   | 0.89 ± 0.17 (1.7%) | 1.48 ± 0.33 (2.8%)         | 0.093 (17%)              | 13.8 ± 2.9  | 0.17 ± 0.01   |
| E571D   | 0.32 ± 0.03 (0.61%) | 3.95 ± 0.44 (7.6%)         | 0.13 (25%)               | NAd        | 1.65 ± 0.11   |
| E571Q   | 0.68 ± 0.06 (1.3%) | NA                          | 0.02 (3.9%)              | 8.26 ± 0.45 | 0.35 ± 0.02   |
| R606A   | 1.73 ± 0.09 (3.3%) | 1.75 ± 0.02 (3.4%)         | 0.056 (11%)              | 2.24 ± 0.26 | 0.51 ± 0.01   |

a Overall PDHC activity was measured under conditions optimal for E1ec (0.20 mM ThDP) after reconstitution with E2ec-E3ec subcomplex (see “Experimental Procedures”). The steady state regions of the progress curves for NADH production were analyzed, and activity was expressed as units/mg E1ec (μmol of NADH min−1 mg E1ec−1). Activities for E571D and E571Q variants are from Refs. 15 and 18.

b Specific activity of the E1ec and its variants in the absence of E2ec and E3ec.

c S_{0.5, THDP} presented for the E235A and E237A variants. For the E571A variant, the K_{m,THDP} was obtained in the DCPiP reaction. E571D was not saturated at 2.5–500 μM ThDP.

d K_{m,pyruvate} presented for the E235A and E237A variants. For the E571A variant, the K_{m,pyruvate} was determined in the presence of 1 mM ThDP.

e K_{m,THDP} could be enhanced 2.3-fold (E235A) and 2.6-fold (R606A) by 0.50 mM ThDP.

f Activity measured with 5 mM ThDP.

g NA, not applicable.
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... was performed with the CNS program (14) without imposing any non-crystallographic symmetry, with a random subset of all data (∼5%) set aside for calculation of \( R_{\text{free}} \). After refinements of the protein parts were complete, \( 2F_O - F_c \) composite, simulated annealing, omit maps were calculated and examined. In the case of the E571A-ThDP, the map revealed the active site residues and location of only the diphosphate group of the ThDP cofactor; hence, only those atoms were used for further refinement. For the E571A-ThDP, strong electron density clearly indicated that residues His\(^{142}\) and Tyr\(^{598}\) in the active site were disordered, partially occupying two rotamer conformations. The alternate conformation setup procedure in CNS (14) was followed to provide for refinement of the disorder. Similar to the E1-ThDP structure reported previously, the regions 1–55, 401–413, and 541–557 were completely disordered, and they remain absent in the model. The model was then refined by simulated annealing, and subsequent cycles consisted of positional and \( B \)-factor refinement. Further model building, water molecule addition, and refinement cycles resulted in an \( R \) factor of 19.7% for the E571A-ThDP complex.

For the complexes of E235A with both the high and low ThDP concentrations, the maps revealed the active site residues and locations of the ThDP and Mg\(^{2+}\) cofactors. In the lower ThDP concentration data set, the electron density for the cofactors is very weak, and a difference map showed some negative density for the thiazolium ring in one of the active sites. In the higher ThDP concentration data set, the cofactor electron density is stronger but still shows differences in the two active sites. Average \( B \)-factors for the atoms in the two cofactors for both data sets were significantly higher than the values for the nearby protein residues, suggesting that they may be bound at partial occupancy. To fit the occupancy of the cofactors in the higher concen-
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**RESULTS**

**Steady-State Kinetic Analysis of the E1ec Variants Created for Residues on the Communication Path Reveals that ThDP Binding Is Affected**

As seen in Fig. 1, the residues Glu\(^{571}\), Glu\(^{235}\), Glu\(^{237}\), and Arg\(^{606}\) are on the path connecting the ThDPs in the two active centers of E1ec. Earlier we reported that substitution of the highly conserved glutamate 571 within strong hydrogen-bonding distance of the N1’ atom of ThDP resulted in a significant reduction of the overall activity: E571A (1.7%), E571D (0.6%), and E571Q (1.3%) (18, 19) (Table 3). Upon reconstitution of the E235A, E237A, and R606A variants with E2ec and E3ec components, the overall PDHc activity measured under the optimal conditions for E1ec was significantly reduced: E235A (11.4%), E237A (7.2%), and R606A (3.3%) (Table 3). Surprisingly, increasing the ThDP concentration from 0.20 mM, saturating for E1ec, to 1 mM or even 5 mM enhanced the activity of the variants but not E1ec: E235A (2.4-fold), E237A (1.7-fold), E571A (1.7-fold), and E571D (12-fold). The activity of the R606A variant was unchanged with 0.20 mM or 5 mM ThDP and increased by 2.6-fold with 5 mM ThDP present (Table 3). In the E1-specific reaction, a similar response upon increasing ThDP concentration was observed for the E237A and R606A variants (Table 3), signaling that ThDP binding was affected upon these substitutions in E1ec.

**From the steady-state kinetic analysis of ThDP binding, the values of**

\[ \frac{S_{0.5,\text{ThDP}}}{K_{m,\text{ThDP}}} \]

**were 133 \(\mu\)M (E235A) and 633 \(\mu\)M (E237A), as compared with**

\[ K_{m,\text{ThDP}} = 1.6 \mu\text{M} \] for E1ec (Table 3 and Fig. 2). The \[ K_{m,\text{ThDP}} \] values for E571A, E571Q, and R606A were close to

**FIGURE 2. Steady-state analysis of ThDP binding at 30 °C.** The reaction mixture contained the following in 1 ml: 0.10 M Tris-HCl (pH 8.0), 0.2 mM MgCl\(_2\), 2.5 mM NAD\(^+\), 2.6 mM dithiothreitol, and 2.0 mM sodium pyruvate. The reaction was started by the addition of 0.20 mM CoA and 3 \(\mu\)g of PDHc, resulting from reconstitution of E1ec or its variant with E2ec-E3ec subcomplex at a mass ratio of 1:1:1. The insets show the dependence of the lag phase on ThDP concentration.
that for E1ec; however, for R606A, the progress curves for NADH production displayed an activation lag phase (100 s) over the entire range of ThDP concentrations (1–100 μM) (Fig. 2, bottom). The \( K_{d,\text{pyruvate}} \) was not significantly affected by E571A (0.17 mM), E571Q (0.99 mM), E237A (1.36 mM), and R606A (0.51 mM) substitutions as compared with that for the E1ec (0.29 mM). We also emphasize that con-

**FIGURE 3.** Dependence of the percentage of fluorescence quenching at 330 nm on the concentration of ThDP. E1ec and its variants (0.034 mg/ml, concentration of active centers/0.342 M) in 10 mM KH\(_2\)PO\(_4\) (pH 7.0) containing MgCl\(_2\) (5 mM) and pyruvate (1.0 mM) were titrated with ThDP (0.5–300 μM). The values of \( K_{d,\text{ThDP}} \) were calculated using an equation for ligand binding at two active centers with the Sigma Plot 10.0 program and are presented in Table 4.

**FIGURE 4.** Dependence of the quenching of fluorescence at 330 nm on N1'-methyl-ThDP concentration. Quenching of intrinsic fluorescence of E1ec showing no saturation (top). Quenching of the intrinsic fluorescence of E571A E1ec showing saturation with \( K_d = 66 \, \mu\text{M} \) (bottom). The conditions for both experiments were as follows. E1ec (0.046 mg/ml, concentration of active centers/0.23 M) and E571A E1ec (0.084 mg/ml, concentration of active centers/0.42 M) in 10 mM KH\(_2\)PO\(_4\) (pH 7.0) containing 5 mM MgCl\(_2\) and 2 mM pyruvate were titrated by 1–300 μM N1'-methyl-ThDP.

**TABLE 4**

| Substitution | \( K_{d,\text{ThDP}1} \) (μM) | \( K_{d,\text{ThDP}2} \) (μM) |
|--------------|-----------------|-----------------|
| None         | 1.8 ± 0.10\(^a\) | 99.4 ± 39.5 |
| E235A        | 5.97 ± 1.65     | ND              |
| E237A        | ND              | ND              |
| E571A        | 2.39 ± 0.78     | ND\(^a\)        |
| E571D        | 24.5 ± 5.5      | ND\(^a\)        |
| R606A        | 1.57 ± 1.19     | 210 ± 47.5 |

\(^a\) Value represents the average for two sites.

\(^b\) Data for the E237A variant are not presented because its \( S_{0.5,\text{ThDP}} \) value is 633 μM, and maximum concentrations of ThDP used in fluorescence experiment are below 300 μM.

\(^c\) Not presented on account of the large error.
version of Glu to Ala or to a charge-neutralized substituent Gln appears to make little difference in activity, $K_m$, or $K_{w,\text{pyruvate}}$ (Table 3).

**Effect of Substitutions along the E1ec Active Center Communication Pathway on Individual Reaction Steps**

The individual reaction steps are shown in Scheme 1.

**Substitutions Do Not Affect Dimerization**—We demonstrated using an analytical size exclusion column that all E1ec variants are eluted from the analytical column as dimers with retention times of 15.6 min (E235A), 15.6 min (E237A), 15.5 min (E571A), and 15.7 min (R606A), similar to E1ec (15.7 min, corresponding to an $M_r$ of 208,500, as compared with the theoretical mass of 198,948 Da).

**Fluorescence Experiments Reveal That the Substitutions Affect ThDP Binding at the Second Site**—The binding of the ThDP to E235A, E571A, E571D (not shown), and R606A variants using percentage quenching of intrinsic fluorescence versus [ThDP] gave a biphasic plot (Fig. 3), indicating negative cooperativity, not observed for E1ec. For E1ec, a similar plot was hyperbolic with $S_{0.5,\text{ThDP}} = 1.84 \mu M$ and Hill coefficient ($n_H$) = 1.78, indicating positive cooperativity of the two active centers (not shown). For the E235A, E571A, E571D, and R606A variants, the second active center was not saturated even at 300 $\mu M$ ThDP (Fig. 3). The calculated $K_{d,\text{ThDP}}$ values for the first active center were not very different from $S_{0.5,\text{ThDP}}$ for E1ec; however, the $K_{d,\text{ThDP}}$ values for the second one were 17-fold (E235A) and 134-fold (R606A) higher than that for the first active center (Table 4), clearly indicating negative cooperativity upon ThDP binding. With the large $S_{0.5,\text{ThDP}}$ estimated for the E237A variant, quenching data could not be analyzed because ThDP concentrations above the usable range (>600 $\mu M$) would have been needed. The results affirm that binding of ThDP to the second active center is impaired in all of the variants here discussed.

**Effect of E571A Substitution on Binding N1'-MeThDP, an Electrostatic APH+ Analog**—We next investigated whether or not the enlarged ThDP site in the E571A variant could accommodate N1'-MeThDP, a stable analog of the N1’-protonated 4’-aminopyrimidinium form of ThDP (APH+ in Scheme 1). Remarkably, N1'-MeThDP binds to E571A even better than to E1ec; quenching of intrinsic fluorescence gave $K_{d,N1'-\text{MeThDP}} = 66 \mu M$ with E571A, whereas with E1ec no saturation was evident even at a 300 $\mu M$ concentration of N1'-MeThDP (Fig. 4). CD spectra of E571A recorded in the presence of 1–300 $\mu M$ N1'-MeThDP did not display any CD bands in the 300–370-nm region, also consistent with the APH+ form of ThDP, for which we do not observe a CD signature in this wavelength range (data not presented) (20).

**Rate of Ylide Formation at C2 of the E1ec-bound ThDP**—Monitoring the rate of hydrogen/deuterium exchange at the thiazolium C2 position of ThDP (Scheme 1) reveals Half-of-the-sites Reactivity—Monitoring the rate of hydrogen/deuterium exchange at the thiazolium C2 position of ThDP provides information about the first requisite step for catalysis (Scheme 1, $k_{\text{obs,AP} \rightarrow \text{Yl}}$) (10). Such rate constants for the enzyme-bound ThDP are presented in Table 5. The experiment revealed that one-half of the enzyme-bound ThDP C2-H is exchanged within 2 s; however, complete exchange resulted after 30 s (Fig. 5, top and bottom). The $k_{\text{obs,AP} \rightarrow \text{Yl}}$ is $160 \pm 67 \text{s}^{-1}$ for the fast exchanging site (activated site) and $0.04 \pm 0.02 \text{s}^{-1}$ for the slow exchanging site.

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**TABLE 5**

| Variant | Active center concentration $\mu M$ | Fast exchanging site $k_{\text{obs}}$ $\text{s}^{-1}$ | Slow exchanging site $k_{\text{obs}}$ $\text{s}^{-1}$ |
|---------|-----------------------------------|-----------------|-----------------|
| None    | 76.2                              | $160 \pm 67$   | $0.04 \pm 0.02$ |
| E235A   | 117                               | $9.8 \pm 5.7$  | $0.14 \pm 0.06^a$ |
| E237A   | NA                                | NA              | NA              |
| E571A   | 160                               | $9.3 \pm 3.2$  | $0.12 \pm 0.02^a$ |
| R606A   | 160                               | $0.08 \pm 0.01$| ND               |

$^a$ The constant represents slow hydrogen/deuterium exchange at C2-H for unbound ThDP (5).

**FIGURE 5. Hydrogen/deuterium exchange kinetics of C2-H of E1ec-bound ThDP.** Top, expansions of selected one-dimensional $^1H$ NMR spectra of acid quench-isolated ThDP after reaction of 1 volume of E1ec (reconstituted with an equimolar amount of ThDP and 1 mM Mg$^{2+}$) in 20 mM KH$_2$PO$_4$ (pH 7.0) with 1 volume of 99.9% D$_2$O for different reaction times at 30 °C. The singlet signals of C2-H (9.68 ppm) and C6'-H (8.01 ppm) are shown. The latter signal serves as an internal non-exchanging standard for quantification. Bottom, the decay of the $^1H$ NMR integral intensity of the ThDP C2-H signal relative to the integral of the non-exchanging C6'-H signal was fitted to a double exponential function for exchange of enzyme-bound ThDP (C) as detailed in Ref. 5. The estimated pseudo-first order rate constants are summarized in Table 5.
(dormant site), clearly demonstrating half-of-the-sites reactivity, indicating that when both E1ec active centers are filled with ThDP, only one of the two is in the activated state (Table 5 and Fig. 5). For the E235A and E571A variants, the rate constants for the fast exchanging site were $k_{\text{obs,AP-y1}} = 9.8 \pm 5.7 \text{ s}^{-1}$ and $k_{\text{obs,AP-y1}} = 9.3 \pm 3.2 \text{ s}^{-1}$, respectively, at least 1 order of magnitude smaller than that for the E1ec (Fig. 6). For these two variants, a large amount of unbound ThDP was detected with the rate constants of hydrogen/deuterium exchange of $k_{\text{obs,AP-y1}}$ is $0.14 \pm 0.06 \text{ s}^{-1}$ for E235A and $0.12 \pm 0.02 \text{ s}^{-1}$ for E571A. No hydrogen/deuterium exchange was detected for a slowly exchanging site, indicating that E235A and E571A variants have only one of the two active centers filled with ThDP. For the R606A variant, the hydrogen/deuterium exchange was very slow, with $k_{\text{obs,AP-y1}}$ of $0.08 \pm 0.01 \text{ s}^{-1}$, and the fast phase was not observed, indicating that within the time frame of the experiment, this variant (Fig. 6, bottom) did not bind ThDP, consistent with the ThDP activation lag phase measurements (Fig. 2, bottom).

**Formation of the First Covalent (Predecarboxylation) Intermediate upon the Addition of the Substrate Analog MAP**—As was reported earlier by our groups, formation of the first covalent tetrahedral intermediate (predecarboxylation; LThDP) can be detected by CD upon the addition of the substrate analog MAP to E1ec (21–23) (Schemes 1 and 2). Upon the addition of MAP to the E235A, E237A, and R606A variants, a positive CD band centered at 305 nm developed and reached a limiting value similar to that for E1ec and indicating the formation of $1',4'-\text{iminophosphonolactyl-ThDP}$ (PLThDP) (Fig. 7 and Scheme 2). Values of $K_{d,\text{MAP}}$ of 0.15 ± 0.05 $\mu$M (E235A), 0.17 ± 0.08 $\mu$M (E237A), and 16.0 ± 1.1 $\mu$M (R606A) were determined as compared with $K_{d,\text{MAP}}$ of 0.10 ± 0.06 $\mu$M for E1ec. The ratio of [MAP]/[E1 active centers] at which the limiting value of CD at 304 nm is reached was 1:1 for E1ec (about 92% of active centers are filled with intermediate; data not shown). For the E235A and E237A variants, the [MAP]/[E1ec variant active centers] ratio of 0.5:1 was determined, clearly indicating half-of-the-sites reactivity (about 49% of the active centers are filled with intermediate, calculated using a quadratic equation as in Ref. 11) (Fig. 7). For the R606A variant, probably both active centers are filled (about 92% is calculated), however at a molar ratio of [MAP]/[R606A active centers] of 20:1 (data not shown). For E571A and E571D, such results were reported and discussed earlier (21).

The rate constants for PLThDP formation were very much affected in the variants. It was demonstrated using stopped-flow CD that upon mixing of E1ec with MAP, a positive CD band at 302 nm developed (reporting formation of the $1',4'-\text{imino-PLThDP}$) and reached a maximum in ~1–1.5 s. The kinetic curve could be resolved into two rate constants: $k_1 = 5.78 \pm 0.31 \text{ s}^{-1}$ and $k_2 = 1.02 \pm 0.09 \text{ s}^{-1}$ (Fig. 8). For the E235A variant, the maximum amplitude of the CD band was

**Figure 6.** Hydrogen/deuterium exchange kinetics of C2-H of ThDP for E1ec variants. Top, E571A; middle, E235A; bottom, R606A. NMR data were fitted to either a double exponential (E235A/E571A) or monoexponential (R606A) function. The estimated pseudo-first order rate constants are summarized in Table 5.
reached within 60 s with rate constants about 17.5-fold and 15-fold smaller as compared with E1ec ($k_1 = 0.38 \pm 0.01$ s$^{-1}$ and $k_2 = 0.068 \pm 0.01$ s$^{-1}$). With the E237A variant (200 s needed to reach maximum) and R606A variant (maximum was not reached within 60 s; data not shown), the rate constants for PLThDP formation were about 250-fold and 165-fold smaller as compared with E1ec ($k_1 = 0.023 \pm 0.001$ s$^{-1}$ (E237A) and $k_1 = 0.035 \pm 0.004$ s$^{-1}$ (R606A)). The values of $k_2$ were very small.

**Effect on Binding of the Stable Enamine Analog Thiamin 2-Thiothiazolone Diphosphate**—CD titration of E571A by thiamin 2-thiothiazolone diphosphate (ThTTDP), an analog of the enamine intermediate in Scheme 1, with C2-H converted to C2-S, showed formation of a positive CD band at 325 nm, indicating ThTTDP binding in the active centers. However, a plot of ellipticity versus [ThTTDP] did not show saturation, indicating that the E571A substitution affects ThTTDP binding (Fig. 9). For comparison, E1ec displayed saturation with ThTTDP ($\lambda_{max}$ at 329 nm) and gave a value of $K_d,\text{ThTTDP} = 8.67 \pm 0.35$ $\mu$M, as determined by steady-state CD titration, and two rate constants were obtained for formation of the E1ec-ThTTDP complex, $k_1 = 1.9 \pm 0.025$ s$^{-1}$ and $k_2 = 0.39 \pm 0.021$ s$^{-1}$ (stopped-flow CD). No signal was detected for E571A on stopped-flow CD. The values of $K_d,\text{ThTTDP}$ for E235A (7.53 ±

**FIGURE 7. Near-UV CD spectra of E1ec variants upon titration by methyl acetylphosphonate.** The E235A (top) or E237A (bottom) variants (2.0 mg/ml; concentration of active centers = 20 $\mu$M) in 20 mM KH$_2$PO$_4$ (pH 7.0) containing 2 mM MgCl$_2$ and 0.50 mM ThDP were titrated with 1–100 $\mu$M MAP. The values of $K_{MMAX}$ were calculated using a quadratic equation (11). The inset shows that the limiting value of the CD band is reached at a 0.5:1 molar ratio of [MAP]/[E1ec variant active centers].

**FIGURE 8. Rates of the 1',4'-iminophosphonolactyl ThDP formation for E1ec and its variants determined by time-resolved circular dichroism.** E1ec or its variants (5–10 mg/ml) in 20 mM KH$_2$PO$_4$ (pH 7.0) containing ThDP (0.50 mM) and MgCl$_2$ (2.0 mM) was mixed with MAP (2 mM) in the same buffer, and reaction was monitored for varied time intervals (5–200 s). The data were treated as a double exponential as in Equation 1.
Information Transfer between Thiamin Cofactors on Enzymes

Structure of E571A-ThDP—In E571A-ThDP, the absence of the Glu571 side chain creates additional space near residue Tyr598, and electron density maps clearly indicate two disordered sites for Tyr598 in both active centers to partially fill this space. The most significant observation, however, is the well defined cofactor electron density present in both active centers only for the diphosphate tail of the ThDP. There is no clear electron density for the thiazolium and 4-aminopyrimidine rings. The observed diphosphate group interaction, nevertheless, is similar to that observed in the E1ec-ThDP structure (12). The octahedrally coordinated Mg$^{2+}$ binding site contains three protein ligands, two oxygen atoms from the diphosphate and one water molecule. Regarding the two alternative conformations of Tyr598 in the active site, one of them is the same as that observed in the E1ec-ThDP, whereas the other is a new conformation seen here for the first time (Fig. 10). An immediate consequence of this feature is the steric hindrance that would now occur between the tyrosine ring and 4'-aminopyrimidine ring of the cofactor, if the latter were indeed present. The distance between the tyrosine and 4'-aminopyrimidine ring would be only 2.3 Å if both were present simultaneously (12). Because there is no clear evidence for electron density defining the cofactor rings in these active centers, the ThDP model from the E1ec-ThDP structure was used in Fig. 10 for interpretation of the results.

In addition, residue His142 occupies the rotamer conformation observed in the E1ec-ThDP complex, an alternative conformation, or both in the E571A active centers as shown in Fig. 10. His142 is disordered in only one of the two active centers, partially occupying two rotamer conformations (Fig. 10b). In the other active center, this residue adopts only one conformation (Fig. 10, top). The new H142 rotamer position would collide with the thiazolium ring of the cofactor, if the latter were present in its usual location.

Structural Analysis of E235A-ThDP Complex at Different ThDP Concentrations—As observed in the E571A-ThDP complex, changes are observed in the active centers of E235A-ThDP relative to the E1ec-ThDP complex but differ in their nature. Significant differences are observed between the cofactor’s degree of order (as indicated by occupancies and electron density features) as well as in the conformations of certain amino acid side chains in the active centers. The structural changes observed in the active centers clearly affect the binding of the cofactors, as indicated in Table 1. The most significant observation is the absence of well defined electron density for the cofactor’s thiazolium ring in both structures (Fig. 11 and Table 1). The average occupancy of 0.5 for the thiazolium rings clearly indicates that the rings are not firmly fixed within the active centers. Even after a 10-fold increase of ThDP concentration (from 0.5 to 5 mM), the occupancy of the thiazolium ring is increased only from 0.36 to 0.53 in one of the active centers (Table 1). As in the E571A-ThDP structure, disorder is also apparent in the His142 side chain of E235A-ThDP, and the occupancy difference is consistent with the His142 alternate rotamer conformation present in one of the active centers, because the alternate conformation would collide with the

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0.25 μM), E237A (6.11 ± 0.25 μM), and R606A (9.71 ± 0.37 μM) variants were similar to E1ec. Approximately 61–68% of the active centers were occupied by ThTTPD when the CD signal reached the maximum intensity, indicating saturation of the first active site, and a 5–10-fold molar excess of ThTTPD is needed to saturate the second active center for E1ec and its Glu235, Glu237, and Arg606 variants. The results with Glu235, Glu237, and Arg606 indicate that their substitution does not affect ThTTPD binding, unlike that of Glu571.

Structural Analysis of the E571A-ThDP and E235A-ThDP Complexes

The three crystal structures studied here are all very similar (in a global sense) to the previously reported crystal structure of the E1ec-ThDP complex (12). The main-chain folds are identical, and two subunits are present in an asymmetric unit. The two independent subunits are almost identical stereochemically and are related by a 2-fold non-crystallographic symmetry axis, with the active sites located at the subunit interfaces.

FIGURE 9. Circular dichroism spectra of E571A and E235A variants upon titration by ThTTPD. E571A (top, 2.1 mg/ml, concentration of active centers = 21 μM) and E235A (bottom, 1.3 mg/ml, concentration of active centers = 6.3 μM) in 20 mM KH$_2$PO$_4$ (pH 7.0) containing 2 mM MgCl$_2$ were titrated by 1–90 μM ThTTPD. The insets show the dependence of the circular dichroism at the maximum on concentration of ThTTPD. Data for E235A were treated using the Hill equation (11).
Information Transfer between Thiamin Cofactors on Enzymes

cofactor thiazolium ring, thereby affecting its binding. This structural information also clearly indicates non-equivalence of active centers, because the alternate conformation for residue His\(^{142}\) is present only in one active center.

**DISCUSSION**

The major conclusion derived from structural, kinetic, and spectroscopic studies using substitutions at the Glu\(^{235}\), Glu\(^{237}\), and Arg\(^{606}\) positions, residues that along with the highly conserved Glu\(^{571}\) residue are hypothesized to form a communication pathway between the two active center ThDPs (2, 3), is that ThDP binding in the second active center is affected by these substitutions according to the following observations. 1) Steady-state kinetic analysis revealed that ThDP binding is affected. 2) Analysis of the quenching of intrinsic protein fluorescence by ThDP revealed negative cooperativity upon ThDP binding in the E1ec variants, in contrast to the positive cooperativity reported for E1ec, indicating that ThDP binds with great preference in one of the two active centers. 3) The hydrogen/deuterium exchange kinetics at the thiazolium C2-H position of the enzyme-bound ThDP clearly demonstrated half-of-the-sites reactivity for the E1ec with a fast exchanging (activated) site and a slowly exchanging (dormant) site. For the E235A and E571A variants, the rate constant for the fast exchanging site was at least 1 order of magnitude slower than that for the E1ec. 4) The rate constants for PLThDP formation were at least 100 times slower than for E1ec. 5) The crystal structures of the E571A-ThDP and E235A-ThDP complexes do not reveal significant changes in the folding of the two subunits but do reveal pronounced differences in the active centers (see below).

It has been suggested that the two active centers in the E1bs PDHc communicate by mostly acidic residues in a hydrated channel (2). In the E1ec (3), the two active center ThDPs are separated by ~20 Å and are interconnected by residues Glu\(^{571}\), Glu\(^{235}\), Glu\(^{237}\), and Arg\(^{606}\) and water molecules. The only direct cofactor contacts through this chain are via the catalytically important and conserved hydrogen bond between the ThDP N1’ nitrogen atoms and the side chains of a conserved Glu\(^{571}\) (Fig. 1). Structural studies presented in this paper show that Glu\(^{571}\) is involved in proper ThDP binding and orientation as well as being directly involved in the catalytic mechanism but is not required for diphosphate binding. This is in accord with spectroscopic studies indicating the compromised binding of ThDP and its analogs N1’-MeThDP and ThTTDP. The site-directed substitution studies clearly indicate that the catalytic activity is greatly impaired upon substitutions of the above mentioned residues, despite the fact that some of them are quite distant from the active center and make no obvious interactions with the cofactors or expected reaction intermediates. In this regard, the crystallographic analysis reported here provides the structural basis for the reduced activity associated with the E235A and E571A variants located on the proposed pathway. It reveals a limited ability to properly bind and orient the ThDP as indicated by the reduced occupancy and apparent disorder of ThDP, especially for the thiazolium ring. It is noteworthy that structural studies of the E1ec-ThDP complex indicated no direct interaction between the cofactor and Glu\(^{235}\) (Fig. 11, top), but there is a strong hydrogen bonding interaction between Glu\(^{235}\) and the conserved residue Glu\(^{571}\); the latter residue interacts directly with the cofactor. The interaction between Glu\(^{571}\) and the N1’ atom of ThDP plays a role in assisting tautomerization of the 4’-aminopyrimidine ring leading to the reactive 1’-4’-iminopyrimidine tautomer, and the latter tautomer is involved in activation of the thiazolium ring to form the active ylide.

The E235A and E571A structural results indicate that the interaction between these residues plays a role in cofactor binding and activation, probably by affecting the protonation state of Glu\(^{571}\) (given their proximity, Glu\(^{235}\) and Glu\(^{571}\) cannot both be ionized at the same time) and therefore affecting its ability to stabilize a protonated N1’ atom in the APH\(^{+}\) ionization state of ThDP (compare Figs. 1 and 10).

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**FIGURE 10. Stereo 2Fo – Fo, omit electron density map contoured at 1σ for the dimer interface environment around the catalytic centers (upper images in subunit a and lower images in subunit b) of the E571A-ThDP complex.** Coordinates for the thiazolium and pyrimidine rings were used from the E1ec-ThDP structure to create the complete cofactor model in the active centers for illustrative purposes. The electron density map indicates dual disordered positions for the His\(^{142}\) and Tyr\(^{208}\) residues.
This conclusion is further supported by the results with the N1\textsuperscript{-}MeThDP; given the additional space in the E571A variant for the methyl group, this stable electrostatic analog of the APH\textsuperscript{+} ionization state of ThDP is indeed stabilized in the active center of E1ec. Because the other three states, AP, IP, and Yl in Scheme 1, can all be derived from the APH\textsuperscript{+} form, this stabilization is very important. The pK\textsubscript{a} for the APH\textsuperscript{+} form has been found to be significantly higher on several ThDP enzymes than in water (20), affirming the need of ThDP enzymes to have all ionization/tautomeric states readily accessible for optimal activity.

The presence of alternate conformations for His\textsuperscript{142} in the E235A structure provides additional evidence for the ability of Glu\textsuperscript{235} to affect remote active site interactions. Furthermore, the observed cofactor binding differences in the two active centers and the alternate conformation for His\textsuperscript{142} in only one of these clearly explains the non-equivalence of active centers implied by the solution studies.

The observed conformational asymmetry and cofactor binding differences in the two active centers, even with a significant excess of ThDP present, are consistent with a communication pathway between active centers outlined in Fig. 1. If so, then the E235A substitution “breaks” the pathway by eliminating the link to Arg\textsuperscript{606} (Fig. 1), thereby disrupting active center communication as is observed, adding credence to the suggested pathway. This is consistent with the solution data presented above. In addition, the structural data reveal that interactions between Glu\textsuperscript{235} and Glu\textsuperscript{571} probably are involved in mediating cofactor binding and catalytic efficiency. However, although we implicate the residues on the signal transduction pathway between the two active centers, an explicit mechanism of information transfer (as suggested by Perham and co-workers (2), such as actual proton transfer between active centers during the reaction) is still unclear.

In conclusion, we present the first experimental evidence for the transfer of information between the two ThDP-containing active centers in a ThDP multienzyme complex via a chain of three acidic residues and an arginine side chain. The result of alteration of any of the residues in this chain impacts on ThDP binding in the second active center with clear non-equivalence of the two active centers.

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