Roles of His$^{291}$-α and His$^{146}$-β in the Reductive Acylation Reaction Catalyzed by Human Branched-chain α-Ketoacid Dehydrogenase

REFINED PHOSPHORYLATION LOOP STRUCTURE IN THE ACTIVE SITE*

Received for publication, June 12, 2003, and in revised form, August 1, 2003
Published, JBC Papers in Press, August 5, 2003, DOI 10.1074/jbc.M306204200

R. Max Wynn‡, Mischa Machius, Jacinta L. Chuang, Jun Li, Diana R. Tomchick, and David T. Chuang§

From the Departments of Biochemistry and Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9038

We report here that alterations of either His$^{291}$-α or His$^{146}$-β in the active site of human branched-chain α-ketoacid dehydrogenase (E1b) impede both the decarboxylation and the reductive acylation reactions catalyzed by E1b as well as the binding of cofactor thiamin diphosphate (ThDP). In a refined human E1b active-site structure, His$^{291}$-α, which aligns with His$^{407}$ in Escherichia coli pyruvate dehydrogenase and His$^{283}$ in yeast transketolase, is on a largely ordered phosphorylation loop. The imidazole ring of His$^{291}$-α in E1b coordinates to the terminal phosphate oxygen atoms of bound ThDP. The N3 atom of wild-type His$^{146}$ plays a structural rather than a catalytic role in the binding of the substrate lipoyl-bearing domain to E1b. The H146A mutation, on the other hand, does not alter the loop structure, but nullifies the reductive acylation activity of E1b. Our results suggest that: 1) His$^{291}$-α plays a structural rather than a catalytic role in the binding of cofactor ThDP and the lipoyl-bearing domain to E1b, and 2) His$^{146}$-β is an essential catalytic residue, probably functioning as a proton donor in the reductive acylation of lipoamide on the lipoyl-bearing domain.

The mammalian branched-chain α-ketoacid dehydrogenase (BCKD)$³$ complex catalyzes the oxidative decarboxylation of branched-chain α-ketoacids (Reaction 1) derived from transamination of leucine, isoleucine, and valine (1).

R$^\text{CO-COOH} + \text{NAD}^+ + \text{CoASH} \rightarrow \text{R-CO-S-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+

\text{REACTION 1}

The BCKD complex is a member of the highly conserved mitochondrial α-ketoacid dehydrogenase multienzyme complexes. This metabolic machine of $4 \times 10^6$ daltons in size is organized around a 24-mer dihydrolipoyl transacetylase (E2b) core, to which multiple copies of branched-chain α-ketoacid dehydrogenase (E1b), dihydrolipoamide dehydrogenase (E3), BCKD kinase, and BCKD phosphatase bind. In patients with heritable maple syrup urine disease, activity of the BCKD complex is deficient, resulting in often fatal acidosis, neurological disorders, and mental retardation (1).

The E1b component is a thiamin diphosphate (ThDP)-dependent enzyme consisting of two α (M$\alpha$ = 45,500) and two β (M$\beta$ = 37,500) subunits. The heterotetramer catalyzes both the ThDP-mediated decarboxylation of the α-ketoacids (Reaction 2) and the reductive acylation of the lipoyl moiety covalently attached to the β-hairpin tip of the lipoic acid-bearing domain (LBD) on the E2b subunit (Reaction 3).

\text{RCOOCOH} + \text{E1b-ThDP} \rightarrow \text{E1b-ThDP} + \text{RCOCHO} + \text{CO}_2

\text{REACTION 2}

E1b-ThDP + LipS2-E2b + \text{RCO-S-Lip(SH)-E2b} + \text{E1b-ThDP}

\text{REACTION 3}

Crystal structures of Pseudomonas E1b (2) and human E1b (3) have been determined. These E1b proteins are heterotetramers each containing two α- and two β-subunits that form two ThDP-binding pockets at the α/β and the α'/β' interfaces. This topology is conserved between E1b proteins and the homodimeric yeast transketolase; in the latter two ThDP-binding sites are formed at head-to-tail interfaces between two identical subunits (4), each corresponding to a fused α and β or α' and β' subunits of E1b. Residues in rat (5) and human (6) E1b, which interact with cofactor ThDP as required for Reaction 2, have been identified and characterized. In contrast, details of the catalytic mechanism for Reaction 3 are not fully understood. Based on chemical studies with P. J.-S.-methylthioperoxy acid methyl ester as a model substrate, Pan and Jordan (7) proposed the presence of a general acid catalyst located on the E1 enzyme, which, during reductive acylation, serves as a proton donor for the S6 atom of lipoamide on the E2 subunit. The Pseudomonas E1b structure shows that for the reductive acylation reaction to occur, the oxidized lipoyl-lysine residue on the LBD needs to penetrate a 20-Å long funnel-shaped active-site channel of E1b (2). The acyl group in the resultant S-acetyldihydrolipoamide is then transferred, in an E2b-catalyzed

---

* This work was supported by Grants DK26758 and DK62306 from the National Institutes of Health and Grant I-1286 from the Welch Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (codes 1OLS, 1OLU, and 1OLX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

§ To whom correspondence should be addressed. Tel.: 214-648-2457; Fax: 214-648-8856; E-mail: david.chuang@utsouthwestern.edu.

The abbreviations used are: BCKD, branched-chain α-ketoacid dehydrogenase; DCTP, 3,4-dichlorothionolipodiphosphonol; E1b, branched-chain α-ketoacid dehydrogenase; E2b, dihydrolipoyl transacetylase; E1p, E1 component of pyruvate dehydrogenase complex; E2p, E2 component of pyruvate dehydrogenase complex; E3, dihydrolipoamide dehydrogenase; KIV, α-ketoisovalerate lipoyl-LBD, lipoylated lipoyl-bearing domain; PDB, Protein Data Bank; ThDP, thiamin diphosphate; ITC, isothermal titration calorimetry.

---

This paper is available online at http://www.jbc.org
Roles of Two Histidine Residues in E1b Active Site

Materials—N-terminally His<sub>146</sub>-tagged wild-type and mutant E1b proteins were produced as described previously, except that primers containing the desired mutations were used for site-directed mutagenesis (10). The following primers (with mutational changes underlined) were used: 5'-TCACTGTTGCTGCTGGCCGGA-TCC-3' (H291A-α); 5'-GTGCTACGTGCTGGCTGCCCAGCCT-5' (H291N-α); 5'-GTGCTACGTGCTGGCTGCCCAGCCT-5' (H291Q-α); 5'-CATGGGCTCTTGATGCTCAGTGGTATAGA-C-3' (H146A-β); and 5'-CATGGGCTCTTGATGCTCAGTGGTATAGA-C-3' (H146N-β). C-terminally His<sub>146</sub>-tagged LBD (residues 1–84 of the E2b subunit) was expressed and purified as described with n,κ-thionic acid (oxidized) LpIA ligation as described previously (12). A C-terminally His<sub>146</sub> (E1b) was prepared also as described previously (3).

Enzyme Assays for Decarboxylation Activities—The co-ke[14C]]isovalerate was carried out with wild-type or mutant E1b in the presence of excess lipoylated E2b (lip-E2b) and E3 as described previously (13). The decarboxylation reaction carried out in the presence of cysteamine (Reaction 2) was assayed spectrophotometrically using 0.1 mM α-ke-toisovalerate (KIV) as a substrate and 0.05 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor as also described previously (13).

ASSAY FOR THE REDUCTIVE ACETYLACTION (REACTION 3) OF LIPOYL-LBD—The assay was modified from that described previously (14). The reaction mixture in a volume of 0.2 ml contained 50 mM potassium phosphate, pH 7.5, 1 mM ThDP, 2 mM MgCl<sub>2</sub>, 0.2 mM [U-<sup>14</sup>C]KIV (specific activity 127,500 cpm/nmol), 132 mM E1b (heterotetramers), and 20 μM C-terminally His<sub>146</sub>-tagged lip-LBD. The reaction was initiated by the addition of the radiolabeled α-ketoadipate. After incubation at 22 °C for 30 min, the reaction was quenched with 0.1 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor in the presence of excess lipoylated E2b (lip-E2b) and E3 as described previously (13). The decarboxylation reaction carried out in the presence of cysteamine (Reaction 2) was assayed spectrophotometrically using 0.1 mM α-ke-toisovalerate (KIV) as a substrate and 0.05 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor as also described previously (13).

ASSAY FOR THE REDUCTIVE ACETYLACTION (REACTION 3) OF LIPOYL-LBD—The assay was modified from that described previously (14). The reaction mixture in a volume of 0.2 ml contained 50 mM potassium phosphate, pH 7.5, 1 mM ThDP, 2 mM MgCl<sub>2</sub>, 0.2 mM [U-<sup>14</sup>C]KIV (specific activity 127,500 cpm/nmol), 132 mM E1b (heterotetramers), and 20 μM C-terminally His<sub>146</sub>-tagged lip-LBD. The reaction was initiated by the addition of the radiolabeled α-ketoadipate. After incubation at 22 °C for 30 min, the reaction was quenched with 0.1 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor as also described previously (13).

Assaying for the Reductive Acetylation (Reaction 3) of Lipoyl-LBD—The assay was modified from that described previously (14). The reaction mixture in a volume of 0.2 ml contained 50 mM potassium phosphate, pH 7.5, 1 mM ThDP, 2 mM MgCl<sub>2</sub>, 0.2 mM [U-<sup>14</sup>C]KIV (specific activity 127,500 cpm/nmol), 132 mM E1b (heterotetramers), and 20 μM C-terminally His<sub>146</sub>-tagged lip-LBD. The reaction was initiated by the addition of the radiolabeled α-ketoadipate. After incubation at 22 °C for 30 min, the reaction was quenched with 0.1 mM 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor as also described previously (13).

Binding Measurements Based on Isothermal Titration Calorimetry (ITC)—Human E1b and lip-LBD, both C-terminally His<sub>146</sub>-tagged, were dialyzed exhaustively against the same reservoir of 50 mM Tris buffer, pH 7.5, 50 mM KCl, 10 mM β-mercaptoethanol, and 0.2 mM EDTA to remove bound Mg<sup>2+</sup> ions and some of the radioactivity incorporated into lip-LBD was radioactively labeled with [14C]-nitritolactic acid (Ni-NTA) resin (Qiagen, Chatsworth, CA). The resin was washed 3 times with the above phosphate buffer containing 200 mM NaCl and 2 mM β-mercaptoethanol. The washed resin containing radiolabeled lip-LBD was added to 2 ml of scintillation mixture and radioactivity was counted.

Binding Measurements Based on Tryptophan Fluorescence Quenching—We used wild-type E1b as well as His<sub>146</sub>- and His<sub>146</sub>His<sub>146</sub>-tagged human E1b, which were expressed and purified as described previously (3). A C-terminally His<sub>146</sub>-tagged human E1b was prepared also as described previously (3).

Values for the association constant.

Binding Measurements Based on Tryptophan Fluorescence Quenching—We used wild-type E1b as well as His<sub>146</sub>- and His<sub>146</sub>His<sub>146</sub>-tagged human E1b, which were expressed and purified as described previously (3). A C-terminally His<sub>146</sub>-tagged human E1b was prepared also as described previously (3).
Roles of Two Histidine Residues in E1b Active Site

TABLE I

Data collection and refinement statistics

|                        | Wild-type | H146A-β | H291A-α |
|------------------------|-----------|---------|---------|
| A. Data collection     |           |         |         |
| Space group            | P3,21     | P3,21   | P3,21   |
| Unit cell (Å)          |           |         |         |
| a = b                  | 144.72    | 145.21  | 145.37  |
| c                      | 69.15     | 69.52   | 69.49   |
| Energy (eV)            | CuKα      | 12,000  | 12,661  |
| Resolution range (Å)   | 39.09–1.85| 32.18–2.25| 23.79–1.85|
| Measurements           | 313,991   | 83,771  | 447,895 |
| Unique reflections     | 71,059    | 37,471  | 66,922  |
| Data completeness (%)  | 99.8      | 93.3    | 93.3    |
| Last shell (Å)         | 99.7 (1.88–1.85 Å) | 99.4 (2.29–2.25 Å) | 95.7 (1.88–1.85 Å) |
| Rmerge (%)             | 4.8       | 9.0     | 10.9    |
| L(σ(I))                | 55.6      | 58.2    | 68.2    |
| B. Refinement          |           |         |         |
| No. reflections Rmerge/Rmerge | 67,330/1,178 | 34,100/1,442 | 62,060/1,423 |
| Non-H protein atoms    | 5,784     | 5,775   | 5,679   |
| No. solvent atoms      | 460       | 380     | 586     |
| Rmerge (%)             | 17.81     | 16.22   | 18.83   |
| Rmerge (%)             | 19.18     | 21.30   | 20.23   |
| R.m.s.d. bond lengths (Å) | 0.008      | 0.010   | 0.007   |
| R.m.s.d. bond angles (°) | 1.36      | 1.44    | 1.36    |
| Mean B value (Å²)      | 28.26     | 19.8    | 18.7    |
| Main chain             | 32.08     | 23.10   | 22.1    |
| Side chain             | 36.33     | 27.8    | 32.3    |
| Solvent atoms          | 20.89     | 15.8    | 27.0    |
| Ligands and ions       | 0.22      | 0.23    | 0.15    |
| Cross-validated a-coordinate error (Å) | 0.15 | 0.15    |
| Missing residues       | A: 1–5, 293–294, 299, 302–307 | A: 1–4, 302–306 | A: 1–6, 288–312 |
| B: 1, 9–13             | B: 7–13   | B: 1, 338–342 |
| Number of alternate conformations | 7        | 4        | 15       |

MgCl₂. Fluorescence readings were corrected for dilution and inner filter effects using Equation 1 (16),

\[ F_{corr} = F_{obs}(V/V_o) \times 10^{\frac{V_o}{V_{sam}}} \]  

(Eq. 1)

where, \( F_{corr} \) is the corrected fluorescence intensity value, \( F_{obs} \) the experimentally measured fluorescence intensity, \( V_o \) the initial volume of the sample, \( V \) the volume after adding ThDP, \( d \) the path length of the cuvette, \( A_o \) the absorption of the sample at the excitation wavelength, and \( A_m \) the absorption of the sample at the emission wavelength. Three different readings were taken and averaged with the experiment conducted three times (\( n = 3 \)). The binding data were fitted by nonlinear regression using the program KaleidaGraph (Synergy Software, Essex Junction, VT) according to Equation 2 describing a bimolecular reaction (17),

\[ \Delta F = \Delta F_{max} \times \frac{K_d}{[E1b] + \frac{[ThDP]}{K_d}} \]  

(Eq. 2)

where \( \Delta F \) is the (corrected) fluorescence change, \( F_c \) the fluorescence intensity prior to the addition of ThDP, \( \Delta F_{max} \) the maximal fluorescence change, \( K_d \) the dissociation constant, and \([ThDP] \) the concentration of ThDP in the cuvette. The parameters determined by the fitting procedure were \( \Delta F_{max} \) and \( K_d \).

Crystalization of Wild-type and Mutant E1b Proteins—Wild-type and mutant E1b proteins (C-terminally His-tagged on the β-subunit) were produced as described (3). Crystals were grown at 20 °C via the vapor diffusion method by mixing equal volumes of E1b (20–25 mg/ml) in 50 mM Na-HEPES buffer, pH 7.5, 500 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 20 mM dithiothreitol, and 5% (v/v) glycerol with a well solution (1.4–1.6 M ammonium sulfate, 0.1 M sodium citrate, pH 5.8, 20 mM β-mercaptoethanol). MgCl₂, MnCl₂, and ThDP at 4 mM each were added to both the well solution and the cryo-buffer (see below). Serially diluted crushed crystals were used after microseeding 1 day after the drops were set up. Crystals appeared 2 days after seeding and grew to a maximum size of 120 × 800 μm within 10 days, which were stabilized for 12 h by soaking in fresh well solution. Crystals were cryo-protected by stepwise exchanges with a cryo-buffer containing 1.6 M ammonium sulfate, 50 mM Na-HEPES, pH 7.5, 100 mM sodium citrate, pH 5.8, 100 mM KCl, 50 mM dithiothreitol, and 20% (v/v) glycerol. Mn²⁺ ions could replace Mg²⁺ required for the binding of ThDP to E1b. The presence of Mn²⁺ ions in crystals resulted in improved x-ray diffraction qualities without affecting the catalytic properties (data not shown). Crystals obtained with this procedure exhibited the symmetry of space group P3,21 with cell parameters of \( \alpha = \beta = 145° \) and \( c = 145° \) and contained one E1b heterodimer per asymmetric unit. They diffracted x-rays significantly better (up to a minimum Bragg spacing, \( d_{min} \) of 1.6 Å) than those described by Varsson et al. (18) with CNS), which were found to be nearly identical to regular A- coordinate error (Å) (3).

X-ray Crystallography—Crystals were flash-cooled in liquid propane and kept at about 100K during data collection at beamlines 19ID and 19BM (Advanced Photon Source (APS), Argonne National Laboratory, Argonne, IL), for the H291A-α and H146A-β mutants. Data from the wild-type crystal were collected at 110K using CuKα radiation from an in-house rotating anode source (Rigaku RU-300, Japan) and an RAXIS-IV detector (MSC Industrial Supply, Houston, TX). Data sets were processed with the HKL2000 package (18). Complete data processing statistics are listed in Table I (part A).

The E1b wild-type structure was determined by molecular replacement using the previously determined lower resolution E1b structure (PDB code 1DTW) as the search model. Mutant structures were subsequently determined by Fourier techniques. Refinement of the models was carried out in the program package CNS 1.1 (19) with a random subset of all data set aside for the calculation of free R factors. The refinement protocol consisted of an initial simulated annealing step to remove model bias, followed by cycles of conjugate gradient minimization and calculation of anisotropic displacement parameters interspersed with manual adjustments to the model using the program O (20). The electron density clearly showed the presence of several glycerol molecules in the crystal structure. After the refinement of the protein part was complete, solvent molecules were added where stereochemically reasonable. The quality of the models was finally checked against composite simulated annealing 2Fo − Fc omit maps (calculated with CNS), which were found to be nearly identical to regular σA-weighted 2Fo − Fc electron density maps. Residues in flexible regions (particularly at the termini and in some surface-exposed loop regions)
that did not have corresponding electron density were excluded from the models. Residues with corresponding electron density for the main chain atoms but not for the side chain atoms were added with the side chains in the most favorable rotamer conformations that did not lead to steric clashes. Single residues with little corresponding electron densities were included in the model, when they were flanked by other residues with well-defined electron density. Two residues (Ile266- and Arg298-β) had main chain φ and ϕ angles that would place them in the disallowed regions in the Ramachandran plot. The corresponding electron densities were, however, very well defined and thus their conformations were dictated by the structural context. Complete refinement statistics are listed in Table I (part B).

RESULTS

Oxidative Decarboxylation (Reaction 1) Catalyzed by Wild-type and Mutant E1b—Both His291-α and His146-β residues were mutated, and effects of these mutations on the overall reaction (Reaction 1) catalyzed by the reconstituted BCKD complex were measured radiochemically using α-keto[1-14C]isovalerate as a substrate as described under “Experimental Procedures.” Kinetic values represent the average of three independent measurements.

Table II shows that His291-α and His146-β substitutions, respectively, whereas H146A-β and H146N-β variants exhibit only 6% and 11% of the wild-type activity, respectively. Activity for the E1b-catalyzed reductive acylation of lip-LBD (Reaction 3) is more severely impeded than ThDP-mediated decarboxylation in these mutants. Rates for Reaction 3 are 3, 7, and 2.2% of the wild type for H291A-α, H291N-α, and H291Q-α substitutions, respectively. Both H146A-β and H146N-β mutants show no detectable reductive acylation activity. The data establish His146β as an essential catalytic residue for E1b-mediated acylation reaction (see “Discussion”).

Afﬁnity of E1b Mutants for Cofactor ThDP—Dissociation constants ($K_d$) of wild-type E1b and the His146-β and His291-α variants for cofactor ThDP were measured by tryptophan fluorescence quenching. Fig. 3 shows the percent quenching versus ThDP concentrations with wild-type (panel A) and mutant (panel B) E1b. Wild-type E1b exhibits a $K_d$ value for ThDP of 1.52 μM, whereas the H291A-α and the H146A-β mutants show signiﬁcantly elevated $K_d$ values for ThDP of 39.3 and 47.1 μM, respectively (Table III). Therefore, substitutions at either H291A-α or H146A-β affect the binding of ThDP.

**His291-α Is an Essential Residue for the Binding of LBD to E1b**—The interactions of wild-type and mutant E1b with lip-LBD were studied by ITC. Fig. 4 shows that wild-type E1b readily binds to lip-LBD, but not unlipoylated LBD, with $K_d = 15.6$ μM, and $ΔH^o = -1.32$ kcal/mol (Table IV). The His146A-β variant binds to lip-LBD with afﬁnity similar to the wild type. The $ΔH^o$ of -0.69 kcal/mol with the His146A-β mutant is smaller than that obtained with wild-type E1b. For the His291A-α E1b, no binding to lip-LBD was observed. The fact that this mutant exhibits 6% of the wild-type overall activity (Table II) suggests that low afﬁnity binding of lip-LBD to this

### Table II

| Wild-type/mutants | $K_m$ (KIV) | $k_{cat}$ | $k_{cat}/K_m$ ($×10^3$) | $K_m$ (ThDP) | $k_{cat}$ | $k_{cat}/K_m$ ($×10^3$) |
|-------------------|-------------|----------|------------------------|-------------|----------|------------------------|
| WT                | 59 ± 4      | 8.65 ± 0.15 | 146 ± 14            | 0.55 ± 0.6  | 6.38 ± 0.95 | 11,600 ± 334         |
| H146A-β’          | 0           | 0         | 0                     | 0           | 0         | 0                     |
| H146N-β’          | 206 ± 16    | 0.58 ± 0.5 | 2.8 ± 0.1             | 24 ± 2      | 0.73 ± 0.3 | 31 ± 5               |
| H291A-α’          | 118 ± 13    | 0.67 ± 0.2 | 5.6 ± 0.2             | 1.4 ± 0.3   | 0.6 ± 0.6 | 420 ± 11             |
| H291Q-α’          | 203 ± 11    | 0.49 ± 0.1 | 2.4 ± 0.3             | 9 ± 2.3     | 0.41 ± 0.12 | 45 ± 3               |

* The reconstituted overall activity of the human BCKD complex was measured radiochemically using α-keto[1-14C]isovalerate as a substrate as described under “Experimental Procedures.” Kinetic values represent the average of three independent measurements.

* Data for H291A-α are taken from Ref. 6.
Roles of Two Histidine Residues in E1b Active Site

**Fig. 3.** ThDP binding to wild-type and mutant human E1b proteins measured by tryptophan fluorescence quenching. Incremental amounts of ThDP were added to a solution of 0.23 μM human E1b. Samples were excited at 290 nm, and emission intensity at 335 nm was measured. Changes in tryptophan fluorescence due to ThDP binding were normalized for the dilution of the sample and for inner filter effects. The data were plotted for wild-type (○), H146A-β' (●), and H291A-α (□) E1b as % quenching of fluorescence versus ThDP concentrations. The % quenching represents ΔF/Fo, where Fo is fluorescence intensity prior to the addition of ThDP and ΔF is the decrease in fluorescence at a given ThDP concentration. The data were fitted as described under "Experimental Procedures." Dissociation constants (Kd) for ThDP are shown in Table III.

E1b variant is likely to occur, however, the affinity is too low to be detected by the present ITC measurements. The ITC data therefore indicate that the His291A-α residue is essential for the interactions of E1b with the lip-LBD of E2b.

**Refined Active-site Structure of Human E1b—**Our crystallization conditions yielded crystals that diffract to a significantly higher resolution (up to 1.65 Å for selected crystals) than those reported by Ävarsson et al. (2.7 Å, PDB code 1DTW). This improvement allowed a more detailed characterization of the conformation at the E1b active site including bound water molecules, which is a pre-requisite to dissect the reaction mechanism of this enzyme (Fig. 5). The crystal structure of holo-E1b with bound Mn-ThDP was determined using data to a minimum Bragg spacing, dmin, of 1.81 Å (Table I). As shown in Fig. 5, there are only two histidine residues, His291-α and His146-β' that are within hydrogen-bonding distance to the bound ThDP in the human E1b active site. The N1 nitrogen atom of His291-α is hydrogen-bonded to the O1 water molecule that in turn interacts with the two terminal phosphate oxygen atoms of ThDP. The same O1 water molecule O2 coordinates directly to N3 nitrogen atom of the cofactor ThDP to wild-type, H291A-α, and H146A-β' E1b as determined by fluorescence quenching

**Fig. 4.** ITC measurements for lip-LBD binding to wild-type, H146A-β and H291A-α human E1b. ITC experiments were carried out in a MicroCal VP-ITC microcalorimeter by consecutively injecting aliquots of 1.5 mM lip-LBD or unlipoylated LBD into the reaction cell containing 25 μM wild-type or mutant human E1b. Binding isotherms for wild-type (○), H146A-β' (●), and H291A-α (▲) were obtained by plotting heat changes against the molar ratio of lip-LBD, as derived from the integrated raw data. The data were fit using the ORIGIN software supplied by the manufacturer. Wild-type E1b and the His146-β' variant show similar affinity for lip-LBD with dissociation constants (Kd) of 2.52 ± 10^-5 M and 1.56 ± 10^-5 M, respectively. The binding of the H291A-α mutant to lip-LBD cannot be detected by ITC as indicated by the absence of heat changes. Binding of unlipoylated LBD (△) to wild-type E1b also cannot be detected.

**TABLE III**

| E1b protein          | Kd μM | Maximal quenching |
|----------------------|-------|-------------------|
| Wild-type            | 1.52 ± 0.01 | 60.1 ± 1.7 |
| H291A-α             | 39.3 ± 5.6  | 40.2 ± 2.1 |
| H146A-β'           | 47.1 ± 2.8  | 47.1 ± 2.8 |

a Dissociation constants represent the average of three independent measurements.
the two rings of ThDP, approaching from the other side of the aminopyrimidine ring. These interactions contribute to the formation of the V-conformation (torsion angles $\Phi_r = 10^\circ$, $\Phi_p = -71^\circ$) for the cofactor (3). Overlapping densities are observed between the N1 atom of the ThDP aminopyrimidine ring and the side-chain carbonyl group of the invariant Glu$^{11003}$-$\beta$. This interaction results in the increased basicity of the $\epsilon^1$-NH$_2$ group, which is required for efficient deprotonation of the C2 atom of the aminopyrimidine ring for the formation of enamine-ThDP (21).

An important structural feature of our E1b crystals is the visibility of the loop region in wild-type E1b between Tyr$^{286}\alpha$ and Gln$^{312}\alpha$ including Ser$^{292}\alpha$ (phosphorylation site 1), with electron density interrupted between Thr$^{293}\alpha$ and Ser$^{294}\alpha$, at Ala$^{399}\alpha$, and between Ser$^{302}\alpha$ and Asn$^{307}\alpha$ (Fig. 6A). Remarkably, the H291$\alpha$ mutation renders the entire phosphorylation loop disordered in the active site of this mutant (Fig. 6B). Residual electron densities observed in Tyr$^{286}\alpha$ and Arg$^{387}\alpha$ are accompanied by the complete absence of densities between Ile$^{398}\alpha$ and Gln$^{312}\alpha$ (Fig. 6B). In contrast, the H146A-$\beta$ substitution does not result in significant conformational alterations from the wild type in the phosphorylation loop region or the vicinity of this histidine residue (Fig. 6C). However, the O4 water molecule hydrogen-bonded to His$^{146}\beta$' in the wild-type human E1b (Fig. 5) is absent from the H146A-$\beta$ variant. This change abolishes water-mediated interactions between Tyr$^{102}\beta$' and His$^{146}\beta$' in the wild-type active-site structure.

**DISCUSSION**

In the earlier study with D,L-S-methylthiolic acid methyl ester, a tetrahedral adduct at the C2 position of ThDP was formed between enamine-ThDP and the methyl ester (7). The isolation of this tetrahedral intermediate led Jordan’s group to postulate the presence of an, if donor in E1, which facilitates the disulfide bond scission of lipoic acid by electrophilic catalysis at the S6 atom (7, 22). The E1b structure of *Pseudomonas* BCKD complex recently showed the presence of two histidine residues (His$^{312}\alpha$ and His$^{131}\beta$') flanking the cofactor ThDP in the active-site channel (2). It was proposed, based on the D,L-S-methylthiolic acid model, that either histidine residue can be a candidate proton donor during reductive acylation of lipoamide attached to the E2b subunit. The present study focused on the equivalent histidine residues in human E1b in order to shed light on their roles during the reductive acylation catalytic cycle.

The role of His$^{391}\alpha$ was investigated by measuring the affinity of the H291A-$\alpha$ mutant for cofactor ThDP. The $K_d$ (39.3 $\mu M$) of this variant, as determined by tryptophan fluorescence quenching, is an order of magnitude higher than that of the wild-type at 1.52 $\mu M$. These values are comparable to the $K_a$ values of 24 $\mu M$ and 0.66 $\mu M$ for the H291A-$\alpha$ variant and wild-type E1b, respectively (6). In addition to electrostatic stabilization of the phosphate group by a putative positively charged His$^{391}\alpha$, the N1 atom of this residue establishes a water-mediated hydrogen bond to the terminal phosphate oxygen atoms (Fig. 5). The reduced affinity of the H291A-$\alpha$ mutant for ThDP likely results from the loss of the stabilizing interaction between the cofactor and the His$^{391}\alpha$ side chain.

**Roles of Two Histidine Residues in E1b Active Site**

**Fig. 5.** Refined structure of the human E1b active site at the interface between $\alpha$- and $\beta$-subunits. 2F$_{o}$ − F$_{c}$ electron densities (in green) are contoured at 1.0. Only two histidine residues are within 5-Å distance from the C2 atom of the bound ThDP. His$^{146}\beta$ is hydrogen-bonded to the O4 water molecule, whereas His$^{291}\alpha$ forms hydrogen bonds to the O1 and O2 water molecules (in red spheres); the former in turn coordinates to the terminal phosphate oxygen of ThDP. The channel leading to the activated C2 atom of ThDP lies at the interface between the $\alpha$- and $\beta$-subunits, such that these two histidine residues flank opposite sides of the channel. A Mn$^{2+}$ ion is bound at the metal ion binding site in place of the common Mg$^{2+}$ ion. Good electron density is present for Ser$^{292}\alpha$ (phosphorylation site 1), which is positioned at the opening of the channel. Carbon atoms are in gold, ThDP in green, oxygen atoms in red, nitrogen atoms in blue, phosphorous atoms in magenta, and sulfur atoms in yellow. Graphics were generated with the programs BobScript (24) and PovRay (Persistence of Vision, v3.02, POV-Team, www.povray.org).

| E1 construct | $K_d$ (M) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (kcal/mol) |
|--------------|-----------|------------------------|------------------------|------------------------|
| Wild-type    | $1.56 \times 10^{-5}$ | $-6.17$ | $-1.32$ | $+4.85$ |
| H146A-$\beta'$ | $2.52 \times 10^{-5}$ | $-6.44$ | $-0.69$ | $+5.75$ |
| H291A-$\alpha$ | N.D.$^a$ | N.D. | N.D. | N.D. |

$^a$ N.D., not detectable.

**TABLE IV**

Thermodynamic parameters for the interactions of Lip-LBD2 with wild-type, H146A-$\beta'$, and H291A-$\alpha$ E1b as determined by isothermal titration calorimetry
The impaired interactions of the H291A-α mutant with ThDP account for the reduced rate of decarboxylation (Reaction 2) with this variant relative to the wild-type. It is of significant interest that the H291A-α mutant exhibits severely impaired binding to lip-LBD. From the striking absence of visible electron density for most of the phosphorylation loop in crystals of this variant, we conclude that the above indirect interactions between the side chain of His291-α and the terminal phosphate oxygen atoms of ThDP are vital for the stabilization of this loop (Fig. 6B). An asparagine residue in place of a histidine at position 291-α would be able to establish direct or indirect hydrogen bonding interactions with ThDP, but it would not exhibit electrostatic stabilization. Partial stabilization of the phosphorylation loop in this variant would explain the modestly reduced affinity for ThDP ($K_d = 1.4 \mu M$) of the H291N-α mutant, compared with the wild type (Table II). Similar mechanisms can be invoked for the H291Q-α variant. Taken together, our results suggest that for binding to lip-LBD, the phosphorylation loop must be in a specific conformation conferred by the interactions between ThDP and His291-α. The markedly decreased rate of reductive acylation with the H291A-α variant most likely results from its inability to bind lip-LBD, and appears to be largely responsible for the marginal overall activity reconstituted with this mutant (Table II). As discussed above, based on the Pseudomonas E1b structure (2), for the reductive acylation to occur the lip-LBD must penetrate the 20-Å E1b active-site channel, so as to facilitate an efficient acetyltransfer from enamine-ThDP to the S8 atom on the dithiolane ring of lipoamide attached to lip-LBD. The current data therefore strongly support a structural role for His291-α in the reductive acylation reaction catalyzed by human E1b. The weak binding of the H291A-α E1b to lip-LBD as measured by ITC is similar to that observed with the equivalent H407A variant of E. coli E1p (8). The $K_d$ value (15.6 μM) and thermodynamic parameters ($\Delta G^\circ$, $\Delta H^\circ$, and $\Delta S^\circ$) for the binding of wild-type human E1b to lip-LBD determined by the same method are also analogous to those measured with E. coli E1p and its cognate lip-LBD (8).

His407 in E. coli E1p was proposed to function as a catalytic residue that assists in the protonation of the dithiolen sulfur atom of lipoamide on the E2p subunit (8). In contrast to this hypothesis, our present data argue against the equivalent His291-α in human E1b serving as a putative proton donor in the E1b-mediated reductive acylation reaction, since substitution of His291-α with an asparagine or glutamine, which cannot be a proton donor, does not nullify the activity for reductive acylation in these mutants. The basis for the discrepancy between the human E1b and E. coli E1p studies is presently unknown. However, the proposed catalytic role of His407 in E. coli in activating E2p-attached lipoamide during reductive acetylation cannot be substantiated from the structural viewpoint, since the region that carries His407 is not visible in the E. coli E1p structure (23).

On the other hand, our data show that the only other conserved histidine residue within the 4.75-Å distance of enamine-ThDP in the E1b active site, i.e. His146-β is a critical catalytic residue. The decarboxylation reaction is severely reduced in both H146A-β and H146N-β mutants, indicating that this histidine is involved in but not essential for this reaction (Fig. disordered in the H291A-α variant (B). The substrate access channel lies to the right with His141-β at its mouth. His291-α in the phosphorylation loop and His146-β flank the end of the channel with the co-factor ThDP located at its bottom. Except for the H291A-α variant, good electron density is observed for Ser292-α (phosphorylation site 1). See Fig. 5 for color designation of atoms and the programs used for the generation of graphics, except that the phosphorylation loops are in cyan.

**Fig. 6.** The phosphorylation loop in the active site of human E1b. 2Fo − Fc, electron densities (green, contoured at 1σ) showing the degree of order of the phosphorylation loop (Tyr239-α to Gln312-α) well ordered in wild-type E1b (A) and in the H146A-β variant (C) and
Roles of Two Histidine Residues in E1b Active Site

2). However, both substitutions completely abolish the activity of the subsequent reductive acylation reaction, establishing that His146-β’ is essential for this step. The block in reductive acylation is apparently responsible for a complete absence of the overall activity reconstituted with these two mutants (Table II). In the refined E1b active-site structure, the N3 atom of His146-β’ coordinates to the O4 water molecule (Fig. 5); the latter is replaced by KIV in the crystal structure of the human E1b-substrate complex (data not shown). The non-protonated N3 atom of His146-β’ is capable of abstracting a proton from a general enzymic base and donating this proton to the S6 atom of the incoming lipoamide during the E1b-mediated reductive acylation reaction. Since neither alanine nor asparagine can serve as a proton donor, a replacement of His146-β’ leads to a complete disruption of the reductive acylation reaction.

A proposed reaction scheme involving His146-β’ is shown in Fig. 7. At the onset of the E1b-mediated decarboxylation reaction, the proton on the OH group of the enamine is likely to be abstracted by a histidine residue in the active site of E1b (6, 7). The H146A-β’ variant for lip-LBD despite a reduced binding enthalpy (ΔHf) (Table IV). The abrogation of the O4 water-mediated interactions between His146-β’ and Tyr102-β’ in the H146A-β’ mutant is expected to cause a displacement or increased mobility of the Tyr102-β’ prior the binding of cofactor ThDP. This putative effect may be associated with the significantly reduced affinity of the H146A-β’ mutant for ThDP, since Tyr102-β’ is required for the correct orientation of the aminopyrimidine ring (δ). In the 2.7 Å structure of Pseudomonas E1b co-crystallized with a substrate analog α-chloroisocaproate, residue His131-β’ corresponding to His146-β’ in human E1b featured additional electron density, which was interpreted to represent a covalent adduct of the bacterial His131-β’ with the substrate analog (2). Such a covalent adduct with α-chloroisocaproate was not observed in the current refined human E1b structures. However, our results do not rule out the possibility that His146-β’ binds a ω-κetoacid through electrostatic interactions, and positions the substrate for a nucleophilic attack by the Carbanion of the covalent adduct. The abrogation of the O4 water-mediated interactions between His146-β’ and Tyr102-β’ may account, in part, for the higher residual decarboxylation rate (6% of the wild-type E1b) exhibited by the H146A-β’ mutant (Fig. 1). An asparagine residue at position 146-β’ could potentially coordinate to Tyr102-β’ or enamine-ThDP through hydrogen bonding. This partial stabilization effect may account, in part, for the higher residual decarboxylation activity in the H146N-β’ variant (11% of the wild type) over its H146A-β’ counterpart.

REFERENCES

1. Chuang, D. T., and Shah, V. E. (2001) in The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Vogelstein, K. B., and Childs, B., eds) 8th Ed., pp. 1971–2006, McGraw-Hill, Inc., New York
2. Ahransson, A., Seger, K., Turley, S., Sokatch, J. R., and Hol, W. G. J. (1999) Nat. Struct. Biol. 6, 785–792
3. Ahransson, A., Chuang, J. L., Wynn, R. M., Turley, S., Chuang, D. T., and Hol, W. G. J. (2000) Structure 8, 277–291
4. Lindqvist, Y., Schneider, G., Ermiller, U., and Sundstrom, M. (1992) EMBO J. 11, 2373–2379
5. Hawes, J. W., Schnepp, R. J., Jenkins, A. E., Shimomura, Y., Popov, K. M., and Harris, R. A. (1995) J. Biol. Chem. 270, 31071–31076
6. Wynn, R. M., Ho, R. Chuang, J. L., and Chuang, D. T. (2001) J. Biol. Chem. 276, 4168–4174

Fig. 7. A proposed reaction cycle for ThDP-mediated decarboxylation and His146-β’-promoted reductive acylation catalyzed by human E1b.
Roles of Two Histidine Residues in E1b Active Site

43410

7. Pan, K., and Jordan, F. (1998) Biochemistry 37, 1357–1364
8. Nemeria, N., Arjunan, P., Brunskill, A., Sheibani, F., Wei, W., Yan, Y., Zhang, S., Jordan, F., and Furey, W. (2002) Biochemistry 41, 15459–15467
9. Fiedler, E., Golbik, R., Schaefer, G., Tittmann, K., Neef, H., Konag, S., and Hubner, G. (2001) J. Biol. Chem. 276, 16051–16058
10. Wynn, R. M., Davie, J. R., Song, J. L., Chuang, J. L., and Chuang, D. T. (2000) Methods Enzymol. 324, 179–191
11. Chuang, J. L., Wynn, R. M., and Chuang, D. T. (2002) J. Biol. Chem. 277, 36995–36998
12. Chuang, J. L., Davie, J. R., Wynn, R. M., and Chuang, D. T. (2000) Methods Enzymol. 324, 192–200
13. Wynn, R. M., Davie, J. R., Chuang, J. L., Cote, C. D., and Chuang, D. T. J. Biol. Chem. (1998) 273, 13110–13118
14. Graham, L. D., Packman, L. C., and Perham, R. N. (1989) Biochemistry 28, 1574–1581
15. Hennig, J., Kern, G., Neef, H., Biswas, H., and Hubner, G. (1966) in Biochemistry and Physiology of Thiamin Diphosphate Enzymes (Biswas, H., and Schellenberger, A., eds) pp. 245–251, A. u. C. Intemann, Frien, Germany
16. Lakowicz, J. R Mahlawal, B. P., Cherek, H., and Balter, A. (1983) Biochemistry 22, 1741–1752
17. Nemeria, N., Yan, Y., Zhang, Z., Brown, A. M., Arjunan, P., Furey, W., Guest, J. R., and Jordan, F. (2001) J. Biol. Chem. 276, 45969–45978
18. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
19. Brzinski, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Goss, P., Grose-Kunste, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D 54, 905–921
20. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
21. Kern, D., Kern, G., Neef, H., Tittmann, K., Killenberg-Jabs, M., Wikmer, C., Schaefer, G., and Hubner G. (1997) Science 275, 67–70
22. Rice, J. L., (1968) Acc. Chem. Res. 1, 58–64
23. Arjunan, P., Nemeria, N., Brunskill, A., Chandrasekhar, K., Sax, M., Yan, Y., Jordan, F., Guest, J. R., and Furey, W. (1999) Biochemistry 41, 5213–5221
24. Esnouf, R. M. (2002) Acta Crystallogr. D Biol. Crystallogr. 58, 938–940