The Two Active Sites in Human Branched-chain \( \alpha \)-Keto Acid Dehydrogenase Operate Independently without an Obligatory Alternating-site Mechanism*

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A long standing controversy is whether an alternating active-site mechanism occurs during catalysis in thiamine diphosphate (ThDP)-dependent enzymes. We address this question by investigating the ThDP-dependent decarboxylase/dehydrogenase (E1b) component of the mitochondrial branched-chain \( \alpha \)-keto acid dehydrogenase complex (BCKDC). Our crystal structure reveals that conformations of the two active sites in the human E1b heterotetramer harboring the reaction intermediate are identical. Acidic residues in the core of the E1b heterotetramer, which align with the proton-wire residues proposed to participate in active-site communication in the related pyruvate dehydrogenase from Bacillus stearothermophilus, are mutated. Enzyme kinetic data show that, except in a few cases because of protein misfolding, these alterations are largely without effect on overall activity of BCKDC, ruling out the requirement of a proton-relay mechanism in E1b. BCKDC overall activity is nullified at 50% phosphorylation of E1b, but it is restored to nearly half of the pre-phosphorylation level after dissociation and reconstituation of BCKDC with the same phosphorylated E1b. The results suggest that the abolition of overall activity likely results from the specific geometry of the half-phosphorylated E1b in the BCKDC assembly and not due to a disruption of the alternating active-site mechanism. Finally, we show that a mutant E1b containing only one functional active site exhibits half of the wild-type BCKDC activity, which directly argues against the obligatory communication between active sites. The above results provide evidence that the two active sites in the E1b heterotetramer operate independently during the ThDP-dependent decarboxylation reaction.

Thiamine diphosphate (ThDP), a derivative of thiamine or vitamin \( B_{12} \), is an essential cofactor for ThDP-dependent enzymes that mediate the decarboxylation of \( \alpha \)-keto acids or the transfer of the glycoaldehyde moiety from a ketone to an aldose (1–3). ThDP-dependent enzymes in the form of homodimers (\( \alpha_2 \)), homotetramers (\( \alpha_4 \)), or heterotetramers (\( \alpha_2\beta_2 \)) contain ThDP-binding pockets that constitute the two or four active sites of these enzymes. A long standing controversy in the literature has been whether these multiple active sites in a ThDP-dependent enzyme communicate with each other to affect the enzymatic reaction. An alternating active-site mechanism, or half-of-the-sites reactivity, was proposed, based on the negative cooperativity of ThDP binding to apotransketolase (4–7). However, the crystal structure of the \( \alpha \)-carbanion of (\( \alpha \beta \)-dihydroxyethyl)thiamine diphosphate in the active sites of yeast transketolase shows that the intermediate is present in high occupancy in both active sites, which does not support the alternating active-site mechanism in this enzyme (8). Yeast pyruvate decarboxylase, an \( \alpha_2 \)-type ThDP-dependent enzyme, was shown kinetically to have two distinct pairs of active sites that alternate during the decarboxylation reaction (9). This kinetic model is supported by the observation that two of the four active sites of yeast pyruvate decarboxylase bind the nonmetabolizable substrate analog pyruvamide and assume a closed conformation in the crystal structure (10). Alternating active sites have also been described for bacterial benzoylformate decarboxylase, according to the spectroscopic detection of transient ThDP-bound intermediates (11).

The mitochondrial \( \alpha \)-ketoadipic acid dehydrogenase multienzyme complexes, comprising the pyruvate dehydrogenase complex (PDC), the \( \alpha \)-ketoglutarate dehydrogenase complex, and the branched-chain \( \alpha \)-ketoadipic acid dehydrogenase (BCKDC), catalyze the oxidative decarboxylation of \( \alpha \)-ketoadipic acids (Reaction 1) for energy production through the Krebs cycle (12, 13).

\[ \text{R-CO-COO}^- + \text{CoASH} + \text{NAD}^+ \rightarrow \text{CO}_2 \uparrow + \text{R-CO-SCoA} + \text{NADH} \]

**REACTION 1**

These macromolecular catalytic machines, ranging from 4 to 10

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2. The abbreviations used are: ThDP, thiamine diphosphate; PDC, pyruvate dehydrogenase complex; BCKDC, branched-chain \( \alpha \)-ketoadipic acid dehydrogenase complex; E1b, the decarboxylase/dehydrogenase component of BCKDC; E1p, the decarboxylase/dehydrogenase component of PDC; E2b, the transacylase core of BCKDC; E3, dihydrolipoamide dehydrogenase; LBD, lipoyl-bearing domain; SBD, subunit-binding domain; KIV, \( \alpha \)-ketosovalerate; VTHDP, 2-(\( \alpha \)-hydroxyisobutyryl)ThDP; DCPIP, 2,6-dichlorophenolindophenol; MPA, 4-methylpentanoate; DSC, differential scanning calorimetry; TMAO, trimethylamine N-oxide; DTT, dithiothreitol; lip-LBD, lipoylated LBD; Strep, strepavidin.
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We have previously described crystal structures of wild-type and mutant human E1b in the absence and presence of decarboxylation reaction intermediates (16, 19–22). For these structure determinations, we used a crystal form that has an αβ heterodimer in the asymmetric unit and exhibited the symmetry of space group P3₁21. The full heterotetramer is generated by a crystallographic 2-fold symmetry operator. This symmetry persists to the observed high resolution limit (1.4 Å) of these crystals. As a result of this packing arrangement, the two half-tetramers must be structurally identical. The electron densities showed that both active sites are occupied with intermediates. We did not detect any structural asymmetry involving the active sites or surrounding regions, either for human E1b in the absence of substrate or when intermediates were bound. Likewise, the crystal structure of the E1b component from *Thermus thermophilus* BCKDC showed similar occupancy of the α-carbanion-ThDP intermediate in both active sites (23). Therefore, these crystallographic properties of both human and *Thermus* E1bs are inconsistent with the half-of-the-sites model for these ThDP-dependent enzymes.

To investigate whether the alternating active-site mechanism occurs in the ThDP-dependent E1b component of human BCKDC, we carried out crystallographic and biochemical studies with both wild-type and mutant E1b heterotetramers. We provide evidence that the two active sites in human E1b, unlike *B. steatornophilus* and human E1p, can operate independently without the obligatory communication between active sites. Moreover, we find that the completely inactive BCKDC, harboring 50% phosphorylated E1b, retains a significant amount of overall activity when the covalently modified E1b is dissociated and re-associated with E2b. We suggest that the inactive BCKDC observed with the phosphorylated E1b results from the specific geometry associated with the BCKDC assembly.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—N-terminally and C-terminally His₆-tagged wild-type and mutant human E1b proteins were generated as described previously (20). The mutations were introduced using the QuikChange site-directed mutagenesis kit provided by Stratagene (La Jolla, CA). Expression of C-terminally His₆-tagged LBD (residues 1–84 of the human E2b subunit) and *in vitro* lipoylation was carried out also as described previously (24). To produce an E1b-hybrid protein with only one functional active site, two plasmids were constructed. The pHisT hE1b plasmid containing the N-terminally His₆-tagged S292Qα subunit and the nontagged wild-type β subunit coding regions were as described previously (16). The pStrep hE1b plasmid was derived from the pACYC.Duet-1 plasmid (Novagen, San Diego) with the mature E1bα and E1bβ coding sequences, both of the wild type, inserted into its two multiple cloning sites. The Strept tag contained a short sequence of eight amino acid residues (WSHPQFEK) that allowed binding to Strept-Tactin resin (IBA GmbH, Göttingen, Germany). The pHisT hE1b and pStrep hE1b plasmids were co-transformed into BL-21 cells; the cells were selected against both ampicillin and chloramphenicol. The hybrid E1b protein (S292Qα/WT-β)β₂, consisting of one His₆-tagged mutant α protein...
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subunit and one Strep-tagged wild-type subunit, was purified from the lysate of co-transformed BL-21 cells by consecutive use of the HisTrap HP column (GE Healthcare) connected to an FPLC system and the Strep-Tactin Superflow gravity column. Elution conditions were chosen based on control experiments with His₆-tagged E1b (with both α subunits carrying the His₆ tag) on a Strep-Tactin column, and with Strep-tagged E1b (with both α subunits bearing the Strep tag) on a HisTrap column to avoid possible contamination by the carryover of single tagged species.

X-ray Crystallography of Mutant Human E1b Proteins—Crystallization of the C-terminally His₆-tagged human S302Pα E1b protein was carried out as described previously (22, 25). The crystals exhibited the symmetry of space group P3₁2₁ with cell parameters of approximately a = b = 145 Å and c = 138 Å, contained an entire heterotetramer per asymmetric unit, and diffracted x-rays to a minimum Bragg spacing, dₘᵦᵢₜ, of 1.88 Å. This crystal form is related to a previously published crystal form that exhibited the symmetry of space group P3₂₁2₁ with cell parameters of approximately a = b = 145 Å and c = 69 Å and that contained an α/β' heterodimer per asymmetric unit (22). For x-ray diffraction experiments, crystals were soaked with Mn-ThDP and KIV and flash-cooled in liquid propane and kept at about 100 K during data collection at beamlines 19ID and 19BM of the Advanced Photon Source (APS), Argonne National Laboratory, Argonne, IL. Data were processed with the HKL2000 package (26).

The crystal structure was determined by molecular replacement using the program Phaser (27) and the hE1b wild-type structure (Protein Data Bank code 1ols) as the search model. Refinement was carried out with the program Refmac5 (28) of the CCP4 package (29) with a random subset of all data set aside for the calculation of free R factors. No noncrystallographic symmetry restraints or constraints were used during refinement. Manual adjustments to the models were carried out with the program Coot (30).

The electron density clearly showed the presence of 2-(α-hydroxyisobutyl)-ThDP (VThDP), the decarboxylation intermediate from α-ketoisovalerate (KIV) in both active sites of the human E1b heterotetramer. A model for this intermediate was included only at the very end of the modeling/refinement process. After the refinement of the protein portions was complete, solvent molecules were added where chemically reasonable. Ramachandran analysis shows that all but 2 out of 1,439 residues in the model fall into the allowed regions. Tyr-113α in both copies in the asymmetric unit is in the disallowed region. The electron density for these residues is well defined. Tyr-113α adopts a unique conformation (referred to as the P-conformation) after a decarboxylation reaction intermediate has been formed in the active sites as described previously (22). Data collection and refinement statistics are listed in Table 1.

Enzyme Assays—The overall activity of BCKDC (Reaction 1) reconstituted with E1b, E2b, and E3 was assayed with KIV, CoA, and NAD⁺ as substrates and ThDP and MgCl₂ as cofactors; the progress of the reaction was followed spectrophotometrically by an increase in absorbance at 340 nm. The activity of E1b-mediated decarboxylation (Reaction 2) was assayed with KIV as a substrate and 2,6-dichlorophenolindophenol (DCPIP) as an artificial electron acceptor; the reaction was monitored by the decrease in absorbance at 600 nm. The E1b-catalyzed reductive acylation reaction (Reaction 3) was measured using [U-¹⁴C]KIV and oxidized lip-LBD as substrates. The methods for the above three activity measurements have been described previously (20).

Differential Scanning Calorimetry (DSC)—All E1b proteins were dialyzed against 50 mM potassium phosphate buffer, pH 7.5, containing 250 mM KCl, 5% (v/v) glycerol, 1 mM β-mercaptoethanol, 0.2 mM EDTA, followed by DSC runs in a VP-capillary DSC system (MicroCal). Each E1b has a final concentration of 5.8 μM, which was used to normalize the scans. Final traces were produced after subtraction of the reference scan and subsequent base-line corrections to determine the melting temperature (Tm) using the MicroCal ORIGIN 7 software package.

Phosphorylation of E1b and Dissociation of E1b from E2b after Phosphorylation—Phosphorylation of S302Aα E1b with the BCKD kinase was carried out in the presence of E2b as described previously (20). Aliquots were taken from the reaction mixture at different time points and mixed with 0.5 mM EDTA and the SDS sample loading buffer, followed by analysis by SDS-PAGE. The amount of ³²P-phosphoryl group incorporated into the E1bα subunit was determined by Phospho-Imaging. The calculation was done by comparing the individual PhosphoImaging bands to a band from standard spotting. To resolve E1b, E2b, and the BCKD kinase proteins after phosphorylation, NaCl was added to the reaction mixture to a final concentration of 1.0 M. After incubation overnight at 4 °C, the mixture was concentrated, and E1b was separated from E2b and the BCKD kinase by size exclusion chromatography using a Superdex 200 (HR 10/30) column.

Western Blotting—Column fractions containing His₆α-tagged E1b, Strep-tagged E1b, and His₆/α-Strep-tagged hybrid E1b proteins were separated by SDS-PAGE in 12% gels; protein bands were transferred to the Immobilon P membrane (Millipore, Bellerica, MA). The protein bands were probed following the manufacturers’ protocol, with either the horseradish peroxidase-conjugated His₆ tag probe supplied by Pierce or the horseradish peroxidase-conjugated Strep probe (Strep-Tactin) provided by IBA GmbH (Göttingen, Germany).

RESULTS

The Presence of Two Identical Active-site Conformations in Human E1b—We have determined the crystal structure of human E1b in the presence of 2-(α-hydroxyisobutyl)-ThDP (VThDP), the decarboxylation intermediate from the substrate α-ketoisovalerate (KIV), in a novel crystal form that contains an entire human E1b heterotetramer in the asymmetric unit (Fig. 1). This crystal form was obtained with a human E1b variant (S302Pα) harboring a substitution at phosphorylation site 2 (Ser-302α). The mutation appears to lead to a small structural change in the phosphorylation loop, which alters the intermolecular interactions in this region and results in a new packing arrangement in the crystals, which is different from that reported previously for human E1b (16, 19–22). The new crystal form exhibits the symmetry of space group P3₂₁₂₁ and shares the same a and b unit cell lengths with the previously obtained counterparts (22), but its c unit cell length is twice as long. Data
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**FIGURE 1.** Identical conformation in the two active sites of the human E1b heterotetramer. Ribbon representation of S320P and the human E1b in the crystal form containing an entire heterotetramer in the asymmetric unit. The N and C termini are indicated. All figures of molecular structures were created with the program PyMol (DeLano Scientific, San Carlos, CA).

| Table 1: Data collection and refinement statistics |
|--------------------------------------------------|
| **Data collection**                              |
| Space group                                      | P3, 21                                           |
| Unit cell dimensions a = b; c (Å)                | 145.3, 138.1                                      |
| Resolution (Å)                                   | 26.64-1.88 (1.91-1.88)                           |
| Completeness (%)                                 | 99.3 (100.0)                                     |
| Rmerge (%)                                       | 10.2 (65.9)                                      |
| I/σ(I)                                           | 15.4 (2.4)                                       |
| Multiplicity (%)                                 | 6.1 (6.2)                                        |
| Wilson B factor (Å)                              | 18.2                                             |
| **Refinement**                                   |
| Resolution (Å)                                   | 26.64-1.88                                      |
| No. of reflections Rwork/Rfree                   | 131,975/1,489                                    |
| Non-hydrogen atoms                               | 12,603                                           |
| Water molecules                                  | 1,080                                            |
| Rwork (%)                                        | 17.19/19.90                                      |
| Average B factor (Å²)                            | 16.8                                            |
| Carbanion-ThDP B factor (Å²)                     | 11.8                                            |
| Root mean square deviations                      | 0.014                                           |
| Bond lengths (Å)                                 | 0.014                                           |
| Angles (°)                                       | 1.655                                           |
| Correlation coefficient Fc − Fw                  | 0.957                                           |
| Ramachandran plot (%)                           | 97.6/2.3/0.1                                    |
| Missing residues                                 | α, α' 1-5, 301-312; β, β' 1-13                   |

*Numbers in parentheses refer to the highest resolution shell. *Fav.* = favored; all. = additional allowed region; dis. = disallowed regions.

The results show no structural asymmetry between the αβ' and the α'β heterodimers; the root-mean-square deviation for all compared 711 C-α atoms is 0.17 Å. The two active sites and their neighboring regions are structurally virtually identical. The B factor for the carbanion-ThDP intermediate in both active sites is 11.8 Å², compared with the average of 16.8 Å². When this heterotetramer is compared with the one generated from the previously reported heterodimeric structure (Protein Data Bank code 2beu) based on crystallographic 2-fold symmetry, the resulting root-mean-square deviation for all 1422 compared C-α carbon atoms is 0.24 Å. Thus, the structures of human E1b in the presence of the intermediate VThDP are virtually identical, irrespective of whether an αβ' heterodimer (22) or an entire heterotetramer (the present study) is in the asymmetric unit. Taken together, there is no crystallographic evidence for a structural asymmetry between the two active sites in human E1b.

**Site-directed Mutagenesis of Candidate Proton-wire Residues in E1b**—Fig. 2A shows the alignment of the potential proton-wire residues among sequences from human E1b, *Pseudomonas putida* E1b, human E1p, and *B. stearothermophilus* E1p. In human E1b, these residues include Asp-200α, Glu-46β, Glu-76β, and Asp-108β. These residues are located in a channel between the two symmetrical E1b active sites, each showing good density for the occupied VThDP intermediate (Fig. 2B). Glu-198α is a candidate proton-wire residue in human E1b, but the equivalent Gln-178α in *B. stearothermophilus* E1p has not been implicated as a proton-wire residue (17). Ala-203α in human E1b aligns with the proton-wire residue Glu-183α of *B. stearothermophilus* E1p, but it cannot assume the putative function because of the absence of negative charges. Moreover, there is no magnesium ion present in the center of the channel, unlike in *B. stearothermophilus* E1p. There is also no residue equivalent to this magnesium ion that could fulfill its role. Instead, there is a pair of Gln-77β residues whose side chains are hydrogen-bonded to each other (Fig. 2B). The 2-fold symmetry axis relating the αβ' and α'β heterodimers in human E1b is located exactly between these two residues. Because of the presence of these nonacidic residues, it is difficult to envision how the shuttling of a proton from one active site to another should occur in human E1b.

The putative role of active-site communication involving candidate proton-wire residues in human E1b was investigated. These residues were altered by site-directed mutagenesis, and enzyme kinetic parameters of these variants were determined. The overall activity of BCKDC was assayed by the reconstitution of the wild-type or mutant E1b protein with E2b and E3 without DTT. As shown in Fig. 2C, D200Na, E198Qα/D200Nα (a double mutant), and E46Qβ substitutions reduced overall activities of the mutants to 25, 45, and 65%, respectively, compared with the wild type. D200Aα and D108Nβ variants show 86 and 116% of wild-type activity, respectively. The Km values of these mutants for substrate KIV are similar to that of the wild type (56.5 ± 3.6 μM), except for the Km value of the D108Nβ variant (127 ± 15 μM), which is twice as high as that of the wild type. The D200α/D108Nβ double mutant exhibits 6.8% of residual wild-type overall activity. The rate for E1b-catalyzed decarboxylation of KIV was also measured in the presence of the electron acceptor DCPIP. The rates of E1b-catalyzed decarboxylation measured by decreased absorbance at 600 nm because DCPIP reduction are identical to those determined radiochemically by 14CO2 evolution using [U-14C]KIV as a substrate because of the absence of negative charges. Moreover, there is no magnesium ion present in the center of the channel, unlike in *B. stearothermophilus* E1p. In human E1b, the putative function because of the absence of negative charges. Moreover, there is no magnesium ion present in the center of the channel, unlike in *B. stearothermophilus* E1p. There is also no residue equivalent to this magnesium ion that could fulfill its role. Instead, there is a pair of Gln-77β residues whose side chains are hydrogen-bonded to each other (Fig. 2B). The 2-fold symmetry axis relating the αβ' and α'β heterodimers in human E1b is located exactly between these two residues. Because of the presence of these nonacidic residues, it is difficult to envision how the shuttling of a proton from one active site to another should occur in human E1b.
FIGURE 2. Sequence alignment, structure, activities, and stability of mutant human E1b in the putative proton-wire channel. A, E1α and E1β residues from human E1b, P. putida E1b, and human E1p are aligned with residues in B. stearothermophilus E1p. Secondary structural elements of human E1b are shown above the sequences. Red arrowheads indicate negatively charged residues conserved between human E1b and B. stearothermophilus E1p. Sequences were aligned with ClustalW (40). The graphical representation was created with ESPript (41). Red box, identical residues; yellow box, conserved residues. B, E1b residues in the putative proton-wire channel. A 2Fo−Fc omit map (contoured at 1.2σ) for the VThDP reaction intermediate is shown in light blue. VThDP, the intermediate from KIV, is shown as a stick-filling model; the α and α' subunits are shown in blue and yellow, respectively; the β and β' subunits are shown in magenta and green, respectively. Carbon atoms are shown with the colors of the subunit to which they belong. For the VThDP reaction intermediate, carbon atoms are shown in gray, oxygen atoms in red, nitrogen atoms in blue, sulfur atoms in yellow, and phosphorous atoms in purple. Water molecules are depicted as red spheres. Hydrogen bonds are represented by dotted lines. C, E1b and overall activities of the proton-wire mutants. Human E1b residues Asp-200, Glu-198, Glu-46, and Asp-108, which are equivalent to proton-wire residues (except Glu-198) of B. stearothermophilus E1p, are altered by site-directed mutagenesis. The overall activity of single and double mutants was measured by reconstitution with E2b and E3 as described under “Experimental Procedures.” Activity for E1b-catalyzed decarboxylation was measured in the presence of an electron acceptor DCPIP. Relative activities are expressed as percentage of the wild type (WT) (0.74 μmol/min/mg for overall activity and 0.06 μmol/min/mg for E1b activity, both measured in the absence of DTT). D, overall activity of TMAO-treated proton-wire mutants. Wild-type and mutant E1b proteins were incubated with 1.0 M TMAO at 23 °C overnight, prior to assay for overall activity of the reconstituted BCKDC. Relative activities are expressed as percentage of the wild type (1.11 μmol/min/mg for overall activity in the presence of 2 mM DTT). E, differential scanning calorimetry of wild-type and mutant E1b proteins. DSC measurements were carried out in a VP-capillary DSC system. Wild-type and mutant E1b proteins (5.8 M) were heated from 20 to 100 °C. All scans were performed in duplicate and averaged.
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FIGURE 3. Overall and reductive acylation activities of 50% phosphorylated human E1b. A, time course for \(^{32}\)P incorporation and the reduction of overall activity for S302A\/E1b. The S302A mutant with phosphorylation site 2 inactivated was incubated at 23 °C with 0.4 mm Mg\(\text{\textsuperscript{2+}}\)ATP, BCKD kinase, and E2b. Aliquots were taken at different times; the ratio of \(^{32}\)P-phosphorylated E1b tetramer and the overall activity of reconstituted wild-type E1b was incubated with 0.4 mm Mg\(\text{\textsuperscript{2+}}\)ATP, BCKD kinase, and E2b. Aliquots were taken at different times; the ratio of \(^{32}\)P-phosphorylated E1b tetramer and the overall activity of reconstituted BCKD were measured. B, dissociation and purification of phosphorylated E1b. The S302A mutant E1b was incubated with 0.4 mm Mg\(\text{\textsuperscript{2+}}\)ATP and BCKD kinase for 20 min. The 50% phosphorylated mutant E1b with no overall activity was dissociated from E2b and BCKD kinase in 1.0 m NaCl, followed by separation on Superdex 200 column. Upper panel, elution profile; lower panel, SDS-PAGE of column fractions. MBP-BCK, maltose-binding protein-fused BCKD kinase. C, activities for the overall reaction (solid bars) and reductive acylation (shaded bars) in the presence of excess exogenous lip-LBD were assayed with nonphosphorylated S302A\/E1b (lane 1) and 50% phosphorylated S302A\/E1b in the phosphorylation mixture prior to dissociation and separation from E2b and BCKD kinase (lane 2). Both activities were also assayed with 50% phosphorylated S302A\/E1b in column fractions 24–27 from B. Upon reconstitution with fresh batches of E2b and E3 (lane 3). Activities of 100% phosphorylated S302A\/E1b after column separation and reconstitution were also measured (lane 4). Relative activity is expressed as percentage of that with the starting nonphosphorylated E1b.

Overall Activity Is Abolished at 50% E1b Phosphorylation—The human E1b contains two phosphorylation sites (site 1 at Ser-292\(\text{\textgamma}\); site 2 at Ser-302\(\text{\textgamma}\)) on the phosphorylation loop (residues Tyr-286 to Pro-315\(\text{\textgamma}\)) located within each active site channel (20). The rapid phosphorylation of site 1 (the major site) alone in E1b is sufficient to completely inactivate BCKDC. Phosphorylation of site 2 (the minor site) is much slower than this residue with an alanine thwarts a proton abstraction at the C-2 atom of ThDP, thereby abolishing E1b activity (25).

The D200A\(\text{\textgamma}\)/D108N\(\text{\textgamma}\) Mutant Is Defective in Folding and Assembly—In the study by Frank et al. (17), double mutants D180A\(\text{\textgamma}\)/E183A\(\text{\textgamma}\) and D180N\(\text{\textgamma}\)/E183Q\(\text{\textgamma}\) of the B. stea-tathermophilus E1p exhibit markedly reduced E1p and overall PDC activity. It was suggested that reduced activities were because of a disruption of the proton-wire channel, preventing communication between the two active sites. To decipher the role of Asp-200\(\text{\textgamma}\) and Asp-108\(\text{\textgamma}\) in the putative proton wiring of E1b, we subject the D200A\(\text{\textgamma}\)/D108N\(\text{\textgamma}\) E1b double mutant to treatment with a naturally occurring osmolyte TMAO. We reported previously that the incubation of E1b proteins carrying certain disease-causing human mutations with TMAO partially correct assembly defects, resulting in the restoration of 30–50% of BCKDC overall activity (31). Fig. 2D shows that the incubation of the D200A\(\text{\textgamma}\)/D108N\(\text{\textgamma}\) mutant, which has no detectable overall activity, with TMAO leads to the recovery of ~50% overall activity compared with the wild type subjected to the same treatment. These results do not support roles of Asp-200\(\text{\textgamma}\) and Asp-108\(\text{\textgamma}\) in a putative proton-wire channel. Instead, these two residues are likely to be involved in the folding and assembly of the E1b heterotetramer. The moderately reduced overall activity of the remaining D200N\(\text{\textgamma}\), E198Q\(\text{\textgamma}\)/D200N\(\text{\textgamma}\), and E46Q\(\text{\textgamma}\) E1b variants compared with wild-type E1b is essentially unchanged after TMAO treatment (Fig. 2D). The data indicate that these mutations may not significantly impede the folding and assembly of the E1b heterotetramer. The higher percentage of BCKDC overall activities reconstituted with D200N\(\text{\textgamma}\) (65%) and E198Q\(\text{\textgamma}\)/D200N\(\text{\textgamma}\) (63%) mutants in the absence of TMAO in Fig. 2D, compared with those of 25 and 45%, respectively, in Fig. 2C, were because of the addition of DTT in the former set of assays. The results suggest that the reducing equivalence preferentially stabilizes the E1b component carrying these two mutations.

The putative adverse effects of the above mutations on the stability of human E1b were dissected by DSC. Wild-type E1b shows two distinct melting temperatures (Tm) at 52.6 and 65.4 °C, which likely represent transitions from heterotetramers to heterodimers and from heterodimers to the random-coil structure, respectively (32). The E46Q\(\text{\textgamma}\) E1b mutant shows two Tm values at 53.3 and 64.0 °C, similar to the wild-type E1b. Notably, both E198Q\(\text{\textgamma}\)/D200N\(\text{\textgamma}\) and D200A\(\text{\textgamma}\)/D108N\(\text{\textgamma}\) double mutants exhibit a single Tm value at 55.4 and 57.4 °C, respectively. The results confirm that these mutant proteins are defective in folding and assembly and undergo a single transition directly from heterotetramers to the random-coil structure. The remaining E1b mutants, D200A\(\text{\textgamma}\), D200N\(\text{\textgamma}\), and D108N\(\text{\textgamma}\) also show decreased Tm values of 53.5, 52.4/59.2, and 54.8 °C, respectively (data not shown), indicating that these E1b proteins are also less stable than wild-type E1b.
site 1 and is without effect on BCKDC activity (16, 33, 34). To
dissect the effect of phosphorylation on the hypothetical com-
munication between the two active sites, the S302Aα E1b var-
iant with phosphorylation site 2 abolished was phosphorylated
by the BCKD kinase in the presence of E2b (33). Fig. 3A shows
that phosphorylation of S302Aα E1b at site 1 reaches 50%
within 10 min, with BCKDC activity largely abolished (<5% ini-
tial activity). The second half of phosphorylation at site 1 is
sluggish, with close to 100% phosphorylation reached and over-
all activity completely lost in 16 h.

The overall activity of the pig heart PDC is also lost at 50% phos-
phorylation of the cognate E1p component (15). Based on
this earlier study, it was proposed that phosphorylation at one
of the two active sites abrogates communication with the other
site in E1p, resulting in the complete inactivation of PDC activ-
ity. To entertain this hypothesis, we dissociated E1b of the
human BCKDC at the 50% phosphorylation level from the E2b
core by incubation in 1 M sodium chloride at pH 7.5, followed by
separation by gel filtration (Fig. 3B, upper panel). The separated
50% phosphorylated E1b (Fig. 3B, lower panel) was assayed for
overall activity by reconstitution with a fresh batch of E2b and
E3. Surprisingly, the BCKDC reconstituted with the 50% phos-
phorylated E1b shows about 40% of wild-type overall activity
(Fig. 3C). The result is consistent with the notion that the 50%
phosphorylated E1b still possesses half of wild-type E1b activity
imparted by the remaining nonphosphorylated active site. The
loss of BCKDC activity at 50% E1b phosphorylation prior to
dissociation and re-assembly may result from the geometric
constraints imposed on E1b and LBD, both tethered to the E2b
scaffold, which prevent the LBD substrate from gaining access
to the nonphosphorylated functional E1b active site
(see “Discussion”).

A Hybrid E1b with One Site Inactivated Exhibits Nearly 40%
Activity—To dissect the requirement for the communication
between the two active sites, a hybrid E1b was produced. To
accomplish this, we generated two plasmids as follows: one
plasmid contained the His6-tagged α subunit sequence harbor-
ing the S292Qα mutation that inactivates BCKDC activity (16)
and the nontagged wild-type β subunit sequence; the other
plasmid consisted of the Strep-tagged wild-type α subunit
sequence and the nontagged wild-type β subunit sequence.
Both plasmids were co-transformed into the same Escherichia
coli BL21 cells and selected simultaneously against appropriate
antibiotics. The hybrid E1b heterotetramer carrying one wild
type, one S292Qα α subunit, and two wild-type β subunits was
purified sequentially using nickel-affinity (Fig. 4A) and Strep-
affinity (Fig. 4B) chromatography. The presence of the His6 tag
and Strep tag in the purified hybrid E1b was indicated by Western
blotting using the respective probes (Fig. 4C). E1b-cata-
yzed decarboxylation rates for wild-type and hybrid E1b were
measured in the presence of DCPIP. The hybrid E1b shows 40%
of wild-type E1b activity (Table 2). Assays for reductive acyla-
tion show a kcat value of 4.0 s−1 for the hybrid E1b, accounting
for 43% of wild-type activity. The Km values for lip-LBD are
similar between wild-type and the hybrid E1b. When assayed
for overall activity in the presence of E2b and E3, the hybrid E1b
shows a kcat of 73.9 min−1, which is nearly 40% of that recon-
stituted with wild-type E1b (Table 2). The Km value for KIV of
the hybrid E1b (118 μM) is twice that of the wild-type E1b (56.5
μM). The S292Qα E1b mutant, which shows no activity for
E1b-catalyzed decarboxylation, reductive acylation, and the
overall reaction reconstituted with E2b and E3 (16), served as
a negative control. Significantly, the catalytic parameters of this
hybrid, despite through a different mechanism of inactivation,
are similar to the catalytic parameters of 50% phosphorylated
E1b. The above results provide direct evidence that one active
site alone can function in the hybrid E1b.

DISCUSSION

The question whether ThDP-dependent enzymes employ an
alternating active-site mechanism during catalysis has been of
intense interest and is controversial in the literature. This work
was prompted by the recent study with B. stearothermophilus
E1p, which suggests the presence of proton wires to mediate
communication between the two active sites (17). We under-
took multiple reinforcing approaches to decipher whether such
active-site communication is also required for ThDP-depend-
ent decarboxylation catalyzed by human E1b. At present, it is not
known whether the presence of two independently operated active
sites in human E1b derived from our studies can be generalized to
other ThDP-dependent enzymes. Nonetheless, the recent NMR
study by Seifert et al. (18) has shown no evidence for chemical
nonequivalence in several related thiamine enzymes, including
yeast transketolase, Lactobacillus plantarum pyruvate oxidase,
and Zymomonas mobilis pyruvate decarboxylase.
The Two Active Sites in Human BCKD Operate Independently

The proposed “slinky cycle” mediated by the proton-relay channel in *B. stearothermophilus* E1p, and therefore an inherent asymmetry, is supported by the presence of both the outer and inner loops in only one of the two active sites upon the binding of ThDP to the E1p heterotetramer in the crystallographic asymmetric unit (17). In contrast, in this study, high occupancies of both ThDP molecules (or the reaction intermediate VThDP; Fig. 2B) and the high degree of order for the phosphorylation loop (equivalent to the outer loop in *Bacillus* E1p) were observed in the two active sites of the E1b of human BCKDC (Fig. 1). Thus, the human E1b structure does not show the two distinct active-site conformations depicted in the *Bacillus* E1p structure. The holo-E1b component studied here exists as a single heterotetramer in the asymmetric unit. In our earlier studies, the holo-E1b component with or without the carboxion-ThDP intermediate is present as heterodimers in the asymmetric unit (16, 19–22). However, the active-site conformation in the heterotetrameric E1b structure built on crystallographic 2-fold symmetry is practically indistinguishable from that in the present structure derived from the heterotetramer present in the asymmetric unit (Fig. 1).

In a related study, the heterotetrameric E1b component of *T. thermophilus* BCKDC was shown to bind a substrate analog, 4-methylpentanoate (MPA), in only one of its two active sites (23). The binding of MPA promotes the movement of a loop region (Gly-121 to Gln-131) in the β subunit to the active site so as to interact with the bound MPA. The authors attribute the different active site conformations in *Thermus* E1b to crystal packing effects. The regions surrounding the bound MPA are in contact with another symmetry-related E1b heterotetramer; this contact is absent in the active site devoid of MPA. The same study shows that the soaking of the *Thermus* E1b crystal with a keto acid substrate 4-methyl-2-oxopentanoate results in a high occupancy of the reaction intermediate α-carbanion-ThDP in both active sites of the heterotetramer, similar to that observed with human E1b. This result is also consistent with the presence of two identical active sites in the crystal lattice prior to the substrate soak.

As shown in Fig. 2A, Asp-200α, Glu-46β, Glu-76β, and Asp-108β in human E1b align with Asp-180α, Glu-28β, Glu-59β, and Asp-91β in *Bacillus* E1p, respectively. The negatively charged side chains of the *Bacillus* E1p residues are suggested to participate in proton relay between the two active sites (17). A double mutant of *Bacillus* E1p, D180Na/E183Qa, exhibits little DCPIP-mediated E1p activity and overall activity of the reconstituted *Bacillus* PDC. It is proposed that removal of the negatively charged side chains disrupts proton relay in the proton-wire channel, which in turn abrogates communication and the switching of their conformations between the two E1p active sites necessary for the alternating reaction mechanism (17). In this study, we show that mutations in the above putative proton-wire residues in human E1b largely result in moderate reductions (D200Aα, D200Na, E198Qα/D200Na, and E46Qβ) and, in some case, even a slightly increase (D108Nβ) in BCKDC overall activity (Fig. 2C). Similar conclusions were reached in a previous study of transketolase (35). Residue Glu-162 in transketolase is part of the glutamic acid cluster at the subunit interface linking the two ThDP molecules. Replacement of this residue results in a mutant with most catalytic properties similar to wild type, which, in retrospect, argues against the presence of a proton wire to mediate communication between two active sites.

Remarkably, the nearly complete inactivation of BCKDC overall activity in the D200Aα/D108Nβ mutant can be rescued to about 50% of the wild type by treatment with the naturally occurring osmolyte TMAO (Fig. 2D). This result suggests that the double mutations in E1b adversely affect the folding and assembly of the mutant protein, similar to that observed with a subset of human mutations causing maple syrup urine disease (31). The putative defective assembly in the D200Aα/D108Nβ mutant is corroborated by the DSC result that shows a significantly reduced single *Tm* (57.4 °C) as opposed to the higher end of the double E1b values (52.6/65.4 °C) shown by wild-type E1b. The single transition exhibited by this mutant strongly suggests that these mutations produce highly unstable heterotetramers that denature directly into random-coil structure without going through the heterodimeric intermediate.

From a structural perspective, side chains of Asp-200α and Asp-108β in human E1b are connected by a water-mediated hydrogen-bonding network in an almost perfect tetrahedral arrangement. This water molecule (Fig. 2B) also participates in a hydrogen-bonding network with two additional water molecules (not shown). Alterations of the Asp-200α and Asp-108β side chains likely disrupt this hydrogen-bonding network required for maintaining the integrity of the α/β interface. The TMAO treatment possibly imparts a compacting effect at the subunit interface via a “solvophobic” mechanism, resulting in a forced assembly and partial restoration of E1b catalysis (31). Therefore, results of the TMAO incubation and DSC measurements make the participation of Asp-200α and

### Table 2

| Protein                  | E1b-catalyzed decarboxylation 100 × mmol/min/mg | E1b-catalyzed reductive acylation | BCKDC overall reaction |
|--------------------------|-----------------------------------------------|----------------------------------|------------------------|
|                          |                                               |                                  |                        |
| Wild type                | 6.0 ± 0.3                                     | 119 ± 14                         | 56.5 ± 3.8             |
| S292Qα (wild type)       | 6.0 ± 0.3                                     | 119 ± 14                         | 56.5 ± 3.8             |
| S292Qα/wild type         | 2.4 ± 0.1                                     | 126 ± 10                         | 118 ± 7                |

* a Data are taken from Ref. 16.
* b NM means not measurable.
The Two Active Sites in Human BCKD Operate Independently

Asp-108β in proton wiring between the two E1b active sites unlikely.

We have shown previously that phosphorylation at Ser-292α results in an order-to-disorder transition of the phosphorylation loop that forms part of the active-site channel (16). This transition renders E1b unable to bind lipoylated LBD, a substrate for E1b-catalyzed reductive acylation (Reaction 3), thereby nullifying the overall reaction of BCKDC. We took advantage of this mechanism to produce the E1b hybrid with one of its two active sites harboring the S292Qo mutation that abrogated both E1b and BCKDC activities (16). The exhibition of 40% wild-type E1b and BCKDC activities with this E1b hybrid provides direct evidence that the remaining unmodified active site can operate independently without communication to the other dysfunctional active site.

As shown in Fig. 3A, the overall activity of the reconstituted BCKDC is essentially lost at 50% phosphorylation of site 1 (Ser-292α, the major site) in the site 2 mutant, in which site 2 (Ser-302α, the minor site) is abolished. The 50% phosphorylation of the two available major phosphorylation sites corresponds to the inactivation of one of the two active sites in E1b. This deduction is based on the notion that if 50% of E1b was phosphorylated at both active sites, the BCKDC reconstituted with this half-phosphorylated E1b sample would exhibit 50% of the starting overall activity. Unexpectedly, about 40% of overall activity was recovered by dissociation of the 50% phosphorylated E1b from E2b and the kinase, followed by reconstitution with a different E2b preparation (Fig. 4C). This result indicates that the remaining nonphosphorylated E1b active site can still function independently, and does not support the interruption of the obligatory alternating active-site mechanism described for the half-phosphorylated E1p of the mammalian PDC (15).

To explain the above results with phosphorylated E1b, we propose a model of sidedness, in which substrate LBD for reductive acylation (Reaction 3) is juxtaposed to the other unmodified active site because of geometric constraints, because E1b is tethered to the SBD on the same E2b chain. Our recent NMR studies show that the tethering of LBD and SBD of human BCKDC significantly retards their overall rotational correlation times, indicating that the presence of the inter-domain linker restricts the free movement of these domains on E2b (36). The sidedness of E1p active sites has been recently suggested based on single-particle cryoelectron microscopy models for PDC from bovine kidney (37) and B. steaothermophilus (38). Thus, the dissociation and reconstitution of the BCKDC with 50% phosphorylated E1b likely reorient the E1b on SBD such that about half of the nonphosphorylated active sites are now pointing toward E2b core and are therefore geometrically readily accessible to LBD for binding, resulting in the partial recovery of BCKDC overall activity. On the other hand, the retention of 37 and 42% reductive acylation activity of 50% phosphorylated E1b in complex with E2b before and after dissociation and re-association, respectively, indicates that the nonphosphorylated active site in E1b is still able to bind free LBD used in the assays and is fully active for reductive acylation. The sidedness of E1b active sites may also account for the markedly slow kinetics in the second half of phosphorylation, which corresponds to the modification of the distal E1b active site relative to the BCKD kinase bound to LBD (Fig. 3A). A similar phosphorylation profile is also observed with E1p, in which only one of the two phosphorylation sites in the E1p heterotrimer can be phosphorylated by pyruvate dehydrogenase kinase 1 bound to the E2p core of the human PDC (39).

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REFERENCES
1. Jordan, F. (2003) Nat. Prod. Rep. 20, 184–201
2. Jordan, F., Nemeria, N. S., and Sergienko, E. (2005) Acc. Chem. Res. 38, 755–763
3. Kern, D., Kern, G., Neef, H., Tittmann, K., Killenberg-Jabs, M., Wikner, C., Schneider, G., and Hübner, G. (1997) Science 275, 67–70
4. Kochetov, G. A., Meshalkina, L. E., and Usmanov, R. A. (1976) Biochem. Biophys. Res. Commun. 69, 839–843
5. Egan, R. M., and Sable, H. Z. (1981) J. Biol. Chem. 256, 4877–4883
6. Kovina, M. V., Selivanov, V. A., Kocheva, N. V., and Kochetov, G. A. (1997) FEBS Lett. 418, 11–14
7. Kovina, M. V., and Kochetov, G. A. (1998) FEBS Lett. 440, 81–84
8. Fiedler, E., Thorell, S., Sandalova, T., Golbik, R., Konig, S., and Schneider, G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 591–595
9. Sergienko, E. A., and Jordan, F. (2002) Biochemistry 41, 6164–6169
10. Lu, G., Dobritszch, D., Baumann, S., Schneider, G., and Konig, S. (2000) Eur. J. Biochem. 267, 861–866
11. Sergienko, E. A., Wang, J., Polovnikova, L., Hasson, M. S., McLeish, M. J., Kenyon, G. L., and Jordan, F. (2000) Biochemistry 39, 13862–13869
12. Reed, L. J. (2001) J. Biol. Chem. 276, 38329–38336
13. Reed, L. J., Damuni, Z., and Merryfield, M. L. (1985) Curr. Top. Cell Regul. 27, 41–49
14. Perham, R. N. (2000) Annu. Rev. Biochem. 69, 961–1004
15. Sugden, P. H., and Randle, P. J. (1978) Biochem. J. 173, 659–668
16. Wynn, R. M., Kato, M., Machius, M., Chuang, J. L., Li, J., Tomchick, D. R., and Chuang, D. T. (2004) Structure (Lond.) 12, 2185–2196
17. Frank, R. A., Titman, C. M., Pratap, J. V., Luisi, B. F., and Perham, R. N. (2004) Science 306, 872–876
18. Seifert, F., Golbik, R., Brauer, J., Lilie, H., Schroder-Tittmann, K., Hinze, E., Korotchkin, L. G., Patel, M. S., and Tittmann, K. (2006) Biochemistry 45, 12775–12785
19. Aevarsson, A., Chuang, J. L., Wynn, R. M., Turley, S., Chuang, D. T., and Hol, W. G. (2000) Structure (Lond.) 8, 277–291
20. Li, J., Wynn, R. M., Machius, M., Chuang, J. L., Karthikeyan, S., Tomchick, D. R., and Chuang, D. T. (2004) J. Biol. Chem. 279, 32968–32978
21. Wynn, R. M., Machius, M., Chuang, J. L., Li, J., Tomchick, D. R., and Chuang, D. T. (2003) J. Biol. Chem. 278, 43402–43410
22. Machius, M., Wynn, R. M., Chuang, J. L., Li, J., Klager, R., Yu, D., Tomchick, D. R., Brautigam, C. A., and Chuang, D. T. (2006) Structure (Lond.) 14, 287–298
23. Nakai, T., Nakagawa, N., Maoka, N., Masui, R., Kuramitsu, S., and Kamiya, N. (2004) J. Mol. Biol. 237, 1011–1033
24. Chuang, J. L., Davie, J. R., Wynn, R. M., and Chuang, D. T. (2000) Methods Enzymol. 324, 192–200
25. Wynn, R. M., Ho, R., Chuang, J. L., and Chuang, D. T. (2001) J. Biol. Chem. 276, 4168–4174
26. Owinski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
27. McCoy, A. J., Grosse-Kunstleve, R. W., Storoni, L. C., and Read, R. J. (2005) Acta Crystallogr. Sect. D Biol. Crystallogr. 61, 458–464
28. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D. Biol. Crystallogr. 53, 240–255
29. Collaborative Computational Project 4 (1994) Acta Crystallogr. Sect. D. Biol. Crystallogr. 50, 760–763
30. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 2126–2132
31. Song, J. L., and Chuang, D. T. (2001) J. Biol. Chem. 276, 40241–40246
32. Wynn, R. M., Davie, J. R., Chuang, J. L., Cote, C. D., and Chuang, D. T. (1998) J. Biol. Chem. 273, 13110–13118
33. Davie, J. R., Wynn, R. M., Meng, M., Huang, Y. S., Aalund, G., Chuang, D. T., and Lau, K. S. (1995) J. Biol. Chem. 270, 19861–19867
34. Zhao, Y., Hawes, J., Popov, K. M., Jaskiewicz, J., Shimomura, Y., Crabb, D. W., and Harris, R. A. (1994) J. Biol. Chem. 269, 18583–18587
35. Meshalkina, L., Nilsson, U., Wikner, C., Kostikova, T., and Schneider, G. (1997) Eur. J. Biochem. 244, 646–652
36. Chang, C. F., Chou, H. T., Lin, Y. J., Lee, S. I., Chuang, J. L., Chuang, D. T., and Huang, T. H. (2006) J. Biol. Chem. 281, 28345–28353
37. Zhou, Z. H., McCarthy, D. B., O’Connor, C. M., Reed, L. I., and Stoops, J. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14802–14807
38. Milne, J. L., Shi, D., Rosenthal, P. B., Sunshine, J. S., Domingo, G. J., Wu, X., Brooks, B. R., Perham, R. N., Henderson, R., and Subramaniam, S. (2002) EMBO J. 21, 5587–5598
39. Korotchkina, L. G., and Patel, M. S. (2001) J. Biol. Chem. 276, 37223–37229
40. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
41. Gouet, P., Courcelle, E., Stuart, D. I., and Metoz, F. (1999) Bioinformatics (Oxf.) 15, 305–308