Roles of Active Site and Novel K+ Ion-binding Site Residues in Human Mitochondrial Branched-chain α-Ketoacid Decarboxylase/Dehydrogenase*

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The human mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD) is a heterotetrameric (α2β2) thiamine diphosphate (TDP)-dependent enzyme. The recently solved human BCKD structure at 2.7 Å showed that the two TDP-binding pockets are located at the interfaces between α and β subunits and between α' and β subunits. In the present study, we show that the E76A-β' mutation results in complete inactivation of BCKD. The result supports the catalytic role of the invariant Glu-76-β' residue in increasing basicity of the N-α' amino group during the proton abstraction from the C-2 atom on the thiazolium ring. A substitution of His-146-β' with Ala also renders the enzyme completely inactive. The data are consistent with binding of the α-ketoacid substrate by this residue based on the Pseudomonas BCKD structure. Alterations in Asn-222-α, Tyr-224-α, or Glu-193-α, which coordinates to the Mg2+ ion, result in an inactive enzyme (E193A-α) or a mutant BCKD with markedly higher $K_m$ for TDP and a reduced level of the bound cofactor (Y224A-α and N222S-α). Arg-114-α, Arg-220-α, and His-291-α interact with TDP by directly binding to phosphate oxygens of the cofactor. We show that natural mutations of these residues in maple syrup urine disease (MSUD) patients (R114W-α and R220W-α) or site-directed mutagenesis (H291A-α) also result in an inactive or partially active enzyme, respectively. Another MSUD mutation (T166M-α), which affects one of the residues that coordinate to the K+ ion on the α subunit, also causes inactivation of the enzyme and an attenuated ability to bind TDP. In addition, fluorescence measurements establish that Trp-136-β in human BCKD is the residue quenched by TDP binding. Thus, our results define the functional roles of key amino acid residues in human BCKD and provide a structural basis for MSUD.

The human mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD)

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The abbreviations used are: BCKD or E1, branched-chain α-ketoacid dehydrogenase/dehydrogenase; E2, dihydrolipoyl transacetylase; E3, dihydrolipoamide dehydrogenase; FPLC, fast protein liquid chromatography; NTA, nitrotriacetic acid; KIV, α-ketoisovalerate; MSUD, maple syrup urine disease; TDP, thiamine diphosphate; TEV, tobacco-etch virus.

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Roles of Amino Acid Residues in Human BCKD

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids for Mutant His<sub>6</sub>-tagged BCKD—The Altered Site in vitro mutagenesis system (Promega, Madison, WI) was used to introduce desired mutations into the cDNA for the α or the β subunit. Detailed protocols for the mutant vector construction and subsequent mutagenesis were described previously (13). Briefly, oligonucleotides for the desired mutations and the β-lactamase repair primer were annealed to the single-stranded form of pAlter-α or pAlter-β vector. After the second strand synthesis and two rounds of ampicillin selection, clones harboring the correct mutations were isolated for plasmid preparation. DNA segments containing the mutations were used for cassette replacements of the expression vector pHis-TEV-E1 for wild-type BCKD (13).

Expression and Purification of Human His<sub>6</sub>-tagged BCKD—The recombinant His<sub>6</sub>-tagged BCKD heterotrimer was efficiently expressed in E. coli strain CG-712 (ES ts) by cotransformation of the pGroESL vector. After the second strand synthesis and two rounds of ampicillin selection, clones harboring the correct mutations were isolated for plasmid preparation. DNA segments containing the mutations were used for cassette replacements of the expression vector pHis-TEV-E1 for wild-type BCKD (13).

plasmid overproducing chaperonins GroEL and GroES as described previously (14, 15). Wild-type and mutant His<sub>6</sub>-tagged BCKD heterotrimers were isolated from cell lysates using a Ni<sup>2+</sup>-NTA-derivatized Sepharose CL-6B column (Qiagen, Chatsworth, CA) as described previously (16). BCKD proteins were further purified on a Superdex-200 gel filtration column (2.6 × 60 cm) connected to an FPLC system from Amersham Pharmacia Biotech. The column buffer consisted of 50 mM potassium phosphate, pH 7.5, 250 mM KCl, 10% (v/v) glycerol, 5 mM dithioerythritol, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. BCKD activity during purification was assayed spectrophotometrically (see below). Protein concentrations were determined using the Coomassie Plus protein reagent from Pierce with absorbance read at 595 nm. Alternatively, during enzyme purification, protein concentrations were determined by the direct measurement of absorbance at 280 nm using a calculated molar extinction coefficient of 1.15 cm<sup>−1</sup> mg<sup>−1</sup> for the αβ<sub>2</sub> heterotrimer.

Assays for BCKD Activity and Kinetic Studies—To determine K<sub>m</sub> for TDP, a radiochemical assay based on activity of the reconstituted BCKD complex was used (15). The rate of decarboxylation of 0.2 mM α-keto[1-<sup>14</sup>C]isovalerate ([1-<sup>14</sup>C]KIV) by BCKD in the presence of an excess of E2 and E3 was measured at varying concentrations of TDP. Double reciprocal plots were used to determine K<sub>m</sub> and V<sub>max</sub> values for cofactor TDP. For the determination of kinetic parameters for substrate KIV, a spectrophotometric assay, also according to the reconstituted BCKD complex activity, was employed. The assay mixture contained 50 mM potassium phosphate, pH 7.5, 100 mM NaCl, 3 mM NAD<sup>+</sup>, 0.4 mM Ca<sup>2+</sup>, 2 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 0.1% Triton X-100, 400 mM TDP, 7 mM reduced bovine E2, and 0.4 µM recombinant human E3 (17). The reduction of NAD<sup>+</sup> absorbance at 340 nm at different substrate concentrations was used to derive the k<sub>obs</sub> value. The plots observed the pseudo-first order decay. The double-reciprocal plots of k<sub>obs</sub> values versus KIV concentrations were used to determine K<sub>m</sub> and V<sub>max</sub> values for KIV.

Measurements of Amounts of TDP Bound to Wild-type and Mutant BCKD—The purified recombinant human BCKD was essentially devoid of TDP. The residual bound TDP was removed by exhaustive dialysis in the presence of 0.2 mM EDTA. The wild-type or mutant apo-BCKD (500 µg each) was incubated for 30 min with 150 µM Mg-TDP at 4 °C. The holo-BCKD was re-extracted with Ni<sup>2+</sup>-NTA resin and eluted with 100 mM imidazole. The BCKD-bound TDP was measured after oxidation to its fluorescent derivative thiochrome diphosphate in the presence of ferricyanide. The thiochrome diphosphate derivative was measured in a PerkinElmer Life Sciences model LS50B luminescence spectrometer.
Roles of Amino Acid Residues in Human BCKD

RESULTS AND DISCUSSION

Catalytic Residues for the TDP-mediated Decarboxylation—In human BCKD, residues that form each Mg-TDP cofactor binding pocket are derived from two separate subunits. Fig. 1 shows that residues from both the α and β subunits are involved in binding to TDP, and the Mg²⁺ ion is held in an octahedral coordination. This topology maintains TDP in a strained “V-shaped” conformation having torsion angles Φ₉ = 10°, and Φ₃ = -71° to facilitate the proton extraction from the C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5). Most of these residues are conserved among TDP-dependent enzymes including Glu-193-C-2 carbon (5).

| Enzyme | KIV | TDP |
|--------|-----|-----|
|        | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^3$ s⁻¹ μM⁻¹) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^3$ s⁻¹ μM⁻¹) |
| Wild type | 62 | 8.25 | 140 | 0.66 | 8.16 | 12,400 |
| E76A-β | 78 | 1.75 | 22 | 0.45 | 6.4 | 14,200 |
| S162A-α | 0 | 0 | 0 | 0 | 0 | 0 |
| H146A-β | 2,508 | 1.8 | 0.65 | 273 | 2.3 | 8.3 |
| S292D-α | 75 | 1.3 | 17 | 1.9 | 2.2 | 1150 |

Kinetic parameters of BCKD mutants affecting residues involved in TDP-mediated decarboxylation

| Enzyme | KIV | TDP |
|--------|-----|-----|
|        | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^3$ s⁻¹ μM⁻¹) | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^3$ s⁻¹ μM⁻¹) |
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| S292D-α | 75 | 1.3 | 17 | 1.9 | 2.2 | 1150 |

(Excitation wavelength, 375 nm; emission wavelength, 430 nm) as described previously (18).

Other Methods—Emission spectra over a range of 300–400-nm wavelengths for bound TDP were obtained using the luminescence spectrometer at an excitation wavelength of 280 nm as described previously (19). The quenching of tryptophan fluorescence by Mg-TDP was studied by adding increments of Mg-TDP to the cuvette containing apo-BCKD. The range of final Mg-TDP concentrations studied was 5–400 μM. Cycles of Mg-TDP addition and fluorescence measurements were repeated until the fluorescence quenched by the TDP bound to BCKD reached a plateau. Circular dichroism measurements were carried out on an AVIV (Lakewood, NJ) model 62 DS spectrometer.

The “isocaproate” moiety modeled into the His-146 β residue of human BCKD shows that the carboxylic oxygen is 4.3 Å away from the N-4’ amino group of TDP (Fig. 1). The data support the possible role of His-146 β in positioning a native ketoacid substrate (e.g. KIV) for the TDP-mediated decarboxylation. This point needs to be confirmed by co-crystallization of human BCKD with α-chloroisocaproate, which has not been successful to date. Recently, the structure of the Desulfovibrio africanus pyruvate:ferredoxin oxidoreductase in complex with pyruvate (23) demonstrates that a carboxylate oxygen from pyruvate interacts directly with the N-4’ amino group of TDP. Together, the data strongly imply that the polarization of the substrate carboxylic moiety by the N-4’ amino group of the cofactor is necessary for activation of the substrate.

Ser-292 α and Ser-302 α are site 1 and site 2 for phosphorylation of human BCKD by the specific kinase. Phosphorylation at site 1 results in inactivation of BCKD, whereas phosphorylation at site 2 is silent. Introduction of a negatively charged Asp residue in the S292D α mutant produces the same inactivation effect as phosphorylation at this residue (Table I). This result confirms an earlier study in which replacement of Glu-76 β with Ala (E76A β, Table I) renders BCKD completely inactive. The data establish Glu-76 β as an essential catalytic residue. On the other hand, the interaction between the carbonyl group of Ser-162 α and the N-4’ amino group is required to orient the latter for proton abstraction from the C-2 atom of the thiazolium ring. Since it involves a main-chain carbonyl group, a replacement with Ala is without effect on the catalytic efficiency for the TDP, although the same parameter for substrate KIV is significantly reduced.

His-146 β aligns with His-131 β in BCKD from P. putida. The latter residue was shown to form an adduct with a substrate analog, α-chloroisocaproate, in the crystal structure of the Pseudomonas enzyme (6). It has been proposed that binding of the substrate analog to His-131 β of the bacterial BCKD mimics a natural α-ketoacid substrate. The complete absence of enzyme activity in H146A β (Table I) is consistent with the role of this residue in binding substrate KIV in human BCKD.

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The side chain of Tyr-102 β is packed against one side of the aminopyrimidine ring of the cofactor with the side chain of...
Roles of Amino Acid Residues in Human BCKD

Leu-164-α approaching the other side of the ring, wedging in between the two rings of the cofactor (Fig. 1). The flanking of both sides of the aminopyrimidine ring by these two hydrophobic residues is important to orient the cofactor in the strained V conformation. Substitution of either residue with an Ala has adverse effects in the catalytic efficiency of the mutant enzymes (Table I). However, L164A-α is markedly more severely affected than Y102A-β. The data suggest that Leu-164-α is more important in providing the hydrophobic environment for the aminopyrimidine and possibly the thiazolium ring of the cofactor.

The amounts of TDP bound to His6-tagged wild-type and mutant BCKD were measured by incubating the enzymes with 150 μM Mg-TDP. Following Ni2+-NTA extraction, the bound TDP in BCKD incubated at saturating concentrations of the substrate and TDP, whereas the catalytic residues required for the TDP-mediated decarboxylation, A, mutants affecting residues that coordinate to the Mg2+ ion of TDP. C, mutants affecting residues that interact with diphosphate oxygens of TDP. D, mutants affecting residues in the K+ ion-binding site on the α subunit.

![Fig. 2. Stoichiometry of bound TDP in wild-type and mutant BCKD.](image)

Wild-type or mutant apo-BCKD (His6-tagged) at 500 μg each was incubated with 150 μM Mg-TDP for 30 min at 4 °C. The holoenzymes were extracted with Ni2+-NTA resin, and eluted proteins were oxidized with ferricyanide. The thiochrome-TDP resin, and eluted proteins were oxidized with ferricyanide. The thiochrome-TDP was determined in the form of thiochrome pyrophosphate by fluorescence emission at 430 nm following an excitation at 375 nm. The results are expressed as mol of TDP bound per mol of BCKD heterotetramers. A, mutants affecting catalytic residues required for the TDP-mediated decarboxylation. B, mutants affecting residues that coordinate to the Mg2+ ion of TDP. C, mutants affecting residues that interact with diphosphate oxygens of TDP. D, mutants affecting residues in the K+ ion-binding site on the α subunit.

Active Site Residues That Coordinate to the Mg2+ Ion—As shown in Fig. 1, the Mg2+ ion is octahedrally coordinated between the cofactor phosphates, the carbonyl of Tyr-224-α (not shown) and the side chains of Asn-222-α and Glu-193-α. A substitution of Glu-193-α with Ala results in complete loss of BCKD activity (Table II). An E193K-α mutation recently identified in an MSUD patient also renders the enzyme inactive (data not shown). The N222S-α mutation, which was identified in an MSUD patient, results in marked increases in Km for KIV and TDP, whereas the kcat values are one half of the wild-type. These data, taken together, indicate that the carbonyl groups in the side chains of Glu-193-α and Asn-222-α are critical for Mg2+ ion binding. The hydroxyl group of the Ser side chain in the N222S-α mutant is probably a poorer ligand than Asn for the Mg2+ ion. The O" of a Ser residue would not be able to extend as far toward the Mg2+ ion as the O1 of the Asn residue; this is likely to decrease affinity for the Mg2+ ion at this site. The less attenuated effect in affinity for KIV and TDP in the Y224A-α mutant, compared with the above two mutants is consistent with the fact that the main-chain carbonyl group of Tyr-224-α coordinates to the metal ion. The critical roles of Glu-193-α and Asn-222-α side chains in the metal ion binding is supported by the markedly diminished levels of bound TDP in the E193A-α, N222A-α, and Y224A-α mutants (Fig. 2B). The Y224A-α mutant also shows a markedly decreased ability to
bind TDP despite an only moderate increase in $K_m$ for the cofactor.

**Residues That Interact with Diphosphate Oxygenes of TDP**—
Gln-112-$\alpha$ and Tyr-113-$\alpha$ are hydrogen-bonded to the proximal and the terminal phosphate oxygenes of TDP, respectively (5). Substitution of Gln-112-$\alpha$ with an Ala residue has marginal effects in catalytic efficiency of BCKD as well as $K_m$ values for KIV and TDP (Table III). The data suggest that either the side chain of the Gln-112-$\alpha$ is not an essential ligand to the Mg$^{2+}$ ion or that a neighboring residue(s) can fill in this function. The introduction of an Ala residue into the Tyr-113-$\alpha$ position results in more than 50- and 100-fold increases in $K_m$ for KIV and TDP, indicating that Tyr-113-$\alpha$ is an important ligand to the terminal phosphate oxygen. Remarkably, a replacement of Arg-114-$\alpha$ or Arg-220-$\alpha$, which also coordinates to the same terminal phosphate oxygen (Fig. 1), with an Ala residue results in a completely inactive BCKD. The R114W-$\alpha$ mutation, which occurs in MSUD patients, also renders the enzyme completely inactive. As for Arg-220-$\alpha$, which coordinates to another distal phosphate oxygen, when changed to Ala, Lys or Trp (an MSUD mutation) also results in complete loss in enzyme activity. The combined results strongly suggest that the ionic interactions between positively charged Arg-114-$\alpha$ or Arg-220-$\alpha$ and the negatively charged phosphate oxygenes are critical in maintaining the conformational integrity of the diphosphate group of TDP (Fig. 1). In the case of R220K-$\alpha$, the positively charged Lys residue is pointing away from the distal phosphate oxygen in the BCKD structure. Therefore, the ionic interactions between the Lys side chain and the phosphate oxygen cannot occur.

His-291-$\alpha$ coordinates to another distal phosphate oxygen in the TDP-binding pocket (Fig. 1). Substitution of this residue with Ala results in a trace amount of BCKD activity (Table III). A previous report showed that the same mutation in the rat BCKD is associated with complete absence of activity (26); however, a spectrophotometric assay was used in the latter study, which is less sensitive than the radiochemical assay employed here. The combined results are thus consistent with His-291-$\alpha$ as an essential ligand to the diphosphate group of TDP. It is noteworthy that the $K_m$ for TDP with the His-291-$\alpha$ mutant is increased by 40-fold over the wild-type (Table III). This is consistent with about 15% of TDP bound to this mutant compared with the wild-type (Fig. 2C). Modifications of the three other residues Arg-114-$\alpha$, Arg-220-$\alpha$, and Gln-112-$\alpha$ that coordinate to the diphosphate oxygenes invariably yield marginal binding of TDP (Fig. 2C). The data

![Fig. 3. The K$^+$ ion-binding site on the $\alpha$ subunit of human BCKD. The metal ion is bound by two main-chain carbonyl groups and by the side chains of Ser-161-$\alpha$, Thr-166-$\alpha$, and Gln-167-$\alpha$. The side chain of Leu-164-$\alpha$ and the main-chain carbonyl group of Ser-162-$\alpha$ make direct contacts with cofactor TDP. The octahedral coordination of the metal ion stabilizes the loop structure on the $\alpha$ subunit (residues 161–167) that is essential for the efficient binding of the cofactor.](image-url)
strongly suggest that the amino acid ligands to the diphosphate moiety of TDP are as important as the ligands to the aminopyrimidine ring in maintaining the V conformation of the cofactor. It should be mentioned that in addition to being a ligand for the TDP diphosphate group, His-291-α has been implicated to function as a catalytic residue (6). As described above, BCKD functions as both a decarboxylase and a dehydrogenase during the oxidative decarboxylation of α-ketoads. His-291-α is proposed to serve as a proton donor during the reduction of the disulfide bond of the E2-attached lipoamide, which occurs during acyltransfer from the enamine-TDP to E2. In the H291A-α mutant, the reduction of lipoamide on E2 by the dehydrogenase activity of BCKD may be disrupted. The additional role of His-291-α as a proton donor to the E2-attached lipoamide will require further studies.

**Residues in the Novel K⁺ Ion-binding Site of the α Subunit**—The crystal structure of human BCKD disclosed that the α and the β subunits each has a distinct K⁺ ion binding fold that has not been described previously in any TDP-dependent enzyme (5). Fig. 3 shows that the K⁺ ion on the α subunit stabilizes a loop containing residues Ser-161-α, Thr-166-α, and Gln-167-α, which are directly involved in ligating to the metal ion through their side chains. The structural integrity of this loop structure is essential for ordering Ser-162-α and Leu-164-α for interactions of these residues with the cofactor TDP as described above. An MSUD mutation, T166M-α, is likely to ablate the coordination of the Thr side chain to the K⁺ ion, resulting in a disorder of this loop with a concomitant disruption of TDP binding. This accounts for the loss of both enzyme activity (Table IV) and the inability of the mutant enzyme to bind TDP (Fig. 2D). It is of interest that the replacement of Thr-166-α with an Ala has little effect on the catalytic efficiency of BCKD. It is possible that the side chain of Ala, which is shorter than that of Met, does not aberrantly protrude into the K⁺ ion binding pocket. As a result, the octahedral coordination is not severely impaired by this substitution. This is reflected by near wild-type catalytic efficiency of the T166A-α mutant when assayed at saturating TDP concentrations (Table IV). However, the binding affinity of the mutant enzyme for the cofactor is still reduced as indicated by an elevated $K_m$ for TDP (Table IV) and by a significant decrease in the amount of the bound cofactor compared with the wild-type (Fig. 2).

**Identification of the Trp Residue Quenched by Bound TDP**—One of the characteristics associated with TDP-dependent enzymes involves quenching of tryptophan fluorescence upon binding of the cofactor (19). This property has been used as a means to determine the level of the cofactor binding by TDP-dependent enzymes (22, 27). To identify the Trp residue quenched by BCKD-bound TDP, we replaced Trp-136-β which is the Trp residue closest to the aminopyrimidine ring of the cofactor at 15–18 Å, with a Phe. As a control, a distal Trp-309-α was also converted to a Phe. The W136F-β mutant BCKD showed $K_m$ values for KIV and TDP, which are similar to those for the wild-type (data not shown). Therefore, Trp-136-β appears to be nonessential for efficient binding of both the substrate and the cofactor. The aponozymes of the wild type, W136F-β, and W309F-α were excited at 280 nm. The fluorescence emission spectra of the wild-type and W309F-α apoBCKD were similar (Fig. 4). The maximal fluorescence emissions at 340 nm for both proteins are progressively quenched when titrated with increasing concentrations of Mg-TDP. In contrast, the maximal emission at 340 nm of the W136F-β mutant enzyme is not quenched over the same concentration range of Mg-TDP. The result established that Trp-136-β is the residue that is quenched upon TDP binding. In pyruvate decarboxylase from Z. mobilis, the Trp-487 was shown to also be quenched by the bound TDP (28). The crystal structure of this bacterial pyruvate decarboxylase shows that Trp-487 is 18 Å away from the aminopyrimidine of a chemically modified cofactor, 2-(1-hydroxyethyl)-TDP (10). Thus, the Trp residues in human BCKD and the bacterial pyruvate decarboxylase, which are quenched upon TDP binding, are in similar conformations relative to the cofactor. In the related pyruvate dehydrogenase, Trp-135-β was reported to be the residue that is quenched in

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**Table IV**

Kinetic parameters of BCKD mutants affecting residues involved in $K^+$ ion binding

| Enzyme | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (X10³) | $K_m$ (μM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (X10³) |
|--------|------------|----------------|----------------------|------------|----------------|----------------------|
| Wild type | 62 | 8.25 | 140 | 0.66 | 8.16 | 12,400 |
| T166A-α | 9.85 | 1.1 | 110 | 2.55 | 0.93 | 370 |
| T166M-α | 9.85 | 1.1 | 110 | 2.55 | 0.93 | 370 |

* Naturally occurring mutation in MSUD patients.
response to TDP binding using chemical modification and magnetic circular dichroism methods (29). Although the structure of the pyruvate dehydrogenase has not yet been determined, Trp-135-β in this enzyme aligns with the Trp-136-β in human BCKD. The data show the conservation of the quenchable Trp residue in TDP-dependent enzymes.

**Conclusion**—The present study defines the functional roles of active site residues in human BCKD, which catalyzes the oxidative decarboxylation of branched-chain α-ketoads. The results depict a high degree of conservation in the structure and function of the TDP binding fold in TDP-dependent enzymes. These enzymes include those that catalyze the TDP-mediated oxidative decarboxylation, such as human BCKD (5), and the TDP-mediated nonoxidative decarboxylation, such as yeast pyruvate decarboxylase (8) and benzoylformate decarboxylate (12), as well as those that promote the two-carbon transfer, for example, yeast transketolase (7), 30. Human BCKD is distinct among TDP-dependent enzymes in that the enzyme is both a decarboxylase and dehydrogenase. The enamine-TDP intermediate becomes a substrate for the acyltransfer reaction during the BCKD-mediated reduction of lipoamide covalently attached to the E2 core of the BCKD complex. The mechanism for the BCKD-catalyzed acyltransfer reaction remains to be elucidated at the structure level.

The human BCKD is tightly regulated by reversible phosphorylation/dephosphorylation (4). Residue Ser-292-α, which is responsible for phosphorylation and the resultant inactivation of BCKD, is located inside the TDP-binding pocket. From a structural standpoint, it is unclear how the specific BCKD kinase enters this TDP binding fold to phosphorylate Ser-292-α. However, the phosphorylation efficiency of BCKD is markedly enhanced when the enzyme is in complex with the E2 acyltransferase (31–33). It can, therefore, be speculated that binding of BCKD to E2 may induce conformational changes that render the TDP-binding pocket more accessible to the kinase. These questions will be addressed by solving the structure of human BCKD in complex with the binding domain of E2.

The utilization of the octahedrally coordinated K⁺ ion site to stabilize an essential loop structure in the TDP binding fold has not been described in other TDP-dependent enzymes. Both BCKD and pyruvate dehydrogenase require high concentrations of the K⁺ ion to stabilize enzyme activity (16, 31, 34). It is very likely that the novel K⁺-binding sites are conserved in the decarboxylase/dehydrogenase components of α-ketoacid dehydrogenase complexes. The presence of K⁺-ion-binding sites explains the dependence of high K⁺, but not Na⁺, ion concentrations on the inhibitions of BCKD phosphorylation by TDP (31). A survey of the literature shows that the *Pseudomonas* dialkyglycine decarboxylase also uses the octahedral K⁺ coordination to maintain the conformation of a loop structure in the cofactor pyridoxal phosphate binding fold (35, 36). This topology appears to be conserved among certain cofactor-dependent decarboxylases.
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