We showed previously that the rat branched-chain α-ketoacid dehydrogenase (BCKD) kinase is capable of autophosphorylation. However, despite its sequence similarity to bacterial histidine protein kinases, BCKD kinase does not function as a histidine protein kinase. In the present study, we report that the rat BCKD kinase exists as a homotetramer of 185,000, based on results of gel filtration and dynamic light scattering. This is in contrast to the related mammalian pyruvate dehydrogenase kinase isozymes that occur as homodimers. The tetrameric assembly of BCKD kinase was confirmed by the presence of four 5'-adenylylimidodiphosphate-binding sites ($K_D = 4.1 \times 10^{-6}$ M) per molecule of the kinase. Incubation of the BCKD kinase with increasing concentrations of urea resulted in dissociation of the tetramer to dimers and eventually to monomers as separated on a sucrose density gradient. Both tetramers and dimers, but not the monomer, maintained the conformation capable of binding ATP and undergoing autophosphorylation. BCKD kinase depends on a fully lipoylated transacylase for maximal activity, but the interaction between the kinase and the transacylase is impeded in the presence of high salt concentrations. Alterations of conserved residues in the ATP-binding domain led to a marked reduction or complete loss in the catalytic efficiency of the BCKD kinase. The results indicate that BCKD kinase, similar to pyruvate dehydrogenase kinase isozymes, belongs to the superfamily of ATPase/kinase.

The mammalian mitochondrial branched-chain α-ketoacid dehydrogenase (BCKD)$^1$ complex catalyzes the oxidative decarboxylation of the branched-chain α-ketoacid derived from leucine, isoleucine, and valine. The mammalian BCKD complex is a macromolecule ($M_r = 4 \times 10^6$) comprising multiple copies of five component enzymes. The three catalytic components are as follows: a thiamine pyrophosphate-dependent branched-chain α-ketoacid decarboxylase (E1) with two E1α ($M_r = 45,500$) and two E1β ($M_r = 37,500$) subunits; a dihydrolipoyl transacetylase (E2), which contains 24 lipoate-bearing polypeptides (monomer, $M_r = 45,000$); and a dihydrolipoyl dehydrogenase (E3) (monomer, $M_r = 55,000$) ($1$). In addition, the BCKD complex contains two regulatory enzymes, a specific kinase (2) and a specific phosphatase (3), which control enzyme activity through a reversible phosphorylation (inactivation)-dephosphorylation (activation) of E1 ($2, 3$). The BCKD complex is organized around the 24-mer dihydrolipoyl transacetylase cubic core ($M_r = 1.1 \times 10^5$), to which E1, E3, the kinase, and the phosphatase are attached through ionic interactions.

The regulation of the activity of the branched-chain α-ketoacid dehydrogenase complex through a kinase-mediated phosphorylation has been extensively studied. Rats fed low protein diets showed low hepatic enzyme complex activity (2). This is associated with a reduction in the activity state, or the percent of total enzyme in the dephosphorylated form, of the enzyme compared with rats fed a normal diet (2, 4). Starvation and diabetes stimulate activity of the BCKD complex in skeletal muscle by increasing the proportion of active or dephosphorylated enzyme complex (5, 6). Administration of glucagon or epinephrine was shown to result in a 4-fold increase in the percentage of active (dephosphorylated) enzyme complex in the liver of rats fed a low protein diet; somewhat lesser effects were observed with administration of insulin and cyclic AMP (7).

The deduced amino acid sequence of the rat BCKD kinase shows little similarity to mammalian serine/threonine protein kinases. Instead a much higher degree of sequence similarity exists between the BCKD kinase and members of the prokaryotic histidine protein kinase family ($8$). The subsequent cloning and characterization of the four isoforms of the pyruvate dehydrogenase (PDH) kinase support the notion that PDH kinases belong to the ATPase/kinase superfamily ($9, 10$). The crystal structure of CheA, a signal-transducing histidine protein kinase, has been recently solved at 2.8-Å resolution (11). The structure resembles GyrB and Hsp90 and, as expected, displays little similarity to serine/threonine/tyrosine kinases. The P1 domain of CheA provides the nucleophilic histidine and activated glutamate that are essential for phosphotransfer. The regulatory domain, which binds the homologous receptor, CheW, topologically resembles two SH3 domains. The dimerization domain forms a central 4-helix bundle about which the ATP-binding and the regulatory domains rotate to modulate transphosphorylation.

To understand structure and function of the mammalian BCKD kinase, we previously expressed and characterized the rat enzyme (12). We showed that the BCKD kinase is autophosphorylated at a serine residue in the conserved P1 region of histidine protein kinases. However, despite its sequence similarity to histidine protein kinase, the BCKD kinase does not

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† To whom correspondence should be addressed: Dept. of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75390

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

§ To whom correspondence should be addressed: Dept. of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, 5222 Harry Hines Blvd., Dallas, TX 75390-8889. Tel.: 214-648-8693; Fax: 214-648-8836; E-mail: wyns@utsw.wmed.edu

¶ The abbreviations used are: BCKD, branched-chain α-ketoacid dehydrogenase; ADP-PNP, 5′-adenylyl-β-γ-imidodiphosphate AMP-PNP; FFLC, fast protein liquid chromatography; MBP, maltose-binding protein; Nt-NTA, Ni$^{2+}$-nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; TEV, tobacco-etch virus.
utilize histidine to mediate phototransfer. In the present study, we further characterize the recombinant rat BCKD kinase. We provide evidence to indicate that, unlike dimeric PDH and CheA histidine protein kinases, the rat BCKD kinase is a homotetramer. Moreover, we carried out site-directed mutagenesis studies, which establish that residues in the ATP-binding domain of BCKD kinase are highly conserved in the context of the ATPase/kinase superfamily.

**EXPERIMENTAL PROCEDURES**

Construction of the His<sub>6</sub>-tagged BCKD Kinase Expression Plasmids—The previously prepared pMAL-TEV kinase vector (12) was treated with XbaI and EcoRI restriction endonucleases, and the resulting 1,182-base pair fragment was cloned into an NheI-EcoRI-treated pTrcHisB (Invitrogen) to generate the N-terminally His<sub>6</sub>-tagged pTrcHis kinase (pHisT kinase) expression vector. A C-terminally His<sub>6</sub>-tagged expression vector (pKinase-His) was also produced by PCR amplification of the pMAL-TEV kinase vector using a 31-mer sense primer (sequence 5′-AAACCATGGGCTCAATACGACCCAGCAG-3′) and a 52-mer antisense primer (sequence 5′-CGAATTCTAGATGATGATGATGACCCCCGATTCTGAAGCTTTCCTCC-3′); the sense primer introduced a unique Ncol restriction site (underlined) into the 5′-coding region and the antisense primer contained a unique EcoRI restriction site (underlined) at the 3′-noncoding region of the amplified product. The 1,371-base pair amplification product was purified and treated with Ncol-EcoRI restriction endonucleases, and the resulting 1,367-base pair fragment was cloned into an Ncol-EcoRI-treated pTrcHisB (Invitrogen) to generate the C-terminally His<sub>6</sub>-tagged pTrc kinase-His<sub>6</sub> expression vector (pKinase-His).

Protein Expression and Affinity Purification—All kinase proteins were expressed in *Escherichia coli* strain C12-71 co-transformed with the pGroESL plasmid for overexpression of GroEL and GroES as described previously (13). After a 12-h expression, cells were harvested by centrifugation at 4 °C and resuspended in 5 volumes (w/v) of a lysis buffer (100 mM potassium phosphate, pH 7.5, 500 mM KCl, 20 mM β-mercaptoethanol, 0.5% (v/v) Triton X-100, 2.0% (v/v) Tween 20, 0.2 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM EGTA) with 2.0 mg/ml of lysozyme added. After incubation on ice for 30 min, the cell lysate was sonicated using a Branson Sonifier model 450 (1-cm tip, power setting 6, duty cycle 60%, for 8 min) and subsequently clarified by centrifugation at 29,000 × g for 30 min at 4 °C. Purification of MBP kinase from clarified cell lysates by amylose affinity chromatography was performed as described previously (13). His<sub>6</sub>-tagged E1 dimers (85.5 kDa), human E3 (110 kDa), His<sub>6</sub>-tagged human E1 tetramers (171 kDa), MBP-tagged E1 (331 kDa), and GroEL (840 kDa) bovine E2 was expressed in *E. coli* strain XL1-Blue (Stratagene) and purified as described previously (16). For the preparation of lipoylated E2, the recombinant apoE2 protein was treated with the *E. coli* lipoyl ligase LplA and DL-6,8-thioctic acid (lipoic acid, Sigma) as described elsewhere (17).

Estimation of Molecular Masses by Dynamic Light Scattering—Dynamic light scattering measurements were carried out using a DP-801 DLS instrument from Protein Solutions (Charlottesville, VA) interfaced with a Mitsuba computer. Samples of N-terminally tagged His<sub>6</sub> kinase (1.0 mg/ml), His<sub>6</sub>-E1 (1.1 mg/ml), bovine serum albumin (5 mg/ml), and lysozyme (3 mg/ml) in 50 mM potassium phosphate buffer, pH 7.5, 250 mM KCl, 2% (v/v) glycerol, 1 mM dithiothreitol, and 1 mM EDTA were filtered through a Whatman membrane of 0.45-μm pore size, and manually injected into the instrument. Samples (250 μl) in the flow cell at 22 °C were illuminated with an incident beam from a 255-milliwatt, 790 nm solid-state laser. The photons scattered by protein molecules moving in the Brownian motion were collected at an angle of 90°. The distribution of diffusion coefficients derived from the autocorrelation function was determined using a method of non-negative least squares laplacian transformation (regularization) contained in the Dynamics 4.0 software package from the manufacturer. The hydrodynamic (Stokes) radius (*R<sub>D</sub>*) of the molecules was derived from the Stokes-Einstein equation (Equation 1).

\[
R_D = kT/6πηD
\]

where \( η \) and \( T \) are viscosity the Boltzmann constant, and the absolute temperature, respectively (18).

The corresponding molecular mass (\( M \)) was calculated according to Equation 2:

\[
R_D = \left(3π/4D\rho N_v\rho_v\right)^{1/3}
\]

where \( N_v \) is Avogadro’s number, \( v \) (0.73 cm<sup>3</sup>/g) is the partial specific volume of the protein, and \( h \) (1.3) is the hydration for soluble proteins.

**Fig. 1. FPLC gel filtration profile of His<sub>6</sub> kinase.** His<sub>6</sub> kinase was expressed in *E. coli* and partially purified by Ni-NTA extraction. The partially purified His<sub>6</sub> kinase was applied to a HiLoad Superdex 200 column (2.6 × 60 cm) in the Amersham Pharmacia Biotech FPLC system. The protein was eluted at a flow rate of 1.5 ml/min and collected in 5-ml fractions. The peak fractions of His<sub>6</sub> kinase were separated on a 12% SDS-PAGE gel followed by Coomassie Blue staining (Inset). The molecular mass markers used for calibration were ovalbumin (44 kDa), His<sub>6</sub>-tagged E1 dimers (85.5 kDa), human E3 (110 kDa), His<sub>6</sub>-tagged human E1 tetramers (171 kDa), MBP-tagged E1 (331 kDa), and GroEL (840 kDa).
Following the incubation, the samples were spun in a microcentrifuge, and the resin was washed with the appropriate urea-containing buffer. Samples bound to the resin were eluted with 10 mM Mg-ATP, and the eluted and flow-through fractions were analyzed by SDS-PAGE. To test its ability to undergo autophosphorylation, His6 kinase (100 µg) in 50 mM potassium phosphate, pH 7.5, 250 mM KCl, and 5% (v/v) glycerol was incubated at the indicated urea concentrations for 1 h at 23 °C. Autophosphorylation was initiated by the addition of 0.4 mM [γ-32P]ATP (specific radioactivity, 0.1 Ci/mmol) and allowed to proceed overnight at 4 °C. The samples were denatured and separated on SDS-PAGE followed by phosphorimaging.

**Kinetic Analysis of BCKD Kinase Activity**—The enzymatic assay for BCKD kinase was based on the kinase-mediated inactivation of E1. For determinations of kinase activity, recombinant proteins of E1 (50 µg/ml, 250 nM), lipoylated E2 (37.5 µg/ml, 34 nM), and MBP kinase (25 µg/ml, 125 nM) with various concentrations of Mg-ATP in 100 µl were incubated at 37 °C for 5 or 10 min. At different times, aliquots were collected and assayed for the BCKD complex activity using a reconstituted spectrophotometric assay in the presence of excess recombinant E2 and E3 (17). Residual BCKD activity were plotted against the time to derive the $k_{obs}$ values at different ATP concentrations. The double-reciprocal plots of $k_{obs}$ versus ATP concentrations were used to determine the $K_m$ and $V_m$ values.

**FIG. 2.** Molecular mass determination of His6 kinase by dynamic light scattering. The His6 kinase (1 mg/ml, □) sample (250 µl) was injected into a flow cell, and dynamic light scattering was measured. The Stokes radius was calculated as described under “Experimental Procedures,” using the software package Dynamic version 4.0. The computer calculated Stokes radius for lysozyme (3 mg/ml, △), bovine serum albumin (5 mg/ml, ○), and His6-tagged E1 (1.5 mg/ml, ▽) were plotted against sample number. The molecular mass markers used for the calibration were as follows: lysozyme (14 kDa), bovine serum albumin (66 kDa), and His6-tagged E1 (171 kDa). The molecular mass for the His6 kinase derived from its Stokes radius is 167 kDa.

**FIG. 3.** Scatchard plot analysis of AMP-PNP binding to MBP kinase. MBP kinase (7.5 µM) in a potassium phosphate buffer, pH 7.5, and 200 mM KCl was incubated with different concentrations (20, 25, 30, 40, and 80 µM) of AMP-PNP for 30 min at 4 °C. Each mixture was transferred to a CentriCon-30 concentrator (Mr 30,000 cut-off) and spun at 2,500 × g for 5 min. The concentration of AMP-PNP in the filtrate ([AMP-PNP]) was determined by $A_{259}$ nm, and the concentration of enzyme-bound AMP-PNP ([AMP-PNP]$_e$) was calculated according to $[AMP-PNP]_t - [AMP-PNP]_b$, where [AMP-PNP]$_t$ is the total ligand concentration.

**FIG. 4.** Sucrose density gradient centrifugation profiles and circular dichroism spectra of urea-treated His6 kinase. A, sucrose density gradient profile. His6 kinase in a potassium phosphate buffer, pH 7.5, was incubated at 23 °C for 30 min with various concentrations of urea as indicated. The incubation mixtures were subsequently applied to a 10–30% sucrose density gradient in the presence of the corresponding urea concentrations, followed by centrifugation at 210,000 × g for 15 h at 4 °C. Fractions (730 µl) from the sucrose density gradient were analyzed by SDS-PAGE and Coomassie Blue staining. B, circular dichroism spectra. MBP kinase (185 µg) was treated with varying urea concentrations as described in A. CD spectra (bandwidth 1.5 nm) were obtained at 23 °C with an Aviv (Aviv Associates, Lakewood, NJ) circular dichroism spectrometer (model 62DS). Spectra represent the average of three scans.
**RESULTS**

**Molecular Weight and Assembly State of His₆ Kinase**—The His₆ kinase of the rat BCKD complex with a His₆ tag and a tobacco-etch virus (TEV) protease site connected to the N terminus of the E1α subunit was expressed in *E. coli*. Following extraction with Ni-NTA resin, the enzyme was further purified by FPLC Superdex-200 gel filtration. An elution profile from the calibrated Superdex-200 column shows a single peak with a molecular weight of 185,000 (Fig. 1). Based on the calculated subunit \(M_r = 46,117\), the His₆ kinase exists as a homotetramer in solution. The apparent tetrameric assembly of His₆ kinase was further studied by dynamic light scattering. Fig. 2 shows the Stokes radius of the same His₆ kinase from eight different preparations was 185,000. The calculated number deviates from a frictional ratio of \(f/f_0 = 1.12\) for a hydrated sphere, suggesting that the tetrameric His₆ kinase has a slightly elongated quaternary structure.

**Tetrameric Assembly and Conservation of BCKD Kinase**

**Urea-induced Dissociation of His₆ Kinase Tetramers**—To study the assembly state of His₆ kinase further, the enzyme was incubated with increasing concentrations of urea at 23 °C for 1 h. The urea-treated samples were subjected to sucrose density gradient centrifugation in the presence of the same concentration of urea, and separated fractions were analyzed by SD-PAGE. As shown in Fig. 4A, His₆ kinase sedimented as a tetramer in up to 1 M urea. In 2 M urea, the enzyme existed almost exclusively as dimers. At 3 M urea concentration, the enzyme still occurred predominantly as dimers but began to dissociate into smaller monomeric species. His₆ kinase was studied by sucrose density gradient centrifugation. The enzyme migrated as a \(M_r = 180,000\) species on the gradient (10–30%) in the presence and absence of 10 mM ATP, ADP, or AMP-PNP (data not shown). These results, taken together, corroborate the notion that the His₆ kinase is a homotetramer. A fractional coefficient ratio \((f/f_0) = 1.26\) for the His₆ kinase was obtained also by dynamic light scattering. This number deviates from a fractional ratio of \(f/f_0 = 1.12\) for a hydrated sphere, suggesting that the tetrameric His₆ kinase has a slightly elongated quaternary structure.

**Stoichiometry and Affinity of ATP Binding to MBP Kinase**—To confirm the homotetrameric structure of the BCKD kinase, the number of ATP-binding sites on MBP kinase was determined by two separate methods. We first incubated MBP kinase with increasing concentrations of AMP-PNP for 30 min at 4 °C. The free ligand was separated from the bound by centrifugation in CentriCon-30 microconcentrators. Scatchard plot analysis indicates 3.7 mol of AMP-PNP bound per mol of MBP kinase (Fig. 3). The result is consistent with four AMP-PNP-binding sites in an MBP kinase tetramer. The same analysis gave rise to a \(K_D = 4.1 \times 10^{-6} \text{ M}\) for AMP-PNP binding to MBP kinase. In a parallel experiment, we found that 3.5 mol of \(\gamma^{35}\text{S}-\text{ATP}\) are bound to 1 mol of MBP kinase (data not shown). These data agree with four ATP-PNP-binding sites in each BCKD kinase tetramer.
dom-coiled monomers. The ability of urea-treated His6 kinase to bind Affi-Gel Blue resin was studied by circular dichroism. At up to 2 M urea concentration, His6 kinase maintained its α-helical conformation, similar to the native untreated enzyme, with minimal changes in molar ellipticity at 222 nm (Fig. 4B). The secondary structure began to unravel in 3 M urea, despite the dimeric assembly state of the enzyme. At 4 M urea concentration, His6 kinase completely existed as random-coiled monomers. The ability of urea-treated His6 kinase to bind ATP was then analyzed by SDS-PAGE and Coomassie Blue staining. A fixed concentration of MBP kinase (0–2 μM) with [γ-32P]ATP, and 12.5 mM (tetramer) S302A His6-E1 (a phosphorylation site 2 mutant) in the absence or the presence of unlipoylated or lipoylated E2 (3.3 μM each, 24-mer). Aliquots of the reaction mixtures were subjected to SDS-PAGE. Radioactivity incorporated into the His6-E1 band was determined by Phosphorimager analysis. A, MBP kinase alone; ⋄, MBP kinase and unlipoylated E2; ⋄, MBP kinase and lipoylated E2. B, autophosphorylation of MBP kinase. The experimental conditions and symbols are as described in A, except that 32P incorporation into the MBP kinase band was determined.

Concentrations of urea completely retained its capacity in autophosphorylation (Fig. 5B). At 3 M or higher urea concentration, where His6 kinase lost its ability to bind ATP, the enzyme was not autophosphorylated.

Requirement of Lipoylated-E2 for Optimal E1 Phosphorylation by MBP Kinase—The availability of recombinant bovine E2 facilitated studies on the effects of apo and lipoylated E2 on kinase activity. MBP kinase was used in these studies based on its higher solubility than the untagged kinase. Moreover, the presence of the MBP moiety doubled the size of the kinase subunit, allowing the separation of autophosphorylated MBP kinase (Mr = 86,000) subunit from the phosphorylated E1α (Mr = 45, 500) subunit on SDS-PAGE. A fixed concentration of human E1 (12.5 μM) was incubated with increasing concentrations of MBP kinase (0–2 μM) with [γ-32P]ATP in the presence or absence of bovine apoE2 or lipoylated E2 (3.3 μM). At the end of incubation, the radiolabel incorporated into E1 or MBP kinase was analyzed by SDS-PAGE and phosphorimaging. Fig. 6A shows that the presence of apoE2 results in 2-fold increase in the rate of 32P incorporation into E1α subunit over the MBP kinase alone. Incubation with lipoylated E2 resulted in a 4.3-fold increase compared with the absence of lipoylated E2. The data established that BCKD kinase requires lipoylated E2 for efficient phosphorylation of E1, similar to PDH kinases. The highest 32P incorporation into E1α subunit was obtained at the molar ratio of MBP kinase:E1:lipoylated E2 = 2:12.5:3.3. This stoichiometry was used throughout subsequent kinetic studies with MBP kinase. As shown in Fig. 6B, MBP kinase also catalyzes autophosphorylation in proportion to the enzyme concentration. However, in contrast to phosphotransfer to E1, autophosphorylation of MBP kinase is not affected in the presence of apoE2 or lipoylated E2.
Adverse Effects of Increased Salt Concentrations on Kinase Activity—The above kinetic studies establish that interactions between E1, E2, and MBP kinase are essential for maximal kinase activity. We next investigated the effects of salt concentrations on these interactions. As suspected, increasing ionic strengths inhibit MBP kinase activity, with a complete loss of kinase activity occurring at 217 mM salt concentration (Fig. 7). At this salt concentration, the MBP kinase is dissociated from the E1 and E2, as determined by Ni-NTA extraction and SDS-PAGE (Fig. 7, inset). This dissociation disrupts the interaction of MBP kinase with E1 and E2, resulting in the inability of the kinase to phosphorylate E1.

Site-directed Mutagenesis of Conserved Residues in the ATP-binding Domain—As described above, the sequence in the ATP-binding domain is highly conserved between BCKD kinase and bacterial histidine protein kinases (20), and the crystal structure of CheA, a histidine kinase from *Thermotoga maritima*, was recently solved (11). Fig. 8 shows the sequence alignment in the ATP-binding domain among BCKD kinase, CheA, and other bacterial histidine protein kinases. Residues in BCKD kinase designed for site-directed mutagenesis Asn-249, Asp-285, Tyr-301, and Gly-339 are located in the conserved N box, the G1 box, helix 6, and the G2 box, respectively. These residues except Tyr-301 are invariant in this superfamil of ATPases. As shown in Table I, the N249A and D285A resulted in a complete loss of MBP kinase activity. The Y301A and G339A substitutions caused drastic decreases in affinity for ATP and $k_{cat}$ compared with the wild-type MBP kinase. This resulted in a markedly lower catalytic efficiency $k_{cat} / K_m$ of both Y301A and G339A mutants than the wild-type kinase. N249A and D285A substitutions inactivate kinase activity completely. The data indicate that Asn-249, Asp-285, Tyr-301, and Gly-339 are essential active site residues in the conserved ATP-binding domain of the BCKD kinase.

**TABLE I**

| Amino acid residue | $K_m$ (ATP) | $k_{cat}$ | $k_{cat}/K_m$ |
|--------------------|-------------|-----------|--------------|
| Wild type          | 9.1         | 4.16      | 0.45         |
| N249A              | 0           | 0         | 0.0006       |
| D285A              | 0           | 0         | 0.0006       |
| Y301A              | 255         | 0.17      | 0.0006       |
| G339A              | 136         | 0.14      | 0.001        |

**DISCUSSION**

The present study was undertaken to shed further light on structure and function of the rat BCKD kinase. The mammalian BCKD kinase has been highly purified from rat liver (21) and bovine kidney (22). However, the assembly state of the native mammalian kinase has never been shown. In our earlier studies, the MBP kinase expressed in *E. coli* appeared to exist as a homotetramer. Since the related PDH kinases, with 30% sequence identity to BCKD kinase, were shown to be dimeric proteins, we suspected that the apparent homotetrameric assembly of BCKD kinase might have been an artifact caused by the presence of an MBP ligand. In the present study, we replaced the MBP moiety with a His6 tag, and we showed that the His6 kinase clearly migrated as a tetrameric species during FPLC gel filtration (Fig. 1) and sucrose density gradient centrifugation (Fig. 4A). In addition, dynamic light scattering measurements confirm that the His6 kinase has a Stokes radius corresponding to a non-spherical 180-kDa protein. The results clearly indicate that BCKD kinase is a homotetramer, which occurs irrespective of the presence of an MBP ligand or a His6 tag. The tetrameric assembly of BCKD kinase is further supported by the presence of four ATP-binding sites as measured by AMP-PNP and $\gamma^{35}$S-ATP binding. The presence of ATP apparently has no effect on the tetrameric assembly of BCKD kinase during sucrose density gradient centrifugation. This is in variance with dodecamer trimeric GroEL, which tends to dissociate in the presence of ATP on sucrose density gradients (23). Thus, our data unequivocally show that...

2 R. Max Wynn, J. L. Chuang, C. D. Cote, and D. T. Chuang, unpublished observations.
the rat BCKD kinase, unlike PDH kinases, is a homotetrameric protein.

The urea-induced dissociation of His<sub>6</sub> kinase into stable homodimers is also consistent with the tetrameric assembly of the enzyme. It is of interest that the dimer maintains its secondary structure, as analyzed by circular dichroism, and is capable of binding ATP to catalyze autophosphorylation of the enzyme. The data suggest that the dimer possesses the conformation that is necessary and sufficient for ATP binding. The capacity of the dimer to mediate phosphotransfer to E1 was not studied, because the presence of 2 M urea required to maintain the dimeric state of the kinase impedes the assembly of E1. The kinase dimer does not occur in the absence of a denaturant and presumably possesses exposed hydrophobic surfaces that need to be shielded from the solvent through the assembly of two dimers.

The difference in the assembly state between PDH kinases and the BCKD kinase may have important implications in catalysis between these two highly conserved mitochondrial kinases. PDH kinases have been shown to depend on lipoylated PDC-E2 for efficient phosphorylation of PDC-E1 (24). Each of the PDC-E2 chains contains an outer and an inner lipoyl domain. The PDH kinases were shown to bind to the inner lipoyl domain of PDC-E2 (26), and the reductive acetylation of the inner lipoyl domain further augments the kinase activity (25). A "hand-over-hand" model has been proposed in which both subunits of the PDH kinases interact with different inner lipoyl domains by repeated dissociation and reassociation, thereby allowing the kinase to move along the surface of the 60-meric PDC E2 core (26). It was suggested that this mechanism facilitates the efficient phosphorylation of PDC E1 by the limited number of the kinase molecules on the E2 dodecahedron of PDC (27). In the present study, we show that the BCKD kinase also depends on lipoylated E2 for efficient phosphorylation of BCKD E1, similar to PDH kinases. The necessary interactions between BCKD kinase and E2 are disrupted by high salt concentrations, resulting in a marked reduction in phosphorylation of E1. However, unlike PDC-E2, the BCKD-E2 has a 24-meric cubic structure with a single lipoyl domain, which is equivalent to the inner lipoyl domain of PDC-E2. If the hand-over-hand movement of BCKD kinase is to occur along the surface of the E2 cube, the model has to take into account the presence of four binding sites for the lipoyl domain in the kinase tetramer. At present, it is not known how many lipoyl domains bind to the single tetramer of BCKD kinase. If one assumes that the four sites in the BCKD kinase have equal affinity for the lipoyl domain, the tetrameric structure may have evolved to allow the kinase to efficiently reach dispersed E1 molecules and lipoyl domains on the faces of the BCKD-E2 cubic core. Our preliminary data indicate that 2–3 molecules of BCKD kinase are needed to phosphorylate 12 molecules of E1 per E2 core (not shown). The results support the movement of the kinase on the surface of the E2 cube.

The ATP domain are highly conserved between the BCKD kinase and members of the ATPase/kinase superfamily of bacterial histidine protein kinases, as shown by the presence of regions of similar amino acid sequences N, G1, F, and G2 boxes (Fig. 8) (20). As seen in the recently solved CheA structure (11), the back wall of the ATP-binding domain is formed by four β-sheet strands (β3–β6). The side elements consist of three helices (α4, α7, and α8) as well as two loops. Amino acid side chains of Asn-249, Asp-285, and Gly-339 in BCKD kinase, which are located in N, G1, and G2 boxes, respectively (Fig. 8), are modeled into the CheA structure. B, conformation of the MgATP-binding pocket based on the E. coli GyrB (28). Graphics were prepared using Gl render in the Swiss PDB-Viewer program and POV-ray for Windows 3.1.

The G1 box in the BCKD kinase contains the ATP-binding motif (\(^{25D}XGXG^{269}\)), which is present in histidine protein...
kinase CheA and GyrB (Fig. 8). The F box is less conserved in the BCKD kinase when compared with CheA or GyrB. In the CheA structure, the back wall of the ATP-binding domain is mainly hydrophobic except for two conserved Asp and Asn residues. These two residues are also conserved in the BCKD kinase sequence, i.e. Asp-285 and Asn-249 (Fig. 8). By analogy with the x-ray structure of the complex between GyrB and ADPnP, an analog of ATP, Asp-285 in the BCKD kinase is likely to hydrogen-bond to the adenine amine, whereas Asn-249 putatively coordinates to the Mg²⁺ ion, which in turn binds to the three phosphate oxygen atoms (Fig. 9B). Substitution of either residue with an Ala results in complete loss of kinase activity (Table I). The data confirm the essential role of Asp-285 and Asn-249 in ATP binding of the BCKD kinase. Tyr-301, which is distal to the G1 box, is conserved between BCKD and PDH kinases. Replacement of this residue with an Ala also resulted in a marked increase in the $K_m$ (ATP) (28-fold) (Table I). The data indicate that Tyr-301 is also involved in ATP binding of the BCKD kinase; however, its exact role is less clear. By analogy with the crystal structure of GroEL $\gamma$-S-ATP complex (29), it is possible that the hydroxyl group of Tyr-301 is also hydrogen-bonded to a phosphate oxygen of the ATP. Asn-249 in the N box and Asp-285 in the G1 box of BCKD kinase correspond to Asn-247 and Asp-282 in PDH kinase 2, respectively. Recent studies with PDH kinase 2 showed that substitution of Asn-247 and Asp-282 with an alanine also results in a complete inactivation of PDH kinase 2 activity (9). These results, taken together, strongly indicate that both BCKD and PDH kinases belong to the ATPase/kinase superfamily. The precise roles of the above residues in ATP binding must be confirmed by the solution of the crystal structure of the complex formed between BCKD kinase and an ATP analog.

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