Roles of Amino Acid Residues Surrounding Phosphorylation Site 1 of Branched-chain \(\alpha\)-Ketoacid Dehydrogenase (BCKDH) in Catalysis and Phosphorylation Site Recognition by BCKDH Kinase*

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John W. Hawes, R. Jason Schnepf, Anne E. Jenkis, Yoshiharu Shimomura, Kirill M. Popov, and Robert A. Harris

From the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202-5122

Branched-chain \(\alpha\)-ketoacid dehydrogenase is regulated by reversible phosphorylation of serine 293 (site 1) on the E1\(\alpha\) subunit. Alanine-scanning mutagenesis was used to examine the roles of residues surrounding serine 293 in catalysis by the dehydrogenase and in substrate recognition by branched-chain \(\alpha\)-ketoacid dehydrogenase kinase. Alanine substitution of serine 293 resulted in a 10-fold increased \(K_m\) for \(\alpha\)-ketoisovalerate, a less increased (2.8-fold) \(K_m\) for \(\alpha\)-ketoisocaproate, but no change in \(V_{\text{max}}\) or the \(K_m\) for thiamine pyrophosphate. Alanine substitutions of arginine 288, histidine 292, and aspartate 296, residues highly conserved among \(\alpha\)-ketoacid dehydrogenases, resulted in inactive enzymes. Each of the inactive E1 mutants bound to the E2 core subunit with equal affinity as wild-type E1, and each produced circular dichroism spectra identical to that of wild-type E1. Two mutations, H292A and S293E, abolished the ability of E1 apoenzyme to reconstitute with thiamine pyrophosphate. Each alanine-substituted E1 was phosphorylated at site 1 by branched-chain \(\alpha\)-ketoacid dehydrogenase kinase with similar rates, with the exception of the R288A mutant, which displayed no detectable phosphorylation. Thiamine pyrophosphate inhibited the phosphorylation of all mutant enzymes with the exception of H292A, the mutant E1 that did not bind thiamine pyrophosphate.

The activity state of mammalian branched-chain \(\alpha\)-ketoacid dehydrogenase (BCKDH)

site 1, serine 293 in rat E1\(\alpha\) (3–5). Furthermore, phosphorylation of site 2 occurs at a significantly slower rate (3–5), suggesting that the site 2 serine, serine 303 in rat E1\(\alpha\), is a poor substrate either due to the primary or secondary structure surrounding this residue or the positioning of this serine residue relative to the kinase active site. BCKDH kinase is regulated in part through inhibition by branched-chain \(\alpha\)-ketoacids and thiamine pyrophosphate, all of which are substrates for the BCKDH-catalyzed reaction (6, 7). Inhibition of the kinase by branched-chain \(\alpha\)-ketoacids is believed to occur in vivo, resulting in a fully active BCKDH in the liver of rats fed a high protein diet (2). However, the exact molecular basis for these inhibitory effects is not clearly understood. BCKDH kinase has also been reported to be activated by its interaction with the E2 subunit of the BCKDH complex, although the molecular basis for this mode of regulation is not understood (8, 9). Another important feature of BCKDH kinase is its high degree of substrate specificity, resulting in phosphorylation only of BCKDH E1\(\alpha\) but not the highly homologous E1\(\alpha\) subunit of pyruvate dehydrogenase (PDH). The amino acid sequences surrounding the phosphorylation site 1 serine residues of BCKDH and PDH are approximately 50% conserved. Whether the specificity of BCKDH kinase or any part of its catalytic mechanism requires a specific part of this sequence is currently not known. The recent development of a dicistronic expression system for recombinant E1 subunit of BCKDH (5, 10) provides a means for the analysis of the interaction of BCKDH kinase with reconstituted BCKDH complex containing specific mutant forms of E1. The present study utilizes alanine-scanning mutagenesis to examine the roles of amino acid residues surrounding serine 293 of rat BCKDH E1\(\alpha\) in dehydrogenase activity and in phosphorylation site recognition by BCKDH kinase. Site-directed mutagenesis of protein kinase substrates has been successfully used in several studies of phosphorylation site recognition by cytosolic serine/threonine protein kinases and tyrosine protein kinases (11). The majority of these kinases have been found to require in their substrates the presence of specific amino acid functional groups such as acidic, basic, or hydrophobic residues at defined locations either N-terminal or C-terminal to the phosphorylation site serine. Our data indicate that BCKDH kinase has a strict requirement for the arginine residue present at the position –5 relative to serine 293 of E1\(\alpha\).

EXPERIMENTAL PROCEDURES

Materials—The pET28a expression vector and the HMS174(DE3) Escherichia coli cell line were from Novagen, Inc. (Madison, WI). Ni\(^{2+}\)–NTA resin was from Qiagen Inc. (Chatsworth, CA). All restriction enzymes were from Life Technologies, Inc. The site-directed mutagenesis kit was from Amersham Corp. The DNA sequencing kit was from United States Biochemical Corp. The pBlueScript II SK—plasmid, VCSM13 helper phage, and the TG1 E. coli cell line were from Strat-
agene Cloning Systems (La Jolla, CA). γ-[32P]ATP was from DuPont NEN. All other chemicals were from Sigma. BCKDH E2 was purified from rat liver according to previously published methods (12).

Plasmids—The pGroESL plasmid was kindly supplied by Dr. Anthony Gatbyen (Central Research and Development, DuPont). The construction of the diol stranded expression vector for production of recombinant rat BCKDH E1 was exactly as previously reported (15). "His-tagged" vector for prokaryotic expression of rat BCKDH kinase was constructed as follows. The original rat BCKDH kinase cDNA was ligated to the EcoRI sites of pBluescript II SK−. Single-stranded DNA was produced by helper phage rescue procedures according to the manufacturer's protocol (Stratagen). In order to remove the DNA coding for the polylinker, digestion, SfiI was used in combination with NotI to add an SfiI restriction site just prior to the first codon of the mature protein reading frame. Mutant plasmids were analyzed by restriction analysis, and the cDNA containing the mature protein reading frame was isolated by treatment with SfiI and NotI. This 2-kilobase pair cDNA fragment was ligated to the SfiI and NotI sites of pET28a (Novagen). The reading frame of this construct was verified by double-stranded DNA sequencing.

Site-directed Mutagenesis—Single-stranded S303A pET-E1 was produced by VCSM13 helper phage rescue according to the manufacturer's protocol (Stratagen). Each alanine substitution was produced using single-stranded cDNA containing the S303A mutation (5) in order to limit subsequent phosphorylation to serine 293 only. Synthetic oligonucleotides were designed to direct the synthesis of the desired mutations and were synthesized by the Biochemistry Biotechnology Facility of Indiana University School of Medicine. Site-directed mutagenesis was performed using the Sculptor system according to the manufacturer's protocol (Amersham Corp.). Each mutant pET-E1 plasmid was analyzed by polymerase chain reaction amplifications specific for rat E1 and rat E2 to insure the integrity of the diol stranded plasmids, and by single-stranded DNA sequencing.

Recombinant Enzyme Purification—Recombinant E1 was expressed and purified exactly as described previously (5). Recombinant BCKDH kinase was expressed and purified as follows. HMS174 (DE3) E. coli cells were double-transformed with the pET-S303A (kanamycin-resistant) and the pGroESL plasmid (chloramphenicol-resistant). Double transformants were selected by growth at 37 °C in TY media containing 70 μg/ml of both kanamycin and chloramphenicol. When the cell cultures obtained an optical density of 0.8, isopropyl-1-thio-D-galactopyranoside was added to a final concentration of 0.5 mM, and growth of the cultures was continued for 18–20 h at 37 °C. The induced bacterial cultures were pelleted, then resuspended in 10 mM HEPES-KOH, pH 8.0, 0.5 mM NaCl, 10 mM 2-mercaptoethanol, 5 mM imidazole containing 0.5% Triton X-100, and 100 μM benzamidine and phenylmethanesulfonyl fluoride. The cells were lysed by sonication with a Sonifier Cell Disruptor W185 (Branson Sonic Power Co., Plainview, NY). The resulting homogenate were centrifuged at 20,000 × g for 30 min at 4 °C, and the supernatant was removed. The remaining 1.5 ml of Ni2+-NTA-agarose (Qiagen Inc.) previously equilibrated in buffer A. Following sample loading the columns were washed successively with three volumes of buffer A containing 20 mM imidazole, 40 mM imidazole, and 60 mM imidazole, followed by elution of the kinase with buffer A containing 200 mM imidazole. The BCKDH kinase expressed and purified by this procedure appeared to be phosphorylated as isolated from E. coli and was dephosphorylated by treatment with broad-specificity protein phosphatase isolated from bovine heart (6). The phosphatase-treated BCKDH kinase was repurified by precipitation with ammonium sulfate (75% saturation) followed by Ni2+-NTA agarose chromatography as described above.

BCKDH Activity and Thiamine Pyrophosphate Binding Capacity of Mutant BCKDH E1s—To examine the relative importance of residues surrounding phosphorylation site 1 of BCKDH E1, each residue spanning from Arg-288 to Ser-298 was mutated to alanine. Each of these alanine mutations was produced with Ser-303 also mutated to alanine in order to limit subsequent phosphorylation to serine 293 only. The S303A mutant E1 was previously shown to be enzymatically active and kinetically indistinguishable from wild-type E1 (5). The double mutant S293A/S303A, containing both site 1 and site 2 alanine substitutions, was catalytically active with no change in maximal velocity or the Km for thiamine pyrophosphate but displayed a 10-fold increase in Kcat for α-ketoisovalerate, resulting in a 10-fold decrease in catalytic efficiency as judged by Kcat/Km (Table I). This finding is in agreement with the previously reported single mutant, S293A (5). The activity with this mutant was also analyzed with α-ketoisocaproate as the ketoacid substrate. Interestingly, the alanine substitution at position 293 produced only a 2.8-fold increase in the Km for this α-ketoisocaproate (68 ± 15 μM for wild-type and 190 ± 25 μM for S293A). As with α-ketoisovalerate, the Vmax and Kcat values were not changed by the S293A mutation when α-ketoisocaproate was used as substrate (4.8 units/mg protein and 12.8 s−1, respectively, for wild-type; 5.0 units/mg protein and 13.2 s−1, respectively, for S293A). Thus the Kcat/Km values with α-ketoisocaproate as substrate were 0.188 and 0.067, respectively, for wild-type and S303A, indicating a 2.8-fold decrease in the catalytic efficiency with this substrate.

Further alanine substitutions were produced for each residue located at positions 288–298 relative to Ser-293. Of these mutations, seven produced enzymes that were similar to wild-type in their catalytic properties (Table I). However, three specific alanine substitutions, R288A, H292A, and D296A, produced enzymes that were completely inactive under all conditions tested (Table I). All of these mutant E1 forms were expressed and purified with an equal ratio of E1α and E1β subunits, suggesting proper formation of the E1 complex in spite of mutations in the E1α component.

Measurements of the amount of thiamine pyrophosphate showed that each of the E1 mutants, as well as wild-type enzyme, were initially produced as apoenzymes containing no measurable cofactor. Whether these enzymes are expressed in E. coli as apoenzymes or our purification procedure, consisting of Ni2+-chelation chromatography and polyethylene glycol precipitation, results in the production of apoenzyme is not known. Nevertheless, it was found that the control recombinant E1 as well as the R288A and D296A mutant enzymes were readily reconstituted with thiamine pyrophosphate. Thus, incubation of E1 (2 mg) with 200 μM thiamine pyrophosphate and MgCl2 followed by removal of unbound cofactor by gel filtration chromatography resulted in stoichiometries of 1.7, 1.8, and 1.7 nmol of thiamine pyrophosphate/nmol of E1 for control, R288A, and D296A, respectively. Two mutations that are contiguous in
sequence. H292A and S293E, produced enzymes which were unable to reconstitute with thiamine pyrophosphate, remaining as apoenzymes after incubation with cofactor and MgCl₂ (≤ 0.05 nmol of cofactor/nmol of E1). This result suggests that these mutations specifically affect thiamine pyrophosphate binding, thereby explaining their lack of enzymatic activity. Previously, the S293E mutant E1 was reported to be enzymatically inactive but reconstituted normally with E2 (5), but this mutant had not been analyzed with respect to binding of thiamine pyrophosphate.

Phosphorylation of Alanine-substituted BCKDH E1s by BCKDH Kinase—Most protein kinases studied to date require the presence of specific amino acid functional groups, such as hydrophobic or charged side chains, at specific locations either N-terminal or C-terminal to the site of phosphorylation (11). To analyze the possible roles of any amino acid side-chain surrounding Serine 293 in phosphorylation site recognition by BCKDH kinase, each mutant E1 produced for this study was tested as substrate for phosphorylation of serine 293 by recombinant BCKDH kinase. As shown in Table II, all alanine substitutions in the range of −4 to +5 resulted in E1 mutants, which were phosphorylated with equal efficiency as compared to S303A as control, with no significant change in either $V_{\text{max}}$ or $K_m$ values. Since aspartate residues are present at both positions +3 and +4 relative to serine 293, a triple mutant (D296A/D297A/S303A) was also produced to remove all acidic residues from this region. This mutant was also phosphorylated with equal efficiency as the single aspartate mutants (data not shown), indicating that no acidic residues C-terminal to Ser-293 are required for the interaction of E1 with BCKDH kinase. In contrast, one alanine substitution, R288A/S303A, completely abolished phosphorylation of E1 by BCKDH kinase, even when the mutant E1 was present at relatively high concentrations (Table II). Thus, arginine 288 appears to be critical to the interaction of BCKDH kinase with the phosphorylation site 1 of BCKDH. The lack of phosphorylation of the R288A mutant E1 was confirmed when analyzed by SDS-PAGE (Fig. 1). Each of the mutant E1s with alanine substitutions in the range of −4 to +5 relative to Ser-293 were found to be phosphorylated to the same extent, with stoichiometries between 0.9 and 1.1 nmol of phosphate incorporated/nmol of E1 (Fig. 1). That no other serine residues other than Ser-293 were phosphorylated is indicated by the stoichiometry as well as by the absence of phosphorylation in the S293A/S303A mutant E1.

Inhibition of BCKDH kinase by the thiamine pyrophosphate cofactor of E1 was analyzed using several mutant E1s as substrates. The phosphorylation of S303A and D296A/S303A mutant E1s was dramatically inhibited with an IC₅₀ of approximately 1.0 µM, and 90% inhibition at 12 µM (Fig. 2). Phosphorylation of H292A mutant E1 was not affected by thiamine pyrophosphate in this concentration range, consistent with the lack of cofactor binding by this mutant.

E2 Binding—Binding of mutant E1 to the E2 core subunit of BCKDH was analyzed by inhibition of reconstitution of BCKDH activity with wild-type E1 and native E2. In these
that it may be positioned in a site important for this residue does not have a crucial catalytic role and suggests phorylation of site 2, serine 303 in rat BCKDH kinase activity was measured as described under “Experimental Proce­dures.” Each value is the average of three determinations. □, control; ●, H292A; △, D296A.

approximately 50% inhibition was observed for each mutant with an equal molar ratio of mutant to wild-type E1, suggesting equal affinity for the E1 binding site on E2.

Circular Dichroism Spectropolarimetry—Each of the inactive mutant E1s, R288A, H292A, and D296A, produced circular dichroism spectra identical to that of the control E1, suggesting that no dramatic changes in secondary structure occurred as a specific result of these three mutations. Estimation of secondary structure contents by comparison to a library of peptides with defined structures (17) revealed a high degree of secondary structure. By this method, each of the E1 forms analyzed was predicted to contain 39% a-helix, 42% b-sheet, and 19% other structures.

**DISCUSSION**

Little is known about the mechanism responsible for inactivation of BCKDH by phosphorylation. It is known that phosphorylation of site 2, serine 303 in rat E1a, is silent with respect to regulation of BCKDH activity (3–5). Furthermore, mutations of serine 293, the phosphorylation site 1, result in kinetically altered enzymes (5). Mutation of serine 293 to glutamate results in complete loss of enzymatic activity (5), suggesting that the absence of the negative charge of glutamate or phosphate are responsible for the loss of activity. The present study provides further evidence that the region of phosphorylation site 1 of BCKDH may be part of the dehydrogenase active site by showing that specific mutations in this region result in kinetically altered E1 enzymes. Interestingly, the alanine substitution of serine 293 resulted in different kinetic effects with two different a-ketoacid substrates. This result indicates that this residue does not have a crucial catalytic role and suggests that it may be positioned in a site important for a-ketoacid substrate binding. That two different a-ketoacids with only a slight difference in the branched-chain structure should display such different kinetic changes with this mutant suggests that this residue may provide a site of interaction with the branched-chain portion of the substrate. Completely inactive mutants included R288A, H292A, and D296A. Examination of the amino acid sequences of other a-ketoacid dehydrogenases, including pyruvate dehydrogenases and a-ketoglu­tarate dehydrogenases from a wide variety of species, revealed that these three residues are very highly conserved, forming a distinct domain common to all of these enzymes (Fig. 4). Only the pyruvate dehydrogenase of E. coli lacks these conserved residues. This level of sequence identity suggests that these residues might have critically important functions common to the structures or mechanisms of a-ketoacid dehydrogenases in general. Little data are presently available concerning active site residues of BCKDH, with most work to date focused on the active site of the highly homologous enzyme, PDH. Chemical modification and circular dichroism studies have suggested the presence of unidentified arginine, histidine, and lysine residues in the vicinity of the active site of PDH (19–21). A specific cysteine residue was identified as an active site residue in PDH E1a by differential chemical modification in the presence and absence of substrates (22), although this cysteine is not conserved in the sequence of BCKDH E1a. A specific tryptophan residue of the E1a subunit was also identified in the active site of PDH (23), but this residue is also not conserved in the BCKDH E1a sequence. Active site residues of BCKDH and PDH have also been inferred from modeling studies of the thiamine pyrophosphate binding site as compared to other thiamine pyrophosphate utilizing enzymes, such as transketolase and pyruvate decarboxylase (24). The models proposed for multisubunit enzyme complexes such as BCKDH and PDH predict that the E1a subunits provide residues that bind specifically to the thiazolium ring portion of thiamine pyrophosphate. E1a, however, contains conserved residues believed to be important for binding the diphosphate portion of thiamine pyrophosphate and to a divalent metal atom associated with the pyrophosphate moiety (Ref. 24 and Fig. 4). These modeling studies have clearly identified conserved residues common to these enzymes (Fig. 4), which were also predicted to bind thiamine pyrophosphate from x-ray crystallographic studies of transketolase (25). Thus, these studies would predict a placement of the conserved residues of the phosphorylation site 1 of BCKDH in the near vicinity of the enzyme-bound thiamine pyrophosphate and that histidine 292 of rat BCKDH, adjacent to the phosphorylation site 1 serine, would be involved in
binding to the diphosphate moiety of thiamine pyrophosphate. This theory is supported by our findings that the H292A mutant BCKDH is enzymatically inactive, does not reconstitute with exogenously added thiamine pyrophosphate, and is phosphorylated by BCKDH kinase without inhibition in the presence of thiamine pyrophosphate. That the region of phosphorylation site one contains components of the thiamine pyrophosphate binding site is also consistent with our present finding that S293E mutant E1 does not reconstitute with thiamine pyrophosphate. Loss of thiamine pyrophosphate binding may explain the lack of enzymatic activity in this mutant. Since the S293A mutant E1 is active and does not show an altered K_m for thiamine pyrophosphate, it seems likely that the negative charge introduced by mutating serine 293 to glutamate may neutralize the ability of histidine 292 to interact with thiamine pyrophosphate. That the current structural models for thiamine pyrophosphate binding sites may also apply to pyruvate dehydrogenases was demonstrated by the effect of one of the reported human genetic defects of PDH (26). The defect was found to be the result of a naturally occurring leucine substitution of the conserved histidine residue, adjacent to the phosphorylation site 1 serine residue, in the thiamine pyrophosphate binding motif of PDH E1α (26). This leucine substitution, in a heterozygous individual, resulted in less than 2% of normal PDH activity compared to controls (26). However, direct evidence of an actual role of this conserved residue in thiamine pyrophosphate binding by α-ketoacid dehydrogenases has not previously been provided.

From the present data we propose that the three conserved residues, Arg-288, His-292, and Asp-296, occupy important positions related to the active site of rat BCKDH E1. Histidine 292 clearly serves a critical role in the binding of thiamine pyrophosphate. The exact roles of Arg-288 and Asp-296 in catalysis by E1 are not yet known, although both the R288A and D296A mutant E1s bound normal amounts of thiamine pyrophosphate. We speculate that the positively charged Arg-288 residue could serve as an anion binding site for the carboxyl group of α-ketoacids. This function would be common to all α-ketoacid dehydrogenases, consistent with the level of sequence homology observed for this residue. It is noteworthy that the functional role of an arginine residue in pyruvate binding to PDH was suggested by arginine-specific chemical modifications in the presence and absence of pyruvate (19), although this residue has not been identified and does not necessarily correspond to Arg-288 of BCKDH. Only speculative roles can be assigned to aspartate 296, such as the extraction of a specific proton or a critical hydrogen bonding for stabilization of a transition state structure.

None of these conserved residues are predicted to serve critical structural roles. This conclusion is supported by the observations that the R288A, H292A, and D296A mutant forms of E1 were expressed and purified as a complex with equal amounts of E1α and E1β subunits and that each of these mutant E1s bound to the E2 core of the complex with equal affinity as wild-type E1. Furthermore, each of these mutants produced circular dichroism spectra identical to that of wild-type E1, suggesting that no gross structural perturbations occurred as a result of these mutations.

The identification of these residues, particularly histidine 292, as important to the active site of BCKDH suggests that inactivation of the dehydrogenase by phosphorylation may be due to the placement of the negatively charged phosphate group within the active site of the dehydrogenase. This regulatory scheme is used by only a few known protein kinases but is well established to be responsible for the inhibition of bacterial isocitrate dehydrogenase by phosphorylation (27), and is consistent with previous studies concerning the mechanisms of inactivation of BCKDH and PDH. Furthermore, the present data conclusively show that the well-known inhibition of BCKDH kinase by thiamine pyrophosphate occurs through binding of this cofactor to E1 rather than through direct binding to the kinase. This observation also suggests a close proximity of the dehydrogenase active site and the kinase binding site. Early studies with native PDH suggested that phosphorylation of PDH increases the binding constant for thiamine pyrophosphate but not to an extent sufficient to result in complete inactivation (28). A more extensive study of PDH revealed that phosphorylation inhibits all of the reactions, forward and backward, leading to the formation of the enzyme-bound 2-hydroxyethyl-thiamine pyrophosphate carbanion from enzyme-bound thiamine pyrophosphate (29). Similar conclusions were drawn from circular dichroism studies of PDH (30). Such a mechanism for the inactivation of BCKDH would be consistent with our present data.

The present study also indicates that arginine 288 of rat BCKDH E1α may have a dual role in catalysis by the dehydrogenase and in binding of BCKDH kinase to E1. Previous studies of cytosolic serine/threonine protein kinases show that many protein kinases require charged residues, particularly arginines, for high affinity binding to their respective substrates (11). We propose that the presence of the Arg-288 residue in the phosphorylation site of BCKDH may be critical to the correct positioning of the kinase for specific phosphorylation of serine 293 rather than other nearby serine and threonine residues. The second site of E1α phosphorylation (serine 303) has an N-terminal arginine residue situated much closer in the primary structure, providing a possible explanation for its much slower rate of phosphorylation (5). No other mutations in the phosphorylation site affected the phosphorylation of serine 293. We propose from this finding that secondary structural elements and specific binding of the kinase and E1 subunits to the E2 core may also play major roles in the substrate specificity of mitochondrial protein kinases. Clear understanding of the mechanisms responsible for inactivation of BCKDH...
and PDH by phosphorylation will require more complete knowledge of the enzymatic mechanisms of \( \alpha \)-ketoacid dehydrogenases as well as determination of the structures of these dehydrogenases and their respective kinases.

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