Regulation of the branched chain α-ketoacid dehydrogenase complex, the rate-limiting enzyme of branched chain amino acid catabolism, involves phosphorylation of 2 amino acid residues (site 1, serine 293; site 2, serine 303). To directly assess the roles played by these sites, site-directed mutagenesis was used to convert these serines to glutamates and/or alanines. Functional E1 heterotetramers were expressed in Escherichia coli carrying genes for E1α and E1β under control of separate T7 promoters in a dicistronic vector. Mutation of phosphorylation site 1 serine to glutamate inactivated E1 activity, i.e. mimicked the effect of phosphorylation of site 1. Replacement of the site 1 serine with alanine greatly increased \( K_a \) for the α-ketoacid substrate but had no effect on maximum velocity. The site 1 serine to alanine mutant was phosphorylated at site 2, but phosphorylation had no effect upon enzyme activity. Mutation of site 2 serine to either glutamate or alanine also had no effect upon enzyme activity, but phosphorylation of these proteins at site 1 inhibited enzyme activity. E1 mutated to change both phosphorylation site serines to glutamates was without enzyme activity. The binding affinity of E1 to the E2 core was not affected by mutation of the phosphorylation sites to glutamates, suggesting no gross perturbation of the association of E1 with the E2 core. The results provide direct evidence that a negative charge at phosphorylation site 1 is responsible for kinase-mediated inactivation of E1. Site 2 is silent with respect to regulation of activity by phosphorylation.

Phosphorylation is an important mechanism for the regulation of the branched chain α-ketoacid dehydrogenase complex (BCKDC).\(^1\) Regulation has been studied in some detail with respect to regulation of activity by phosphorylation.

The abbreviations used are: BCKDC, branched chain α-ketoacid dehydrogenase complex; BCKDH, branched chain α-ketoacid dehydrogenase; E1α, the α subunit of 2-oxoisovalerate dehydrogenase (lipoamide) (E.C. 1.2.4.2); E1β, the α subunit of 2-oxoisovalerate dehydrogenase (lipoamide) (E.C. 1.2.4.4); PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; His-Tag, a consecutive stretch of 6 histidine residues.

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\(3\) The abbreviations used are: BCKDC, branched chain α-ketoacid dehydrogenase complex; BCKDH, branched chain α-ketoacid dehydrogenase; E1α, the α subunit of 2-oxoisovalerate dehydrogenase (lipoamide) (E.C. 1.2.4.2); E1β, the β subunit of 2-oxoisovalerate dehydrogenase (lipoamide) (E.C. 1.2.4.4); PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; His-Tag, a consecutive stretch of 6 histidine residues.

Site-directed Mutagenesis of Phosphorylation Sites of the Branched Chain α-Ketoacid Dehydrogenase Complex\(^*\)

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Clal digestion, T4 DNA polymerase blunting, and Spbi digestion. pET-21a-E1β was cut with BglII, blunt-ended with T4 DNA polymerase, and digested with Spbi. A diestronic expression construct containing both E1α and E1β coding regions was obtained by ligation of the E1α fragment into pET-21a-E1β treated as described above. The final pET-E1 expression vector was modified by oligonucleotide-directed mutagenesis to introduce 6 extra bases from the 5' end of the E1β cDNA. E1α in this final construct had 11 amino acids left from the leader peptide sequence and 19 extra amino acids from the vector, including the starting Met, 6 histidine residues for the His-Tag sequence, 6 amino acids for the thrombin cleavage site, and 6 additional amino acids. One extra amino acid (Met) was present at the N terminus of the E1β. pGroESL construction was described by Goloubinoff et al. (18).

Site-directed Mutagenesis—Single-stranded pET-E1 was rescued with the helper phage R408 following the manufacturer's instructions (Promega). Site-directed mutagenesis was carried out following the manufacturer’s instructions (Amerham Corp.). Mutants were identified by double-stranded sequencing.

Cell Growth—E. coli HMS174 (DE3) was double-transformed with pET-E1 and pGroESL plasmids and selected by growth in M9ZB media containing 200 μg/ml ampicillin and 50 μg/ml chloramphenicol. One-liter cultures were grown at 37 °C until OD600 = 0.8. To these cultures iso-propyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the growth of the cultures continued for 15–18 h at 30 °C. Bacterial cells were pelleted and washed with phosphate-buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 1.2 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.5). The pellets were resuspended in 10 volumes of buffer A (50 mM potassium phosphate, pH 7.0, 2 mg/ml MgCl2, 0.2 mM thiamine phosphate, 0.25 mM histidine, and 500 mM NaCl), containing 0.1% Triton X-100, phenylmethylsulfonyl fluoride (100 μg/ml), and benzamidine (100 μg/ml). The cells were disrupted by sonication with a Sonifier Cell Disruptor W185 (Branson Sonic Power Co., Plainview, NY). The resulting homogenates were centrifuged at 20,000 × g for 30 min at 4 °C.

Recombinant E1 Purification—Extracts (80 mg of protein) were adjusted to pH 7.9, made 10 mM in β-mercaptoethanol, and applied to a 2.5-ml column of His-Bind™ metal chelation resin (Novagen) previously charged with five volumes of 50 mM NaSO4 and washed with five volumes of buffer A. The column was washed with buffer A until unbound protein was completely removed (approximately 10 volumes). The column was then washed with five volumes of buffer A containing 4 mM histidine followed by five volumes of buffer A containing 25 mM histidine. Polyethylene glycol (PEG 8000) was added to the 25 mM histidine eluate to a final concentration of 6%. Precipitated protein was collected by centrifugation and resuspended in one-third volume of a solution 50 mM in potassium phosphate, pH 7.0, 2 mM in MgCl2, 10 mM in β-mercaptoethanol, 100 mM in NaCl, and 20% (w/v) in glycerol (about 0.5 mg protein/ml). Protein concentration was determined by the Bradford method (19).

Enzyme Assay—The recombinant E1 protein (1 μg of protein) was reconstituted with native purified BCKDC E2 (0.75 μg of protein). Purified E3 (58 μg of protein, 7.5 units (Sigma) was added in excess to the assay mixture. Assay of BCKDC activity was based on that previously described by Goodwin et al. (20). Kinetic constants were calculated from initial rates by using the computer program Enzfitter (Elsevier-Biosoft, Cambridge, United Kingdom), which is based on the method of Cleland (21). For the kinase-mediated inactivation assay (22), recombinant E1 protein (1 μg of protein) was reconstituted with native purified BCKDC E2 (0.75 μg of protein) and native purified BCKDH kinase (0.05 μg) in a solution 50 mM in potassium phosphate, pH 7.5, 5 mM in diethio- tol, 0.02% (w/v) in Triton X-100, 7.5 mM in MgCl2, and 0.4 mM in ATP. At different time intervals of incubation at 20 °C, the remaining BCKDH activity was determined, and the percentage of initial activity was plotted as a function of time. A control incubation that contained all reconstituion components except ATP was used for each time point. The [32P]phosphate incorporation experiments were performed in the same way as the inactivation assay except that γ-32P-ATP (200–300 counts/ min/nmol) was included in the reaction mixture. The reaction was stopped at the indicated times by adding a solution 2% (w/v) in SDS, 10 mM in KCl, pH 6.8, 0.1 mM in EDTA, 10% (w/v) in glycerol, and 5% (w/v) in β-mercaptoethanol. Samples were boiled for 2 min prior to analysis by SDS-PAGE with 12% polyacrylamide gels. Gels were stained with Coomassie Blue prior to autoradiography. Quantitation of radioactivity was done with an AMBIS β-scanner. Radioactive standards were used to establish the range of linearity under the conditions of these experiments.

Cell Filtration Chromatography—Aliquots of 250 μg of each purified E1 preparation were applied to a column of Sephacryl S-300 (1.25 x 55 cm) to determine the size of the E1 complex. The column was calibrated with molecular mass markers: β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa). The void volume was determined with blue dextran. Samples were applied in a volume of 0.5 ml and elution fractions of 0.75 ml were collected with a flow rate of 2.5 ml/h.

RESULTS

Expression and Purification of Recombinant BCKDC E1—Davie et al. (23) demonstrated previously that coexpression of E1α and E1β subunits in E. coli was necessary to achieve the assembly of functional E1 components. For this reason cDNAs encoding for both subunits were inserted into a pET-21a vector under control of two separate T7 promoters. To facilitate purification of the wild-type and mutant E1 components, a stretch of consecutive histidine residues (His-Tag) was added at the N-terminal end of the E1α subunit. This sequence binds to divalent cations, allowing the simple one-step purification of the target protein with a His-Bind metal chelation resin containing immobilized Ni2+.

Induction with IPTG of E. coli cells transformed with the diestronic pET-E1 and pGroESL vectors resulted in expression of both E1α and E1β subunits identified in cell lysates by Western blot analysis (data not shown). Recombinant proteins were purified on His-Bind metal chelation resin as described under “Experimental Procedures.” A representative SDS-PAGE analysis of the protein fractions obtained at various stages of purification of wild-type E1 is given in Fig. 1. Identical patterns were found for the other mutant recombinant proteins produced in this study. Nearly homogeneous E1α and E1β proteins were eluted from the affinity column by 25 mM histidine in the last step. The last traces of chaperonin proteins were removed by precipitation of E1 with polyethylene glycol. Since only the E1α protein was tagged with a stretch of histidine residues, whereas the E1β subunit was expressed as a non-fusion protein, copurification of E1α and E1β in equivalent amounts suggests a strong association between these components. To determine if the association between E1α and E1β subunits reflected the assembly of E1 heterotetramers, purified recombinant E1

FIG. 1. Purification of recombinant E1 by metal chelation chromatography. E. coli HMS174 (DE3) was double-transformed with pET-E1 and pGroESL plasmids. Cells were cultured, collected, and homogenized as described under “Experimental Procedures.” An extract of the cells was applied to a column of His-Bind™. Samples were taken from each fraction for protein analysis by SDS-PAGE with Coomassie Blue staining. A representative gel is shown for wild-type E1. Molecular mass markers (in kDa) and positions of E1α and E1β subunits are indicated.
components were fractionated by size on Sephacryl S-300. Apparent molecular masses of 160 kDa for native, wild-type, and each of the mutant E1 proteins suggested proper assembly of the E1 proteins as α2β2 heterotetramers. Recombinant E1 was also found to assemble as a heterotetramer by Davie et al. (23).

Activities of the Wild-type and Mutant E1 Proteins—To determine whether the association between E1 and E1b reflected the assembly of functionally active E1 heterotetramers, wild-type E1, and E1 proteins with mutant Elα subunits were reconstituted with purified native E2 and E3 components. Activities of the reconstituted complexes were measured spectrophotometrically by NADH reduction as described under "Experimental Procedures."

Three out of the five recombinant E1 components with mutant Elα subunits were found to reconstitute catalytic activity in the BCKDC assay (Table I). V_max values of these enzymes were comparable to that of wild-type E1. Replacement of the serine at phosphorylation site 1 of the Elα subunit by glutamate (S293E) resulted in a completely inactive enzyme, consistent with an important role of phosphorylation site 1 in the inactivation of BCKDC. Replacement of the same serine with alanine (S293A) caused a nearly 12-fold increase in K_m for α-ketoisovalerate without a significant change in V_max. In contrast, replacement of the serine at phosphorylation site 2 of the Elα subunit by either glutamate (S303E) or alanine (S303A) had no effect upon enzyme activity, suggesting this phosphorylation site is silent with respect to inactivation of BCKDC. As expected from the finding that an inactive enzyme resulted from replacing serine 293 with glutamate, no activity was detected with the double phosphorylation site mutant obtained when the serines at both phosphorylation sites were replaced with glutamates (S293E/S303E).

Interaction of S293E and S293E/S303E Mutant Elα with the BCKDC E2 Core—To determine whether the inactive mutant E1 components with glutamate substitutions at the phosphorylation sites still associated with E2, their ability to compete with wild-type E1 for binding with E2 was measured. Reconstitution of BCKDC activity occurs immediately upon mixing of the component enzymes (E1, E2, and E3) of the complex (24). Tight binding between E1 and the E2 core is necessary for BCKDC activity, and active E1 will replace inactive (phosphorylated) E1 from sites occupied on the E2 core (25, 26). Thus, inhibition of the reconstitution of BCKDC activity from its component enzymes by an inactive form of E1 assesses relative binding affinities of active versus inactive forms of E1 (24).

For these experiments a constant amount of native E2 was preincubated with various ratios of active to inactive E1 (Fig. 2). E1 was used in excess relative to E2 to assure that E2 was rate limiting for BCKDC activity and that all E2-binding sites were saturated by E1. Lack of any decrease in BCKDC activity with a decrease in amount of wild-type E1 established that E1 was present in excess (Fig. 2). It was found that the catalytically inactive S293E and S293E/S303E mutant E1s were equally effective in inhibiting reconstitution of BCKDC activity with wild-type E1 (Fig. 2), suggesting that the mutations of the Elα components of these S1αs do not affect binding to E2. Moreover, in both cases, 50% inhibition occurred at equal amounts of the inactive mutant protein and active wild-type E1 protein, suggesting comparable affinities of the wild-type E1 and the E1s with mutated phosphorylation sites for the E2 core.

Phosphorylation and Inactivation of Recombinant E1 Components—Since the BCKDC E2 component greatly stimulates BCKDH kinase activity (27), these studies were conducted with BCKDC complexes reconstituted by mixing recombinant E1 preparations with purified E2 and BCKDH kinase. Incubation with ATP resulted in a time-dependent inactivation of the complexes reconstituted with wild-type Elα and the S303A and S303E mutant Elα proteins (Fig. 3, upper panel). Over the course of this experiment 1.2 pmol of phosphate from 32P-labeled ATP was incorporated per pmol of Elα protein (Fig. 3, lower panel), which is comparable to the stoichiometry found previously by this laboratory for native complexes isolated from several tissues (1.2-1.6 pmol 32P/pmol Elα protein, with approximately half of the label in each of the two phosphorylation sites) (10). Approximately half as much phosphate was incorporated into the site 2 mutant proteins (0.5 and 0.7 pmol 32P/pmol Elα protein into S303E and S303A, respectively) (Fig. 3, lower panel), consistent with only site 1 being open for phosphorylation in these proteins. In contrast to these proteins, incubation of the S293A Elα mutant enzyme with ATP caused no more loss of activity than incubation without ATP (Fig. 3, upper panel).

Nevertheless, phosphate from labeled ATP was incorporated into this protein (0.25 pmol 32P/pmol protein; Fig. 3, lower panel).
El, molecular chaperonins GroEL and GroES were coexpressed. The activation of the enzyme has not been studied in great detail. To investigate the mechanism of regulation of BCKDC activity, the authors established a system for phosphorylation site 1. The phosphorylation site 1 mutant enzyme was reconstituted with native E2 and wild-type E1a subunit by BCKDH kinase. The autoradiogram shows incorporation from labeled ATP into the recombinant Ela protein for the S293E mutant and the S293E/S303E double mutant E1. The data were reproduced in independent experiments with different preparations of the recombinant proteins.

![ATP-dependent inactivation of recombinant El proteins](image)

**Fig. 3.** ATP-dependent inactivation of recombinant El proteins by BCKDH kinase. ATP-dependent inactivation of BCKDC activity by BCKDH kinase (upper panel) and phosphorylation of the Ela subunit by BCKDH kinase (lower panel) were assayed as described under "Experimental Procedures." For activity measurement, BCKDC was reconstituted with native E2 and wild-type E1 (○), S293A mutant E1 (□), S303A mutant E1 (■), or S303E mutant E1 (▲). The autoradiogram shows incorporation from labeled ATP into: 1, no recombinant El protein; 2, wild-type Ela; 3, S293A Ela; 4, S303A Ela; 5, S293E Ela; 6, S303E Ela; and 7, S293E/S303E Ela. Data shown were reproduced in independent experiments with different preparations of the recombinant proteins.

Inactivation as a consequence of phosphorylation is a well-established mechanism of regulation of BCKDC activity. The protein kinase tightly associated with the complex phosphorylates 2 consecutive serine residues within Ela subunit by BCKDH kinase. However, the role of individual phosphorylation sites in inactivation of the enzyme has not been studied in great detail. To address this problem, the E1 component of BCKDC along with several mutants carrying amino acid substitutions within phosphorylation sites was expressed in *E. coli* utilizing a dicistronic expression vector. Two subunits of the E1 component were coexpressed by using a dicistronic expression vector under the control of T7 promoters. To facilitate the proper folding of the recombinant Ela, molecular chaperonins GroEL and GroES were coexpressed with Ela and E1β subunits. However, the need for overexpression of chaperonins was not rigorously examined in the present study. Expression in *E. coli* cells of the dicistronic vector constructed for this study resulted in the formation of functional Ela as determined by capacity of the recombinant protein to reconstitute BCKDC activity when mixed with the E2 and E3 components of the complex.

Expression of Ela as a fusion protein with a His-Tag (sequence of 6 histidine residues) at its N-terminal end rendered purification of recombinant Ela as an αβ heterotetramer facile by metal chelation matrix chromatography. The high imidazole concentration most frequently used to elute His-tagged proteins from the Ni²⁺-chelation resin was found inhibitory of Ela activity. However, histidine proved effective for eluting His-tagged Ela from the resin, and no inhibition of Ela activity occurred at the relatively low concentration of histidine required for elution.

Replacement of the serine at phosphorylation site 1 of Ela with a glutamate resulted in a recombinant protein without activity, indicating that a negative charge at this site, introduced by either phosphorylation or an amino acid substitution, results in inhibition of enzyme activity. An increase in amino acid side chain size at site 1 may also be a factor, since glutamate is larger than serine, and the addition of a phosphate group to serine substantially increases its size. Replacement of the serine at phosphorylation site 2 (serine 303) with glutamate did not affect enzyme activity, suggesting that this site is silent with respect to inactivation of the complex by the introduction of a negative charge or a change in residue size. Direct phosphorylation studies with mutant Ela proteins support these conclusions. The phosphorylation site 2 mutant enzymes (S303E and S303A) were active in their dephosphorylated states, but readily inactivated upon phosphorylation at phosphorylation site 2. The phosphorylation site 1 mutant enzyme with serine replaced by alanine was also active, but no inactivation of this enzyme occurred upon phosphorylation at phosphorylation site 2. These findings, therefore, provide direct evidence in support of the previous work indicating phosphorylation of site 1 of the Ela subunit is entirely responsible for kinase-mediated inactivation of BCKDC (9, 10).

No evidence was found for differences in affinity of the wild-type Ela and inactive mutant Ela enzymes for the E2 core of the complex. Thus, introduction of a negative charge at phosphorylation site 1 (S293E) does not appear to cause a gross conformational change in Ela that perturbs its ability to associate with E2. This finding was not expected since Cook et al. (24) reported that phosphorylation induced a 5-fold decrease in the affinity of Ela for E2. The possibility that negative charges at both phosphorylation sites might be required for an effect upon binding of Ela by E2 was ruled out by the finding of an unaltered affinity of the double phosphorylation site mutant (S293E/S303E) for the E2 core.

The increase in $K_a$ caused by replacement of serine at phosphorylation site 1 (serine 293) by alanine along with the complete loss of enzyme activity caused by replacement of this serine with glutamate may indicate that the α-ketoacid binding domain is at or near phosphorylation site 1 of this enzyme. If this should be the case, the introduction of a negative charge at this site would have to be remarkably effective in blocking substrate binding, since no enzyme activity could be detected with the S293E mutant at α-ketoisovalerate concentrations as high as 200 mM (data not given). Changes in activity of enzymes in response to phosphorylation likely result from induced long range conformational changes from an allosteric phosphorylation site (29). On the other hand, the isocitrate dehydrogenase of *E. coli* provides one example in which phosphorylation inhibits catalytic activity by a direct effect within the substrate binding site.

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3. J. Hawes, unpublished observations.
(30). Replacement of the serine at the phosphorylation site of isocitrate dehydrogenase with various amino acid residues had effects analogous to those found with El in the present study (31). However, determination of whether inactivation of El by phosphorylation occurs without conformational perturbation of the ketoacid-binding site will require detailed structural studies. Experiments designed to find suitable conditions for crystallization of recombinant BCKDC El have been initiated.

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