Site-Directed Mutagenesis and Functional Analysis of the Active-Site Residues of the E2 Component of Bovine Branched-Chain α-Keto Acid Dehydrogenase Complex†

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ABSTRACT: The catalytic domain of dihydrolipoamide transacylase (E2c) of bovine branched-chain α-keto acid dehydrogenase complex (BCKAD) was overexpressed in *Escherichia coli*. The E2c catalyzes a reversible acyl transfer reaction between acyl-CoA and dihydrolipoamide, which also occurs spontaneously with a much slower rate. The benzene extracts of both the enzyme-catalyzed and the spontaneous reactions mixture have identical ultraviolet absorbance spectra with a maximum at 233–234 nm, which is characteristic of *S*-acyldihydrolipoamide. The spontaneous reaction rate of various acyl-CoA is in the order of acetoacetyl-CoA > acetyl-CoA > isobutyryl-CoA > isovaleryl-CoA. In other words, the spontaneous acyl transfer is faster when the substituent (R) of acyl-CoA (R-CO-S-CoA) is a more electron-withdrawing group. This result indicates that a negative charge occurs in the substrate during the acyl transfer process. The function of the active-site histidine (His391) and serine (Ser338) of bovine E2c was analyzed by site-directed mutagenesis. Substitution of His391 or Ser338 with alanine caused drastic decreases in catalytic efficiencies by 3–4 orders of magnitude. The residual activity of H391A increased as the pH of the reaction buffer was elevated. These data support the base-catalyzed mechanism inferred from that of chloramphenicol acetyltransferase (CAT). In this reaction, the active-site histidine acts as a general base, and the active-site serine provides a hydrogen bond to the putative negatively charged tetrahedral transition state. Moreover, when Ala348 was changed to valine, the catalytic efficiency for isovaleryl-CoA decreased about 10-fold, and that for acetyl-CoA increased about 3-fold. Ala348 presumably contacts the isobutyl group of isovaleryl-CoA in the acyl transfer reaction. Our results indicate that this residue plays a key role in the substrate preference of bovine E2c.

The family of α-keto acid dehydrogenase complexes comprises pyruvate dehydrogenase complex (PDC),[1] α-ketoglutarate dehydrogenase complex (KGDC), and branched-chain α-keto acid dehydrogenase complex (BCKAD) (Reed, 1974). These multienzyme complexes catalyze the oxidative decarboxylation of α-keto acids with PDC specific for pyruvate, KGDC specific for α-ketoglutarate, and BCKAD specific for branched-chain α-keto acids derived from valine, leucine, and isoleucine. Each enzyme complex is composed of multicopies of three catalytic components: α-keto acid decarboxylase (E1), dihydrolipoamide transacylase (E2), and dihydrolipoamide dehydrogenase (E3). E3 is a common component of the three multienzyme complexes. Eukaryotic PDC and BCKAD also contain specific kinases and phosphatases that regulate the activity by reversible phosphorylation–dephosphorylation of their respective E1 (Reed & Yeaman, 1987; Randle et al., 1987). Such macromolecular organization results in the molecular mass of α-keto acid dehydrogenase complexes in the range of several million daltons. The structural core of the complex is provided by E2, which consists of three distinct domains: i.e., the N-terminal lipoyl-bearing domain, the peripheral E1/E3 subunit-binding domain, and the C-terminal catalytic domain. (Reed & Hackert, 1990; Perham, 1991). Between each domain is a highly flexible segment (Radford et al., 1989) rich in alanine, proline, and charged residues. The catalytic domain of E2 confers the core of α-keto acid dehydrogenase complexes, and catalyzes the reversible acyl transfer reaction: CoA + LipSHS-acyl → acyl-CoA + Lip-$(SH)_2$. The catalytic domains of E2 of the α-keto acid dehydrogenases complex family share considerable homologies in amino acid sequences (Russell & Guest, 1991).

The crystal structure of the catalytic domain of E2 of PDC from *Azotobacter vinelandii* was solved (Mattevi et al., 1992). It showed that eight trimers assemble as a hollow cube with an edge of 125 Å, forming the core of the multienzyme complex. The active-site channel is located at the interface of two 3-fold-related subunits such that each trimer has three active-site channels. Mattevi et al. also found that the trimer has a topology similar to that of chloramphenicol acetyltransferase (CAT) (Leslie, 1990). Both enzymes have a long active-site channel with a histidine and a serine positioned in the center. The active-site histidine acts as a general base (Kleanthous et al., 1985), and the active-site serine provides a hydrogen bond to the transition state (Lewendon et al., 1990) in the CAT-catalyzed acetyl transfer reaction. The E2-catalyzed acyl transfer has been proposed to be mediated by an analogous base-catalyzed mechanism, according to conservation of the region containing the active-site histidine (Guest, 1987) and the similar topology between the trimer of E2 catalytic

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[1] Abbreviations: BCKAD, branched-chain α-keto acid dehydrogenase complex; PDC, pyruvate dehydrogenase complex; KGDC, α-ketoglutarate dehydrogenase complex; E1, α-keto acid decarboxylase; E2, dihydrolipoamide transacylase; E2c, E2 catalytic domain; E3, dihydrolipoamide dehydrogenase; CAT, chloramphenicol acetyltransferase; $Lip(SH)_2$, dihydrolipoamide; MBP, maltose-binding protein; RF DNA, replicative-form DNA; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid.

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domain and CAT (Mattevi, 1993). The function of the active-site histidine of E2 catalytic domain has been studied by mutagenesis experiments, but the results are controversial. Activity was abolished by the substitution of histidine with asparagine or glutamine in $E2_c$ of bovine BCKAD (Griffin & Chuang, 1990). The substitution with cysteine in *E. coli* PDC-E2 also abolished activity (Russell & Guest, 1990). In contrast, there was no significant impact on the activity of E2 catalytic domain of PDC from *Saccharomyces cerevisiae*, when this histidine was changed to alanine or to asparagine (Niu, et al., 1990).

In order to clarify this controversy, we overexpressed the catalytic domain of E2 of bovine BCKAD and substituted alanine for the active-site histidine (His391) and serine (Ser338) to avoid introducing any possible improper hydrogen bond. Effects of these mutations on kinetic constants of the enzyme were studied. On the other hand, although the amino acid sequences of the E2 component of the family of α-keto acid dehydrogenase complex are highly homologous, each subtype enzyme has its own substrate specificity. For instance, favorable substrates of E2 of mammalian BCKAD are branched-chain acyl-CoA, whereas E2 of PDC has strong preference for acetyl-CoA. We hypothesize that the steric effect may be the key factor responsible for substrate specificity. In the present study, we also attempted to decipher which residue(s) was (were) the key structural determinant for E2 of BCKAD with respect to preference for various acyl-CoA. The choice of the target amino acids for site-directed mutagenesis was based on sequence alignment and the crystal structure of the E2 catalytic domain of *A. vinelandii* PDC.

MATERIALS AND METHODS

Strains, Plasmids, and Chemicals. Escherichia coli strain XL1-Blue [*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac (F'proAB, lacI^{q}ZDM15,* Tn*10(tet^r)*)] was the host for expression of the maltose-binding protein (MBP) fused E2 catalytic domain. *E. coli* strain TG1 [*Δ(lac-pro), supE, thi, hsdD5/F'rad36, proA+B+, lacIq, lacZΔM15*] and bacteriophage M13mp19 were used for the preparation of single-strand DNA and replicative-form (RF) DNA. The expression vector for the MBP fusion protein, pMALc, was obtained from New England Biolabs. Isovaleryl-CoA, isobutyryl-CoA, acetyl-CoA, acetoacetyl-CoA, DL-6,8-thioctic acid amide, and malic dehydrogenase were purchased from Sigma. Restriction protease factor Xa was purchased from Boehringer Mannheim. ATP–citrate lyase was purified from rat liver according to the procedure of Srere et al. (Linn & Srere, 1979). Dihydrolipoamide was prepared by sodium borohydride reduction of the oxidized DL-6,8-thioctic acid amide as described previously (Reed et al., 1958). Amylose resin was prepared from corn starch as described previously with modifications (Ferenci & Klotz, 1978). The cross-linking temperature was maintained at 50 °C, and the cross-linking time was reduced to 13 min to increase porosity and improve binding capacity for the megadalton-size MBP-fusion protein.

Construction of Expression Vectors for Normal and Mutant E2 Catalytic Domains. A DNA fragment encoding the entire catalytic domain of E2 of bovine BCKAD (residues 161–421) was generated by PCR and inserted into the cloning site of plasmid pMALc. This expression vector (pMALc-E2c) codes for a fusion protein containing MBP at the N-terminus and the E2 catalytic domain at the C-terminus. Between MBP and E2c is a linking segment containing the factor Xa recognition sequence. Details of the construction were described by Wynn et al. (1994). For the preparation of single-strand DNA, pMALc-E2c was digested with the restriction enzymes *Sac*I and *Sal*I, and the smaller DNA fragment (ca. 0.9 kb) of the digestion mixture was ligated to M13mp19 vector which had been treated with the same restriction enzymes. The single-strand DNA of M13mp19-E2c was subjected to mutation using the oligonucleotide-directed mutagenesis kit from Amersham (Sayers et al., 1988). The nucleotide sequence of the mutated replicative-form (RF) DNA was confirmed by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using *taq* polymerase from Promega. The confirmed RF DNA was digested with *Sac*I and *Sal*I again, and the fragment containing the mutated E2c sequence was reinserted into the pMALc vector. To construct combined mutations, oligonucleotide-directed mutagenesis was carried out on the single-strand DNA of pMalc-E2c(A348F) which contains the mutation Ala348 → Phe in the E2c sequences.

Expression and Purification of Recombinant Catalytic Domains. A 10 mL overnight culture of *E. coli* strain XL1-Blue harboring pMALc-E2c was inoculated into 1 L of LB medium (containing 50 μg/mL ampicillin) and incubated at 37 °C. IPTG (final concentration 0.5 mM) was added to the culture when the cell density reached 0.5 ODU to induce the expression of fusion protein. Cells were harvested after 15 h postinduction by centrifugation. Cell pellets harvested from 1 L of culture were frozen at −70 °C for at least 2 h, and resuspended in 25 mL of potassium phosphate buffer (25 mM, pH 7.5) containing 100 mM NaCl, 0.2 mM EDTA, 0.02% NaN_3, and 1 mM phenylmethanesulfonyl fluoride. This cell suspension at 4 °C was sonicated 8 times with a Branson sonifier with the power setting at 3 and in the 70% cycle for 30 s. The cell extract was clarified by centrifugation at 20000*g* for 20 min (Figure 1, lane 1). The clarified supernatant was mixed with an equal volume of amylose resin slurry and was shaken gently at 4 °C for at least 3 h. Potassium phosphate buffer (25 mM, pH 7.5) containing 100 mM NaCl, 0.2 mM EDTA, and 0.02% NaN_3 was used to remove all unbound material after the mixed slurry was packed into a 50 mL column. Maltose (final concentration 10 mM) was subsequently included in the washing buffer to elute the MBP-fusion protein (lane 2). Approximately 100 mg of the fusion protein was produced from 1 L of culture. To remove MBP, 0.15% factor Xa (w/w) was added to the purified MBP-E2c preparation and incubated at 4 °C overnight (lane 3). The factor Xa digestion product was passed through a 200 mL Sephacryl-S300 HR (Pharmacia) column to allow the separation of E2c from MBP (lane 4). Centriprep 100 (Amicon) ultrafiltrates were used to concentrate the purified E2c in some cases. The protein concentration was determined by the Lowry method (Lowry et al., 1951), with bovine serum albumin (Pierce) as a standard.

Activity Assay. The rate of the acyl transfer reaction was assayed by the method developed by Angier et al. (1987). Briefly, the acyl transfer reaction (eq 1) was coupled with the reactions catalyzed by ATP–citrate lyase (eq 2) and malic dehydrogenase (eq 3).

$$\text{acyl-CoA} + \text{Lip(SH)}_2 \rightarrow \text{CoA} + \text{acyl-S(Lip)SH} \quad (1)$$

$$\text{ATP} + \text{CoA} + \text{citrate} \xrightarrow{\text{ATP–citrate lyase}} \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{P}_i \quad (2)$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{malic dehydrogenase}} \text{NAD}^+ + \text{malic acid} \quad (3)$$

The rate of the acyl transfer reaction at 30 °C was determined

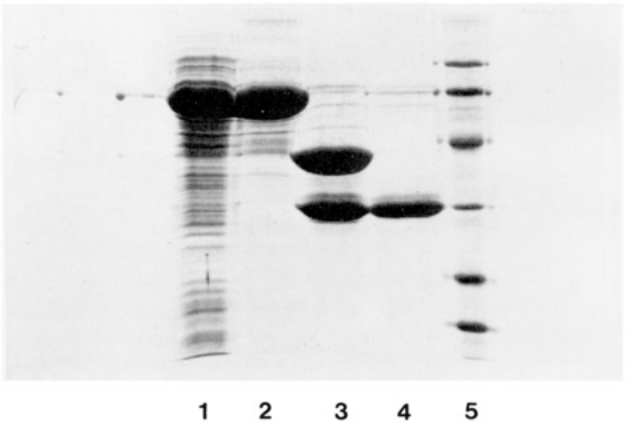

FIGURE 1: Purification of recombinant E2c of bovine BCKAD. The MBP–E2c fusion protein was expressed in *E. coli* and purified as described under Materials and Methods. The enzyme preparations at different purification steps were analyzed by SDS–polyacrylamide (12% w/v) gel electrophoresis, and visualized by Coomassie-blue staining. Lane 1, supernatant of cell extract (30 μg). Lane 2, sample after amylose resin purification (20 μg). Lane 3, factor Xa digestion of MBP–E2c fusing protein (25 μg). Lane 4, E2c (10 μg) after the removal of MBP with an Sephacryl S-300 gel filtration column. Lane 5, low molecular weight standards (Bio-Rad).

by monitoring the decline of absorbance at 340 nm with a Gilford Response scanning spectrophotometer. The reaction mixture contained E2c, Tris buffer, acyl-CoA, dihydrolipoamide, 20 mM sodium citrate, 10 mM $MgCl_2$, 5 mM ATP, 0.2 mM NADH, 10 units of malic dehydrogenase, and 0.1 unit of ATP–citrate lyase in a final volume of 0.5 mL. The blank contained the same reactants except for the exclusion of E2c.

Confirmation of S-Acyldihydrolipoamide. The product of the acetyl transfer reaction, *S*-acetyldihydrolipoamide, was confirmed by the method described previously (Chuang et al., 1984). Three reactions were carried out. In the first reaction, 0.8 μg of wild-type E2c, 1.5 mM dihydrolipoamide, 1.5 mM acetyl-CoA, and 50 mM Tris buffer (pH 7.0) were included in the reaction mixture. In the second reaction, 1.5 mM dihydrolipoamide, 1.5 mM acetyl-CoA, and 50 mM Tris buffer (pH 8.0) were present without E2c. The third reaction included 1.5 mM dihydrolipoamide and 50 mM Tris buffer (pH 8.0). The three reactions were incubated at 30 °C for 25 min. One milliliter of benzene was added to each reaction mixture, and the benzene mixture was vortexed for 20 s. The benzene extract (0.5 mL) was dried under a stream of nitrogen, and the dry residual was dissolved into 0.5 mL of ethanol. The ethanol solutions of the first two reactions were scanned in the ultraviolet range (220–280 nm), and compared with the third using a Gilford Response scanning spectrophotometer.

Determination of Kinetic Constants. The Lineweaver–Burk plot and the Eadie–Hofstee plot were used to determine K_M and V_{max} (Fersht, 1985). Substrate concentration was in the range of 0.2–2.5K_M. The catalytic constant (k_{cat}) was calculated from the equation $V_{max} = k_{cat}[E2c]_0$, where $[E2c]_o$ is the molar concentration of the monomer of E2c.

RESULTS

Nonenzymatic Acyl Transfer Reaction. A nonenzymatic acyl transfer reaction was observed in the blank during the determination of E2c activity. The magnitude of this non-E2c-catalyzed acyl transfer reaction was not altered by increased concentrations of malic dehydrogenase and ATP–citrate lyase. Therefore, the reaction was not caused by contaminants in these two coupling enzymes. At neutral pH,

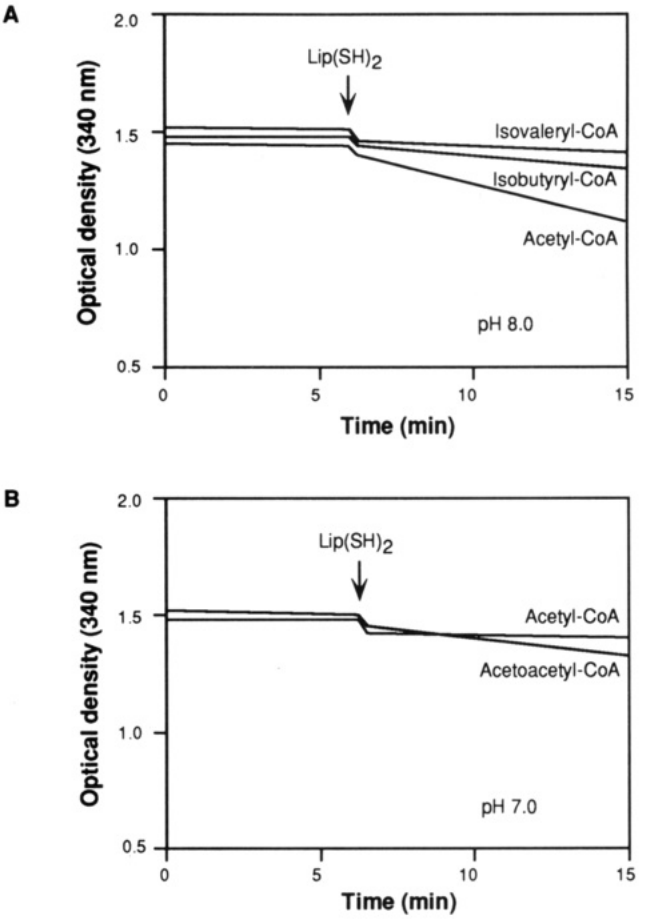

FIGURE 2: Time course of the spontaneous acyl transfer reaction. The spontaneous acyl transfer reaction was carried out at pH 8.0, with 0.5 mM acyl-CoA as substrate (panel A). Since acetoacetyl-CoA is labile at pH 8.0, the reaction was also performed at pH 7.0 with this substrate and acetyl-CoA (panel B). The composition of the reaction mixture was as described under Materials and Methods except that E2c was omitted. Dihydrolipoamide (dissolved in a 25% ethanol solution) at a final concentration of 4.3 mM was added to the reaction mixture to initiate the spontaneous acyl transfer reaction. The abrupt decrease of absorbance upon the addition of dihydrolipoamide was due to the dilution effect.

the coexistence of acyl-CoA and dihydrolipoamide was essential to trigger the decline of absorbance at 340 nm (Figure 2B), and the extent of the reaction was proportional to the concentration of acyl-CoA and dihydrolipoamide (data not shown). As shown in Figure 2A,B, the absorbance declined significantly only after the addition of dihydrolipoamide. Figure 2 also shows that the transfer of the acetyl group is faster at alkaline (panel A) than at neutral (panel B) pH. The second-order rate constant, k_2 (from $v = k_2$[acetyl-CoA][Lip-$(SH)_2$]), of the spontaneous acetyl transfer at pH 7.0 was determined to be 1.0×10^{-7} μM^{-1} min^{-1}, and it increased to 3.2×10^{-7} and 12.0×10^{-7} μM^{-1} min^{-1} at pH 7.5 and pH 8.0, respectively. The pH dependence of the reaction rate indicates that the transfer of the acyl group is a base-catalyzed reaction. The productions of *S*-acetyldihydrolipoamide in the E2c-catalyzed and the non-E2c-catalyzed acetyl transfer reactions were confirmed by the identical UV absorbance spectra with a maximum at 233–234 nm of the reaction products (Figure 3). The thioester was shown to have a maximum absorbance at 232 nm (Daigo & Reed, 1962). Such spontaneous reactions provide an approach to study the structure–activity relationship of substrate. The activity of the spontaneous acyl transfer reaction is dependent on the R group of acyl-CoA, R-C(O)-S-CoA. As shown in Figure 2, the rate of spontaneous acyl

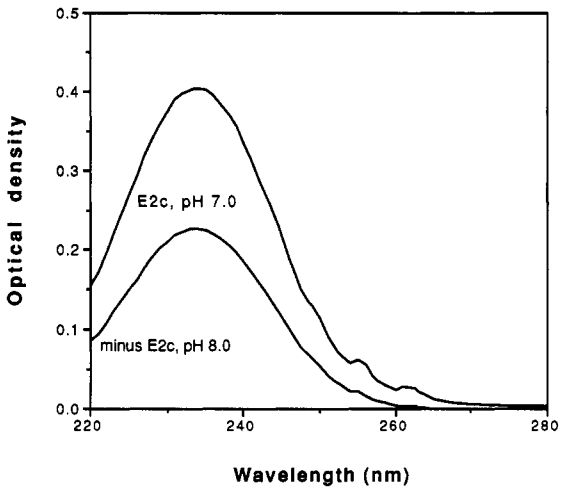

FIGURE 3: Ultraviolet absorbance spectra of *S*-acetyldihydrolipoamide from the E2c-catalyzed and the spontaneous acetyl transfer reaction. The nonenzymatic or spontaneous acyl transfer reaction was carried out at pH 8.0 and the E2c-catalyzed reaction at pH 7.0. Acetyl-CoA (1.5 mM) and dihydrolipoamide (1.5 mM) were used as substrates. The reaction product was extracted with benzene, dried under N_2, and redissolved in ethanol. *S*-Acetyldihydrolipoamide and their difference spectra against the dihydrolipoamide blank were obtained as described under Materials and Methods.

transfer is in the order acetoacetyl-CoA > acetyl-CoA > isobutyryl-CoA > isovaleryl-CoA.

Mutations in the Active-Site Histidine and Serine. Both the active-site histidine (His391) and serine (Ser338) were substituted with alanine in the present study. As shown in Figure 1, wild-type E2c was purified to apparent homogeneity on the Sephacryl-S300 HR column. The mutant and wild-type catalytic domains of E2 had the same elution profile on the Sephacryl-S300 HR column, suggesting that they had identical geometry as a 24-mer. Replacement of H391 with alanine did not totally abolish the activity, and H391A had higher residual activity at alkaline pH. The kinetic constants of wild-type and mutant E2c for acetyl-CoA and isovaleryl-CoA were determined at pH 9.0 as shown in Table 1. The mutations of His391 and Ser338 mainly cause drastic decreases in the catalytic constant, k_{cat}, while having little effect on the Michaelis constant, K_M, for both acetyl-CoA and isovaleryl-CoA (Table 1). The substitution of alanine for His391 and Ser338 also causes the enzyme to lose substrate preference. As shown in Table 1, wild-type enzyme prefers isovaleryl-CoA to acetyl-CoA with a catalytic efficiency ratio of 17. In H391A and S338A, the ratio of catalytic efficiency is reduced to 0.5 and 1.9, respectively. Wild-type enzyme also has identical catalytic efficiencies for acetyl-CoA at pH 7.5 and pH 9.0 (Table 2). H391A has higher catalytic efficiency at pH 9.0 (about 3.3-fold) than at pH 7.5. The drastic decrease and pH dependence of activity agree with the proposed role of H391 as a general base. The base species in the buffer solution (hydroxide ion or/and Tris base) may substitute the function of the imidazole group of His391 in H391A.

Change of Substrate Specificity. Mutagenesis was carried out on several hydrophobic amino acids to identify the key residues conferring the substrate specificity of BCKAD-E2c. The choice of target amino acids was based on the sequence alignment (Figure 4) and the crystal structure of the E2 catalytic domain of *A. vinelandii* PDC (Mattevi et al., 1992, 1993). Two principles were applied: (1) The target amino acids are those conserved (or functionally conserved) in the BCKAD subfamily and have smaller hydrophobic side chains than their counterpart residues in the PDC subfamily. (2) Their counterpart residues in E2 of PDC are located in the active-site channel, especially near the active-site histidine and serine. Several were chosen; they were A348, V351, G360, and A389. The active-site structure of the PDC E2c–CoA complex (Figure 5) shows that all the counterpart residues (F568, I571, L580, and Y608) in PDC E2c are close to the active-site histidine and serine and the thiol group of CoA, and their side chains constitute the surface of the active-site channel except L580, which points to the interior of the protein. A348 was changed to serine, phenylalanine, valine, and leucine. V351 was changed to isoleucine, and G360 to leucine. A389 was changed to cysteine, and to phenylalanine. Multiple mutations were also performed.

The kinetic constants of the wild-type and various mutant E2 catalytic domains were determined at pH 7.5 and are shown in Table 3. In general, the catalytic constant is affected more profoundly than the Michaelis constant by the amino acid substitution. The mutations of Val351 → Ile and Gly360 → Leu slightly increased the catalytic efficiencies toward isovaleryl-CoA and acetyl-CoA with a small change in substrate preference compared to the wild-type E2c. Substitution of Ala389 with Cys or Phe decreased the activity for both substrates; nevertheless, the reduction with isovaleryl-CoA is more than that with acetyl-CoA. This result implies that the size of the side chain of the amino acid in position 389 has an effect on discriminating different acyl-CoA. Substitution of Ala348 with serine decreased the catalytic efficiency toward acetyl-CoA; therefore, A348S was even more specific for isovaleryl-CoA. However, when Ala348 was changed to Val or Leu, the catalytic efficiency for acetyl-CoA increased or was unaffected, but that for isovaleryl-CoA decreased. This differential effect for different substrates results in the loss of substrate preference in A348V. When Ala348 was changed to Phe, the catalytic efficiencies for both acyl-CoAs were reduced. The lesser reduction in acetyl-CoA results in a slight shift in substrate preference. The results of mutation in Ala348 indicate that the size of the side chain of amino acid 348 plays an important role in the selectivity of substrate. A double mutant of E2c, A348F/A389F, shows that the shifts in substrate preference caused by each individual mutation are additive. The amino acid sequence of E2c of mammalian BCKAD is more homologous to that of E2c of Gram-negative bacteria PDC than to that of E2c of mammalian PDC (Russell & Guest, 1991). To investigate whether the neighboring residues in the primary sequence of Ala348

Table 1: Kinetic Constants of Variant E2c for Acetyl-CoA and Isovaleryl-CoA[a]

	isovaleryl-CoA			acetyl-CoA			
	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	$[k_{cat}/K_{M(Iv\text{-}CoA)}]/[k_{cat}/K_{M(Ac\text{-}CoA)}]$
WT	75	130	1.7	210	22	0.1	17
H391A	200	0.07	3.5×10^{-4}	490	0.34	6.9×10^{-4}	0.5
S338A	150	0.1	10×10^{-4}	270	0.14	5.2×10^{-4}	1.9

[a] Reactions occurred in 50 mM Tris buffer (pH 9.0). The concentration of dihydrolipoamide in the reaction mixture was 0.7 mM to reduce the spontaneous acetyl transfer reaction. The kinetic results are the average of two separate experiments with one preparation each of the purified wild-type and mutant enzymes.

Table 2: Catalytic Efficiencies, k_{cat}/K_M, of Variant E2c for Acetyl-CoA at Different pHs[a]

enzyme	pH 7.5	pH 9.0
WT	0.10	0.10
H391A	2.1×10^{-4}	6.9×10^{-4}
S338A	4.2×10^{-4}	5.2×10^{-4}

[a] Reactions occurred in 50 mM Tris buffer with 0.7 mM dihydrolipoamide in the reaction mixture. The kinetic results are the average of two separate experiments with one preparation each of the purified wild-type and mutant enzymes.

play a role in determining substrate specificity, the segment of amino acids from 347 to 351 was therefore changed to the sequence of *Azotobacter vinelandii* PDC. Although such a change reduced the preference for isovaleryl-CoA, the activity for both substrates decreased to a few percent of that of wild-type enzyme.

DISCUSSION

The crystal structure of the E2 catalytic domain from *A. vinelandii* showed that the 30 Å long active-site channel has two entrances; acetyl-CoA approaches from one end, while dihydrolipoamide enters from the other (Mattevi et al., 1993). The active-site histidine and serine, located in the middle of the channel, were proposed to catalyze the acyl transfer reaction by a mechanism analogous to that of CAT. On the basis of the alignment of the amino acid sequence of the E2 family (Figure 4), the active-site histidine and serine of bovine E2c of BCKAD are His391 and Ser338. The substitution of either one with alanine resulted in a drastic decrease in k_{cat} by 10^3–10^4-fold, but a marginal increase in K_M. The small change in K_M implies that the mutation did not cause a significant change in the conformation of the active site. The drastic decreases in activity in H391A and S338A compared to wild-type E2c agree with the proposed catalytic roles of His391 and Ser338 in the acyl transfer reaction. Moreover, the pH dependence of activity found in H391A is consistent with the proposed role of the active-site histidine acting as a general base. The change in the energy of interaction (ΔG_a) between the enzyme and transition state, due to the mutation of S338 → Ala, was calculated to be −4.5 kcal/mol based on the equation: $\Delta G_a = RT \ln\{(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\text{-}type}\}$ (Fersht, 1985). It has been suggested that, in protein–ligand interaction, 3–6 kcal/mol is the expected range of binding energy contributed by a hydrogen bond involving a charge

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E2bBOV   ---TLSNIGSIGGTYAKPVILPPEVAIGAL(362)----WSADHRII(394)---
E2bHUM   ---TLSNIGSIGGTFAKPVIMPPEVAIGAL(357)----WSADHRVI(389)---
E2bPpu   ---TLTSLGALGGIVSTPVVNTPEVAIVGV(366)----SSFDHRVV(397)---
E2pHUM   ---TISNLGMFGIKNFSAIINPPQACILAI(555)----LSCDHRVV(588)---
E2pRAT   ---TISNLGMFGIKNFSAIINPPQACILAI(414)----TLCDHRVV(446)---
E2pAvi   ---TISSLGHIGGTAFTPIVNAPEVAILGV(582)----LSYDHRVI(613)---
E2pEco   ---TISSIGGLGTTHFAPIVNAPEVAILGV(574)----LSFDHRVI(605)---
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FIGURE 4: Alignment of partial amino acid sequences of dihydrolipoamide transacylases. Aligned E2 sequences were taken from Russell and Guest (1991) to depict conservation in the region containing A348, S338, and H391, with the modification that several single gaps between residues are eliminated. E2b and E2p represent E2 of BCKAD and of PDC, respectively. BOV, HUM, RAT, Ppu, Avi, and Eco are abbreviations for bovine, human, rat, *Pseudomonas putida, Azotobacter vinelandii,* and *E. coli*, respectively. The boldface letters in the sequence of the bovine BCKAD complex indicate the amino acids changed in this study. Dashed lines are unlisted residues.

donor or acceptor (Fersht, 1988). Therefore, the active-site serine may act as a hydrogen bond donor to the putative negatively charged transition state. Moreover, in the spontaneous acyl transfer reaction (Figure 2), the rate is higher when the R group is a more electron-withdrawing group (e.g., acetoacetyl-CoA), or lower when the R is a more electron-donating group (e.g., isovaleryl-CoA). On the basis of the transition state theory, the reaction rate depends on the energy difference (G_a) between the transition state and the ground state. The reduction of the energy difference (G_a) by the electron-withdrawing ability of the R group indicates that the reaction involves an increase of negative charge in the transition state.

A simplified mechanism is shown in Figure 6. The $N^{\epsilon 2}$ of imidazole of the active-site histidine abstracts a proton from the reactive thiol group of dihydrolipoamide. The nucleophilic sulfur, strengthened by the action of proton abstraction, attacks the carbonyl carbon of acyl-CoA and forms the putative tetrahedral intermediate. The energy of the transition state is lowered by the active-site serine, which provides a hydrogen bond to the charged transition state. The acyl transfer reaction is completed following the breakdown of the tetrahedral intermediate. It remains unclear whether the formation or the breakdown step of the tetrahedral intermediate is the rate-limiting step.

The wild-type E2c of bovine BCKAD preferentially catalyzes the acyl transfer reaction between isovaleryl-CoA and dihydrolipoamide (Tables 1 and 3). This study was intended to determine which residues of the enzyme are in contact with the isobutyl group of isovaleryl-CoA in the acyl transfer reaction. We asked whether the enlargement of the

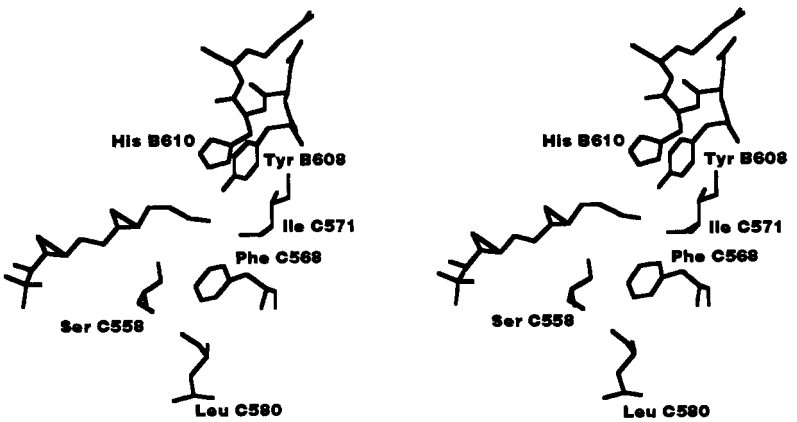

FIGURE 5: Structure of CoA and the active-site residues of E2c of PDC from *A. vinelandii*. The active-site channel is formed at the interface of two neighboring subunits (B and C in this figure). HisB610 and TyrB608 are from the B subunit, while SerC558, PheC568, IleC571, and LeuC580 are from the C subunit. Only the pantetheine arm of CoA is shown. The reactive thiol group of the pantetheine arm points toward the active-site amino acids. The coordinates were provided by Dr. Wim G. J. Hol at the University of Washington, Seattle, and visualized on the Silicon Graphics Computer with the aid of the INSIGHT II graphic program.

Table 3: Kinetic Constants of Variant E2c for Acetyl-CoA and Isovaleryl-CoA[a]

	isovaleryl-CoA			acetyl-CoA			
	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M	$[k_{cat}/K_{M(Iv\text{-}CoA)}]/[k_{cat}/K_{M(Ac\text{-}CoA)}]$
WT	110	400	3.6	370	47	0.13	29
V351I	32	190	5.9	410	72	0.18	34
G360L	56	480	8.6	190	77	0.41	21
A389C	110	150	1.4	250	22	0.088	16
A389F	110	27	0.25	320	4.0	0.013	20
A348S	66	200	3.0	350	19	0.054	56
A348F	130	18	0.14	410	3.2	0.0078	18
A348L	100	56	0.56	410	53	0.13	4.3
A348V	52	19	0.37	230	82	0.36	1.0
A348F/A389F	240	2.3	0.010	650	0.68	0.0010	9.2
Y347A/A348F/K349T/V351I	160	12	0.075	1400	7.8	0.005	13

[a] Reactions occurred in 100 mM Tris buffer (pH 7.5) with 2.5 mM dihydrolipoamide in the reaction mixture. The kinetic results are the average of two separate experiments with one preparation each of the purified wild-type and mutant enzymes.

size of the responsible residues might cause steric effect, which hinders the acyl transfer reaction with isovaleryl-CoA, and renders acetyl-CoA a better substrate for the reaction. Modeling studies based on the structure of enzyme–substrate complex suggested that Phe568 of E2c from *A. vinelandii* (corresponding to Ala348 in E2c of bovine BCKAD) may directly contact the methyl group of acetyl-CoA (Mattevi et al., 1993). When Ala348 was changed to phenylalanine, the substrate preference shifted slightly toward acetyl-CoA, and the catalytic efficiency remained 4% for isovaleryl-CoA and 6% for acetyl-CoA. To proceed the acyl transfer reaction, acyl-CoA and dihydrolipoamide must approach each other to allow the catalytic residues to function efficiently. The phenyl group in A348F may be too large for maintaining the critical distance between the catalytic residues and the reactive groups of acetyl-CoA and isovaleryl-CoA. Alternatively, the phenyl group in the mutant enzyme may block the approach of acyl-CoA and dihydrolipoamide. The side chain of Leu or Val affects the accommodation of isovaleryl-CoA, but has no adverse impact on acetyl-CoA. It is striking that a small change in the size of the side chain (A348V) can decrease the catalytic efficiency for isovaleryl-CoA about 10-fold and increase it for acetyl-CoA about 3-fold. The results indicate that substrate preference is sensitive to alterations in the size of the side chain of amino acid 348, and implicate Ala348 as a major structural determinant for preferential catalysis of isovaleryl-CoA by the bovine E2 of BCKAD. The mutations of Ala348 mainly affect the catalytic constants and not the Michaelis constants, indicating that the steric effect, rendered by the side chain of amino acid 348, influences the accommodation of acyl-CoA in the transition state rather than in the ground state.

Comparison of the activities of the variant E2c (A348V) of BCKAD to that of wild-type PDC-E2c from *Saccharomyces cerevisiae* (Niu et al., 1990) shows that the catalytic efficiency of A348V for acetyl-CoA is about 10% of that of PDC. The inability to convert BCKAD-E2 to PDC-E2 with the same catalytic efficiency indicates that certain other structural elements may be important for creating an optimal environment for efficient acyl transfer with acetyl-CoA. Such structural elements may be the nonconserved active-site residues. A distal region may also affect the substrate specificity. It has been shown that the change of surface loops, which are not the structural components of the active-site pocket, is necessary to convert trypsin to a chymotrypsin-like protease (Hedstrom et al., 1992).

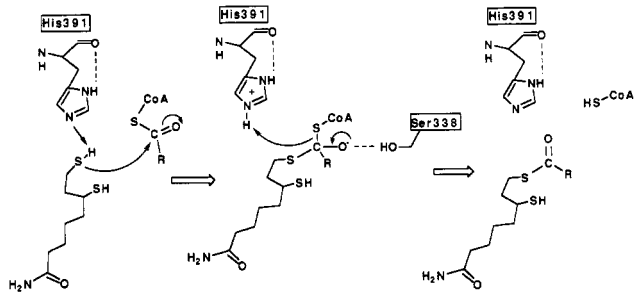

FIGURE 6: Schematic illustration of the proposed mechanism for the E2c-catalyzed acyl transfer reaction. His391 and Ser338 are active-site residues of the E2c domain of the bovine BCKAD complex. Substrate acyl-CoA and dihydrolipoamide are indicated. The dashed line represents the hydrogen bond.

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