Reaction Mechanism of the Heterotetrameric ($\alpha_2\beta_2$) E1 Component of 2-Oxo Acid Dehydrogenase Multienzyme Complexes†

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ABSTRACT: Pyruvate decarboxylase (E1) catalyzes the first two reactions of the four involved in oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase (PDH) multienzyme complex. It requires thiamin diphosphate to bring about the decarboxylation of pyruvate, which is followed by the reductive acetylation of a lipoyl group covalently bound to the N^6 amino group of a lysine residue in the second catalytic component, a dihydrolipoyl acetyltransferase (E2). Replacement of two histidine residues in the E1α and E1β chains of the heterotetrameric E1 ($\alpha_2\beta_2$) component of the PDH complex of *Bacillus stearothermophilus*, considered possible proton donors at the active site, was carried out. Subsequent characterization of the mutants permitted different roles to be assigned to these two particular residues in the reaction catalyzed by E1: E1α His271 to stabilize the dianion formed during decarboxylation of the 2-oxo acid and E1β His128 to provide the proton required to protonate the incoming dithiolane ring in the subsequent reductive acetylation of the lipoyl goup. On the basis of these and other results from a separate investigation into the roles of individual residues in a loop region in the E1α chain close to the active site of E1 [Fries, M., Chauhan, H. J., Domingo, G. J., Jung, H., and Perham, R. N. (2002) *Eur. J. Biochem. 270,* 861−870] together with work from other laboratories, a detailed mechanism for the E1 reaction can be formulated.

The pyruvate dehydrogenase (PDH)[1] multienzyme complex catalyzes the four-step oxidative decarboxylation of pyruvate to generate acetyl-CoA, concomitantly reducing NAD^+ to NADH. It is a member of a family of 2-oxo acid dehydrogenase (2OADH) multienzyme complexes that also includes the 2-oxoglutarate dehydrogenase (OGDH) and branched chain 2-oxo acid dehydrogenase (BCDH) complexes (for reviews, see refs *1* and *2*). For the most part, three main enzymes assemble to form a 2OADH complex: a thiamin diphosphate-dependent 2-oxo acid decarboxylase (E1), a dihydrolipoyl acyltransferase (E2), and an NAD-dependent flavoprotein, dihydrolipoyl dehydrogenase (E3).

The E2 component generates an icosahedral (60-mer) or octahedral (24-mer) assembly of E2 chains as the scaffold around which a 2OADH complex is assembled, the symmetry depending on the type and source of the complex. The E2 chain itself comprises three different types of domain joined by long extended but flexible linker regions: (from the N-terminus) one to three lipoyl domains in tandem array, each with a lipoyl group covalently bound to a specific lysine residue; a peripheral subunit-binding domain; and the core-forming catalytic (acyltransferase) domain responsible for transfer of the acyl group from the reductively acylated lipoyl group to CoA. It is the acyltransferase domain that aggregates to generate the inner core (icosahedral or octahedral) of the multienzyme complex (*3*, *4*). Cryoelectron microscopy of the PDH complexes from *Bacillus stearothermophilus* (*5*) and ox kidney (*6*) has revealed that this E2 inner core is surrounded by an outer shell of E1 and E3 components, with the lipoyl domains confined to the annular space between them where they must make successive journeys between the three types of active sites (E1−E3), which are physically far apart (*2*, *5*, *6*).

The E1 component catalyzes two reactions: the irreversible thiamin diphosphate (ThDP)-dependent decarboxylation of the 2-oxo acid followed by the reductive acylation of the pendant lipoyl group proffered by the E2 chain. The reaction catalyzed by E1 is rate-limiting for the overall activity of

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[1] Abbreviations: BCDH, branched chain 2-oxo acid dehydrogenase; BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenolindophenol; E1, 2-oxo acid decarboxylase; E1α, α-subunit of E1; E1β, β-subunit of E1; E1p, pyruvate decarboxylase (EC 1.2.4.1) of the PDH complex; E2p, dihydrolipoyl acetyltransferase (EC 2.3.1.12); E3, dihydrolipoyl dehydrogenase (EC 1.8.1.4); IPTG, isopropyl β-D-thiogalactopyranoside; OGDH, 2-oxoglutarate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PDH, pyruvate dehydrogenase; PSBD, peripheral subunit binding domain; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance; ThDP, thiamin diphosphate.

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the complex (*7*, *8*), probably at the level of a slow reductive acylation step (*8*, *9*). E3 concludes the sequence by catalyzing the reoxidation of the dihydrolipoyl group remaining after the acyl transfer to CoA, with the concomitant reduction of NAD^+ (*10*). Only lipoic acid covalently attached to the lipoyl domain from its partner E2 chain will serve as a substrate for E1, the attachment of the prosthetic group causing the value of k_{cat}/K_m to increase by a factor of 10^4 and conferring the specificity for the cognate E1 that underlies substrate channeling (*9*, *11*). Thus, the lipoyl domain is the true substrate for the reaction (*2*).

Enzymes that use ThDP as a cofactor are found in all organisms where they catalyze reactions involving the cleavage of a carbon−carbon bond adjacent to an oxo group. In the 50 years since its discovery as a cofactor in pyruvate decarboxylase, much progress has been made in our understanding of how thiamin functions in the active sites of ThDP-dependent enzymes (*12*). The mechanism is common and originates in the generation of the thiamin C2 carbanion, no permanently existing carbanion species of the enzyme-bound cofactor having been detected (*13*). Three essential and evolutionarily conserved elements are known to be required: (1) the pyrimidine N1′ atom juxtaposed with a glutamate side chain, (2) the 4′-amino group of the thiamin, and (3) the V conformation of the ThDP cofactor, which brings the 4′-amino group into direct contact with the C2−H bond. Molecular modeling of the mode of action of yeast pyruvate decarboxylase has given us a good idea of how the first part of ThDP-dependent reactions is carried out (*14*). In all ThDP-dependent enzymes, there is a common sequence motif of ~30 residues (*15*) that generates a common structural motif (*16*) involved in binding the cofactor, but otherwise, there appears to be no set of conserved residues in these active sites not directly involved in binding to the cofactor (*17*).

The reaction mechanism of the E1 component of 2OADH complexes has been the subject of many studies. Alanine replacement of residues surrounding a reversible phosphorylation site (Ser293) in the E1α chains of the E1 ($\alpha_2\beta_2$) component of the rat BCDH complex, implicated in the reversible control of E1 activity in this and other eukaryotic BCDH and PDH complexes, provided evidence of the involvement of histidine, arginine, and aspartate residues in catalysis (*18*). Attention was focused more recently on residues in a loop in the E1α chains of E1 ($\alpha_2\beta_2$) from the *B. stearothermophilus* PDH complex that is particularly susceptible to limited proteolysis (*19*). Systematic replacement of these residues has indicated that they form part of a conserved sequence motif that lies ~50 amino acids to the C-terminal side of the ThDP binding motif, with Arg267 involved in binding the carboxyl group of the 2-oxo acid substrate and Tyr281 and, to a lesser extent, Asp276 and Arg282 affecting the decarboxylation of pyruvate and the reductive acetylation of the tethered lipoyl domain in the active PDH complex (*20*). From the two crystal structures of the homologous E1 ($\alpha_2\beta_2$) from *Pseudomonas putida* (*21*) and human (*22*) BCDH complexes, this region can be placed in the funnel-shaped active site between the E1α and E1*β* subunits, at the bottom of which lies the ThDP (Figure 1).

In the most detailed reaction mechanism to date for the E1 component of 2OADH complexes advanced by Pan and Jordan (*23*), a proton donor residing on E1 is required to protonate S6 of the incoming dithiolane ring in the reductive acylation of the lipoyl group. On the basis of the crystal structures of E1, residues E1α His271 and E1*β* His128 (using *B. stearothermophilus* numbering for convenience here) were identified as possible candidates for this potential proton donor, close in space to the reactive C2 of the ThDP (*21*, *22*). In this paper, we describe the selective replacement of these two histidine residues in *B. stearothermophilus* E1, enabling us to identify His128*β* as a residue playing an essential part as the proton donor and to assign to His271α a less important role, most likely stabilizing the energetically unfavorable dianion formed after nucleophilic attack of ThDP at the 2-oxo group of the substrate.

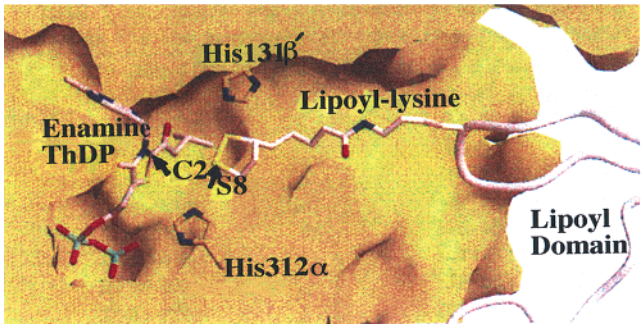

FIGURE 1: Lipoyl-lysine swinging arm of the E2 lipoyl domain visiting the active site of *P. putida* E1b (after ref *21*). The model represents the interaction of the lipoyl domain with E1 just before the reductive acetylation of the dithiolane ring catalyzed by E1. The E1 structure is shown as a solid that has been sliced to disclose the channel leading to the active site, at the interface between the α-subunit and the *β*-subunit. The side chains of the two histidine residues under investigation are labeled. Also labeled are the 2-(1-hydroxyethylidene)-ThDP intermediate, and the incoming lipoyl domain with the lipoyl-lysine arm fully extended down the active site channel. In *B. stearothermophilus* E1p, the residues corresponding to His131*β* and His312α are His128*β* and His271α, respectively.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from Pharmacia Biotech (St. Albans, U.K.) or New England Biolabs (Hitchin, U.K.); *Pfu* DNA polymerase was from Stratagene (Cambridge, U.K.) and T4 DNA ligase from Promega (Southampton, U.K.). IPTG and PMSF were purchased from Melford Laboratories (Chelsworth, U.K.) and bacteriological media from Beta Lab (West Molesely, U.K.) and Duchefa (Haarlem, The Netherlands), and ampicillin was from Beecham Research Laboratories (Brentford, U.K.). Pyruvate, NAD^+, ThDP, DCPIP, CoA, and FAD were from Sigma (Poole, U.K.). Centrifugal filter devices were purchased from Millipore.

Bacterial Strains and Plasmids. Escherichia coli host strain TG1recO and plasmids pKBstE1a and pKBstE1b expressing genes encoding the *B. stearothermophilus* E1α and E1*β* subunits, respectively, have been described previously (*24*). *E. coli* host strain BL21(DE3) [B, F^-, *omp*T, *hsd*S_B, (r_B-, m_B-), *gal*, *dcm*, (DE3)] from Novagen (Madison, WI) was employed to express the genes encoding the E3 (from pBSTNAV/E3) and E2 (from pETBstE2) components of the *B. stearothermophilus* PDH complex (*25*) and a subgene encoding a didomain (from pET11DD) that comprises the lipyol domain, the peripheral subunit-binding domain, and the linker region between them (residues 1−171 of the *B.*

stearothermophilus E2p chain) (*26*). Lipoate-protein ligase A was purified from *E. coli* BL21(DE3) cells transformed with the plasmid TM202 (*27*).

Recombinant DNA Techniques and Mutagenesis. Recombinant DNA techniques were carried out as described elsewhere (*28*). DNA fragments were isolated from gels using the QIAquick gel extraction kit, and plasmids were prepared by means of the QIAGEN plasmid kit, both from Qiagen (Hilden, Germany). Site-specific mutations were introduced into the plasmid pKBstE1a using splicing-by-overlap extension PCR (*29*). The fidelity of the amplified DNA fragments was established by automated DNA sequence analysis after subcloning into the vector.

Protein Purification. Wild-type and mutant E1s (*24*, *30*) and wild-type E2 and E3 (*30*) were purified as described previously. The E2 didomain was purified essentially as described in ref *31*. The apo forms of the E2 component and of the didomain were lipoylated using recombinant *E. coli* lipoate-protein ligase A (*32*, *33*).

Enzyme Assays. The E1 component was assayed for catalytic activity by means of two separate assays.

(1) DCPIP Assay. The DCPIP assay measures the rate of reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP) by the E1 component with pyruvate as a substrate (*34*). The decrease in A_{600} was monitored at 30 °C in a mixture of 0.2 mM ThDP, 2 mM $MgCl_2$, 50 μM DCPIP, 100 mM potassium phosphate (pH 7.0), and 20 μg of E1. The reaction was started by the addition of pyruvate (final concentration of 400 μM) after the assay mixture had been incubated for 10 min at 30 °C.

(2) PDH Assay. The PDH assay measures the rate of formation of NADH at 340 nm and 30 °C after an intact PDH complex had been reconstituted from its single components (*7*, *32*, *35*). The final assay mixture contained 0.2 mM ThDP, 1 mM $MgCl_2$, 2.6 mM cysteine HCl, 2 mM pyruvate, 0.13 mM CoA, 50 mM potassium phosphate (pH 7.0), and the reconstituted PDH complex. The E1 ($\alpha_2\beta_2$):E2 (chain):E3 (α_2 dimer) molar ratio was 3:1:3, and the amount of E2 used per assay was 0.5, 1.0, 1.5, and 2.0 μg. The reaction was started by adding pyruvate and CoA after the reconstituted PDH complex had been incubated for at least 3 min at 30 °C, all as described elsewhere (*32*). Specific activities are expressed as units (micromoles of NADH formed per minute) per milligram of E2 in the assay.

Determination of Kinetic Parameters. The values of K_m for pyruvate and ThDP were determined by using the DCPIP assay, varying the concentrations of pyruvate from 0.2 to 4000 μM and the ThDP concentrations from 0.04 to 200 μM. When the kinetic parameters for ThDP were being determined, the pyruvate concentration was kept at 1 mM. Data were analyzed and fitted to a Michaelis−Menten curve using Sigma Plot.

Temperature Dependence of Catalytic Activity. The dependence of the catalytic activity of E1 on temperature was investigated using the DCPIP assay over a range of 25−85 °C. Assay mixtures were incubated for exactly 10 min at the relevant temperature before the reaction was started by the addition of pyruvate and monitored by observing the decrease in A_{600}.

Interaction of E1 with the Peripheral Subunit-Binding Domain. The formation of a complex between E1 and the didomain of E2 was investigated by submitting appropriate mixtures to nondenaturing PAGE using the Pharmacia Phast System. The kinetics of the interaction of the didomain with E1 was investigated using surface plasmon resonance detection (BIAcore, Pharmacia Biosensor AB). Both experiments were carried out as described in detail elsewhere (*19*, *36*).

Table 1: Specific Catalytic Activities of Wild-Type and Mutant E1s in the DCPIP and PDH Assay

	DCPIP assay		PDH assay	
	units/mg	%	units/mg of E2	%
wild type	0.128 ± 0.004	100	12.4 ± 0.3	100
E1αH271A	0.74 ± 0.02	578	0.83 ± 0.03	7
E1βH128A	0.006 ± 0.001	5	0	0

RESULTS

Generation and Purification of E1 Mutants. Point mutations H128βA and H271αA were introduced into the pKBstE1a (for E1α) or pKBstE1b (for E1β) expression vector on the basis of sequence alignments and structure modeling that identified these histidine residues in *B. stearothermophilus* E1 as being equivalent to His131β and His312α in the crystal structure of the homologous E1 ($\alpha_2\beta_2$) from *P. putida* (*21*). The relevant DNA fragments generated for splicing-by-overlap-extension PCR were digested with *Nco*I and *Hin*dIII to be ligated into pKBstE1a and with *Pst*I and *Hin*dIII to be ligated into pKBstE1b. Mutant and wild-type *B. stearothermophilus* E1α and E1β subunits were purified from *E. coli* TG1*recO* cells and reconstituted to form E1 ($\alpha_2\beta_2$). During purification, no anomalous behavior was observed.

Effect of the Mutations on Catalytic Activity. The effect of the mutations on the catalytic activity was examined by means of the DCPIP and the PDH assays (Table 1). The DCPIP assay measures E1 activity in the presence of an artificial electron acceptor, DCPIP, in place of lipoamide (i.e., two successive one-electron steps instead of one two-electron step); the PDH assay measures the normal catalytic activity of a PDH complex reconstituted from recombinant E2 and E3 and the E1 mutants.

In the DCPIP assay, E1 ($\alpha_2\beta_2$) reconstituted from the E1αH271A mutant displayed a catalytic activity almost 6 times higher than that of wild-type E1. In marked contrast, the E1βH128A mutant retained only 5% of wild-type activity.

In the PDH assay, the complex reconstituted with the E1αH271A mutant was severely impaired, retaining only 7% of wild-type activity, but that with the E1βH128A mutant was totally inactive. In both assays, this behavior resembles that of E1 with proteolytic cleavages (*19*) or amino acid replacements (*20*) in a loop region of E1α at the entrance to the active site.

Kinetic Parameters of the E1 Mutants. Kinetic parameters for pyruvate and ThDP were determined for the E1α mutants by means of the DCPIP assay (Table 2). The values of k_{cat} and K_m for wild-type E1 were essentially the same as those measured previously (*24*). The kinetic constants for the E1βH128A mutant could not be determined, because of its very low catalytic activity. The E1αH271A mutant displayed a K_m for pyruvate 450-fold higher than that of wild-type E1. This caused a substantial drop in catalytic efficiency, as

Table 2: Kinetic Parameters of Wild-Type and Mutant E1s Determined by Means of the DCPIP Assay

	k_{cat} for pyruvate (s^{-1})	K_m for pyruvate (μM)	k_{cat}/K_m for pyruvate ($\times 10^3$ M^{-1} s^{-1})	k_{cat} for ThDP (s^{-1})	K_m for ThDP (μM)	k_{cat}/K_m for ThDP ($\times 10^3$ M^{-1} s^{-1})
wild type	0.48 ± 0.01	1.1 ± 0.1	427	0.46 ± 0.01	23 ± 2	20
E1αH271A	3.80 ± 0.02	442 ± 9	9	2.49 ± 0.03	2.6 ± 0.2	950

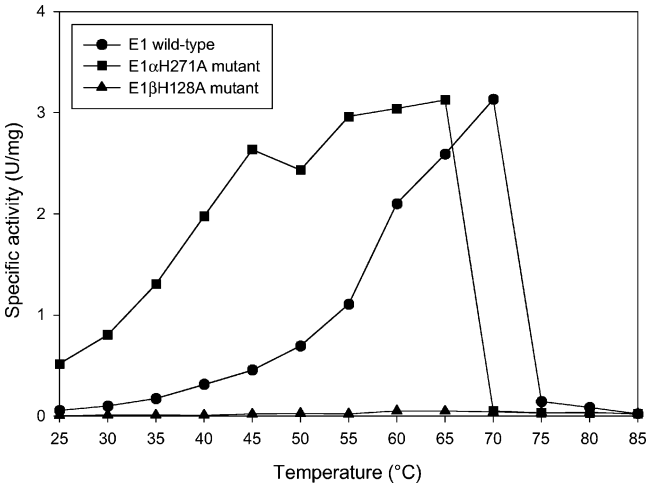

FIGURE 2: Temperature dependence of the catalytic activity in the DCPIP assay of wild-type and mutant E1s.

depicted by the value of k_{cat}/K_m, despite the turnover rate (k_{cat}) of the E1αH271A mutant being some 8 times higher than that of the wild type.

Surprisingly, the apparent K_m for ThDP of the E1αH271A mutant was found to be 2.6 μM, compared with the wild-type value of 23 μM. Thus, the k_{cat}/K_m ratio was significantly increased in this instance.

Temperature Dependence of E1 Activity. To investigate the dependence of E1 activity on temperature, the DCPIP assay was carried out over a temperature range of 25−85 °C (Figure 2). The reaction mixture containing E1 ($\alpha_2\beta_2$) was incubated for exactly 10 min at the relevant temperature, and the reaction was then initiated by adding pyruvate. The E1βH128A mutant exhibited a very low activity over the whole temperature range. However, the E1αH271A mutant demonstrated an increased specific activity up to 45 °C, after which it leveled off until it was inactivated at 70 °C, 5 °C below wild-type E1.

Binding of E1 to the Peripheral Subunit-Binding Domain of E2. The E1 component of the PDH complex of *B. stearothermophilus* is bound to the E2 core mainly by its interaction with the PSBD of the E2 chain. When the mutant E1s exposed to an excess of didomain (lipoyl domain and PSBD, joined by the natural linker region) were examined by means of nondenaturing PAGE (*35*), in neither instance could they be distinguished from wild-type E1 in their ability to bind to the PSBD, as judged by this "band-shift" assay (Figure 3). SPR detection was used to determine the kinetic parameters of the interaction with the PSBD. For this purpose, the lipoylated didomain was immobilized on the detector chip by means of the lipoyl group, leaving the PSBD free to interact with the E1 flowing over it (*35*). Again, no significant differences from wild-type E1 ($k_{on} = 3.27 \times 10^{-6}$ M^{-1} s^{-1}, $k_{off} = 1.06 \times 10^{-3}$ s^{-1}, and $K_d = 3.24 \times 10^{-10}$ M^{-1}) were noted (Table 3).

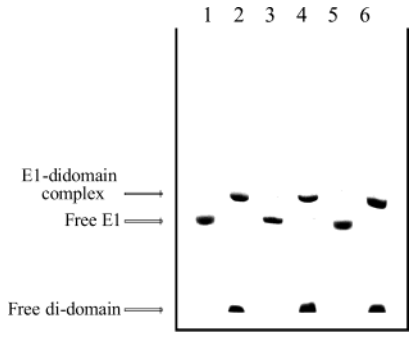

FIGURE 3: Nondenaturing gel electrophoresis of wild-type and mutant E1s with the didomain at a 16-fold molar excess of the didomain over E1: lane 1, wild-type E1; lane 2, wild-type E1 and the didomain; lane 3, E1αH271A mutant; lane 4, E1αH271A mutant and the didomain; lane 5, E1βH128A mutant; and lane 6, E1βH128A mutant and the didomain.

Table 3: Binding Constants for the E1−PSBD Interaction As Determined by SPR

	k_{on}/k_{on}(wild type)	k_{off}/k_{off}(wild type)	K_d/K_d(wild type)
wild type	1.0	1.0	1.0
E1αH271A	0.85	0.93	1.09
E1βH128A	0.87	1.14	1.31

DISCUSSION

The effects of the two mutations, E1αH271A and E1βH128A, in the E1 component of the *B. stearothermophilus* PDH complex described above make it possible to assign to them particular roles in the reaction mechanism of E1. Both are clearly important, and their effects are wholly attributable to changes in the active site of E1 since there was no detectable change in the interaction with E2 (Figure 3 and Table 3). Nor was there any particular effect of temperature on the catalytic activity to cause concern. The E1αH271A mutation did not totally inhibit the enzyme. Indeed, in the DCPIP assay, the activity with this artificial electron acceptor increased 6-fold, but in the overall PDH complex assay, the activity fell by slightly more than 90% (Table 1). This sort of behavior has been noted and discussed before, in studies of proteolytic cleavages (*19*) and amino acid replacements (*20*) in a loop region of the E1α chain at the entrance to the active site of the E1 heterotetramer. It was noted then that such effects may be induced by the DCPIP assay being one in which an artificial electron acceptor replaces the lipoyl domain, whereas the PDH complex activity is a measure of the ability of E1 to catalyze the reductive acetylation of the tethered lipoyl domain in the assembled complex. The K_m for pyruvate in the E1αH271A mutant was increased 450-fold (Table 2), suggesting that this imidazole side chain may have some part to play in recognizing the 2-oxo acid. Replacement of the corresponding residue in the heterotetrameric ($\alpha_2\beta_2$) rat E1b with alanine was reported to cause a loss of catalytic activity as the enzyme could not reconstitute with ThDP (*18*). It could function as a base in the ThDP activation process, according

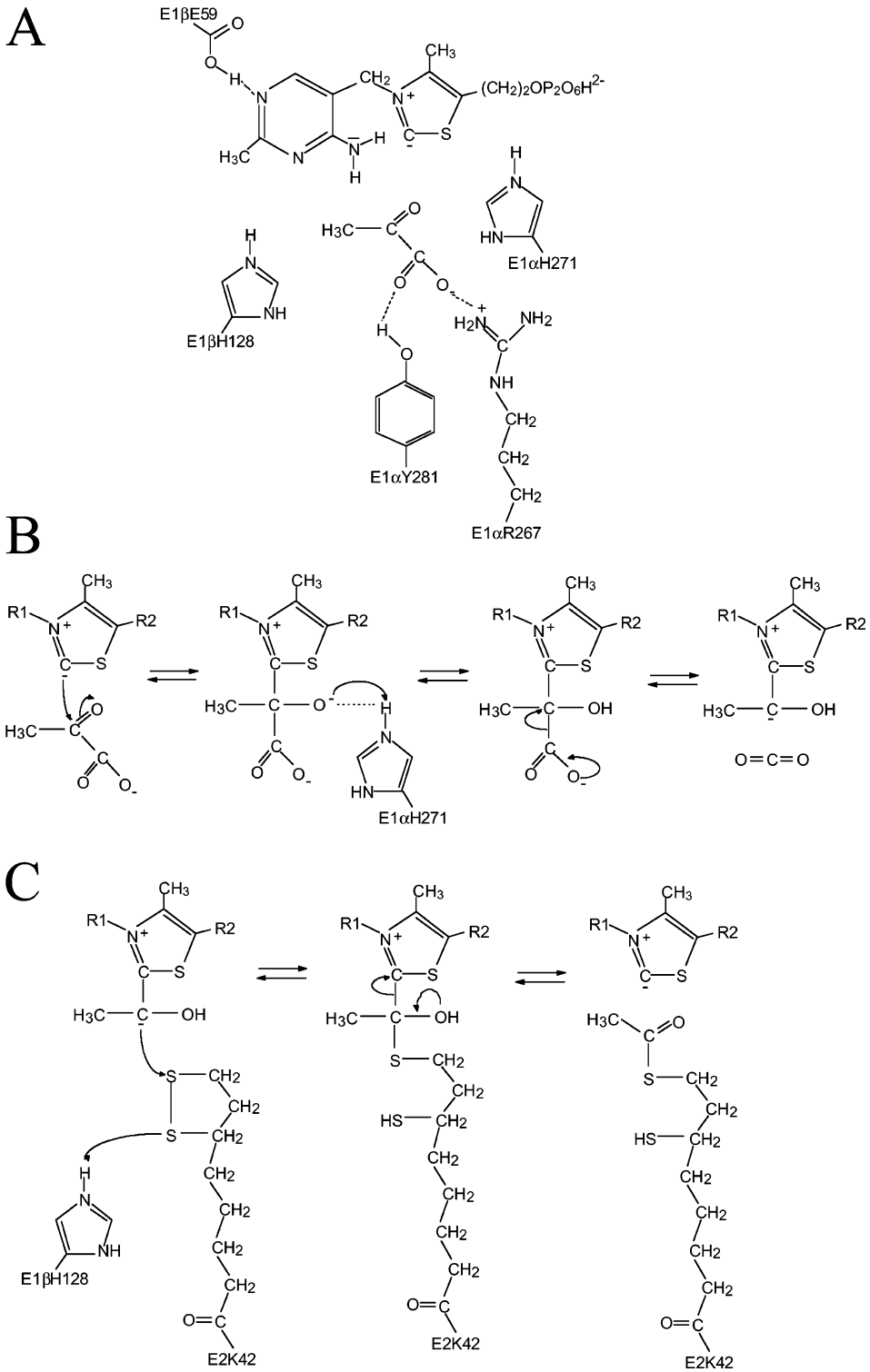

FIGURE 4: Proposed mechanism for the reaction catalyzed by the E1 component of 2-OADH complexes. The numbering refers to the amino acid sequence of the heterotetrameric ($\alpha_2\beta_2$) E1 component of the PDH complex of *B. stearothermophilus*.

to a mechanism proposed by Lindqvist et al. (*37*). However, the lowering of the apparent K_m for ThDP observed here for the *B. stearothermophilus* E1αH271A mutant (Table 2) would argue against such a direct involvement in the binding or the activation process. Further, there is good evidence that this step is catalyzed by ThDP itself (*12*, *38*).

A more attractive possibility is that E1α His271 is involved in proton transfer and stabilization of the transition state during the decarboxylation of the 2-oxo acid. Inspection of the two heterotetrameric E1 ($\alpha_2\beta_2$) crystal structures determined to date (*21*, *22*) suggests to us that it would be in a suitable position to stabilize the negative charge of the carbonyl oxygen, which develops after nucleophilic attack of the ThDP carbanion on the 2-oxo acid.

The E1βH128A mutation turned out to have a very severe effect on the E1-catalyzed reaction, totally inhibiting the PDH complex activity. It is known that E1 catalyzes the rate-limiting reaction in the PDH complex, probably the reductive acetylation of the lipoyl group on the lipoyl domain (*7*−*9*). More recent studies on a chemical model have indicated the

necessity for a proton donor in this part of the reaction, to protonate S6 of the incoming dithiolane ring of the lipoyl group (*23*). Our results strongly suggest that this proton donor is E1β His128, which in the crystal structures of heterotetrameric E1 is located in a suitable position, close to the thiazole of the ThDP (see Figure 1). Why the E1 activity is much diminished in the DCPIP assay by this mutation (Table 1) is not immediately apparent. It is conceivable that E1β His128 has an additional role in an earlier step of the E1 reaction, in either the ThDP activation process or the oxidative decarboxylation, perhaps complementing E1α His271. E1β His128 may conceivably interact directly with DCPIP. The DCPIP assay, which simulates the E1 reaction in the decarboxylation of pyruvate, follows a ping-pong mechanism (*34*), as does the reaction of E1 in the reductive acetylation of the lipoyl domain (*39*).

On the basis of the results presented here and a separate examination of the roles of amino acids [residues Arg267−Tyr282 in the loop region of the E1α chain leading into the active site of *B. stearothermophilus* E1 (*19*, *20*)], it is possible to deepen our understanding of the mechanism of the E1 reaction, following on that summarized by Pan and Jordan (*23*). This is summarized in Figure 4. At the beginning of the reaction, the active site of E1 is in an open conformation (*20*) with ThDP bound. The reactive C2 carbanion is generated according to the mechanism reviewed by Schellenberger (*12*), with a conserved glutamate (residue E1β Glu59 of the PDH complex from *B. stearothermophilus*) close to N1′ of ThDP involved in this step. As shown by Kern et al. (*13*), the contribution of the enzyme is to accelerate the rate at which this activation takes place. The 2-oxo acid (pyruvate) binds to the active site; E1α Arg267 contributes crucially to the binding by interacting with the carboxyl group, and E1α Tyr281 may help bind the 2-oxo acid by providing a hydrogen bond to one of the oxygens of the carboxyl group (*20*). The active site then closes, developing the hydrophobic environment and bringing the 2-oxo carbon of the substrate into the functional proximity of the C2 carbanion of ThDP. After the nucleophilic attack of the carbanion at the 2-oxo carbon of the substrate, an energetically unfavorable dianion is formed, which is stabilized and protonated by E1α His271 of the *B. stearothermophilus* PDH complex. This intermediate is converted to 2-(2-hydroxypropionyl)-ThDP, and decarboxylation yields the carbanion enamine of 2-(1-hydroxyethyl)-ThDP. The active site then reopens, allowing release of the first product, CO_2, and transient binding of the second substrate, the lipoylated lipoyl domain (*11*). Reductive acylation commences with activation of the dithiolane ring of the lipoyl group by a key proton donor (*23*), which is E1β His128 in the case of the PDH complex from *B. stearothermophilus*. This promotes nucleophilic attack of the 2-hydroxyethyl-ThDP at S8 of the lipoyl group, resulting in formation of a tetrahedral adduct, which collapses to yield the reductively acylated lipoyl domain, and regenerates the ThDP cofactor.

It has been postulated (*40*) that the E1α subunit of E1 ($\alpha_2\beta_2$) of the PDH complex from ox kidney catalyzes the first part of the E1 reaction, whereas the E1β subunit catalyzes the second part. Although it is firmly established that a glutamate in the β-subunit of E1 is crucial for the activation of ThDP, this postulate could still hold if activation of ThDP, decarboxylation of the 2-oxo acid, and reductive acylation are regarded as distinguishable processes. Both subunits contribute to the activation of ThDP to form the C2 carbanion, but the decarboxylation is essentially carried out by E1α; on the other hand, E1β is more responsible for the reductive acylation.

It has recently been reported (*41*) that a histidine residue in the active site of the dimeric E1 component of the *E. coli* PDH complex (*42*) plays a role in protonating a sulfur atom in the incoming dithiolane ring during the reductive acetylation of the pendant lipoyl group on its cognate lipoyl domain. Thus, it appears to be the direct analogue of E1β His128 in the heterotetrameric E1 described here. Moreover, a comparable histidine residue could well adopt the role of proton donor in the "ligation" reaction catalyzed by transketolases (*41*), a pretty example of conservation of active site chemistry in enzymes catalyzing superficially different reactions.

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