Identification of the catalytic glutamate in the E1 component of human pyruvate dehydrogenase

Rui Fang[a], Peter F. Nixon[b], Ronald G. Duggleby[b,*]

[a] *Department of Molecular Biochemistry, Jilin University, Changchun 130023, PR China*
[b] *Centre for Protein Structure, Function and Engineering, Department of Biochemistry, University of Queensland, Brisbane, QLD 4072, Australia*

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Abstract The pyruvate dehydrogenase complex catalyzes the conversion of pyruvate to acetyl-CoA. The first component (E1) converts pyruvate to bound acetaldehyde using thiamine diphosphate (ThDP) and Mg^{2+} as cofactors. There is no 3D structure of E1 available but those of other ThDP-dependent enzymes show some similarities including a glutamate residue that assists in ThDP activation. Eukaryotic E1 has an $\alpha_2\beta_2$ structure and the conserved Glu^{89} of the β-subunit was identified as a possible catalytic residue by sequence alignment. Human E1 was expressed in *Escherichia coli* and purified. Mutating Glu^{89} to glutamine, aspartate and alanine markedly reduces catalytic activity and the affinity for ThDP, consistent with a role as the catalytic glutamate.

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Key words: Active site; Catalytic mechanism; Enzyme activity; Pyruvate dehydrogenase; Site-directed mutagenesis; Thiamine diphosphate

1. Introduction

The pyruvate dehydrogenase complex plays a central role in the process and regulation of glucose metabolism in both prokaryotes and eukaryotes. It catalyzes the irreversible oxidative decarboxylation of pyruvate and synthesis of acetyl-CoA:

$$\text{pyruvate} + \text{HS-CoA} + \text{NAD}^+ \rightarrow$$

$$+\text{CH}_3\text{CO-S-CoA} + \text{H}^+ + \text{CO}_2$$

The core structure of the complex contains multiple copies of three enzymes [1,2]: E1 (pyruvate dehydrogenase, EC 1.2.4.1), E2 (dihydrolipoyl transferase, EC 2.3.1.12) and E3 (dihydrolipoyl dehydrogenase, EC 1.8.1.4). E1 catalyzes the first and the rate-limiting step [3] of the overall reaction, decarboxylation of pyruvate and the oxidative transfer of an acetyl group to the lipoyl moiety of E2, using thiamine diphosphate (ThDP) and magnesium ion as cofactors. In eukaryotes and Gram-positive bacteria, E1 is a heterotetramer of two α- and two β-subunits. The activity of E1 is regulated by phosphorylation (inactivation catalyzed by pyruvate dehydrogenase kinase, EC 2.7.1.99) and dephosphorylation (activation catalyzed by pyruvate dehydrogenase phosphatase, EC 3.1.3.43) of the α-subunit. The ratio of the active to the inactive forms of E1 is regulated by both nutritional status and hormones (for review see [4]). Mutations in the α-gene of E1 are responsible for severe defects of glucose metabolism with syndromes such as fatal neonatal lactic acidosis, psychomotor retardation with or without neurodegeneration and a male-only syndrome of ataxia, mild mental retardation and carbohydrate sensitivity (for review see [5]). Many chemical modification studies of E1 have been performed. Cysteine [6–8], histidine [7], tryptophan [9,10], arginine [11,12] and lysine [13] residues have been suggested to be located in or near the active site, but the role of these residues in the mechanism of E1 is far from clear.

Since the pioneering work of Breslow [14], it has been generally accepted that ThDP-dependent enzymes apply the same chemical mechanism to catalyze a wide range of reactions. The dipolar carbanion (or ylid) is the active form of the coenzyme, and the negatively charged C2 initiates a nucleophilic attack on the carbonyl carbon of the substrate. The determination of the X-ray structures of transketolase [15], pyruvate oxidase [16,17], pyruvate decarboxylase [18,19] and benzoylformate decarboxylase [20] has clarified the mechanism of enzymatic catalysis. These crystal structures reveal some common features that are necessary for the binding and activation of ThDP. In each case, the ThDP-binding site is located in a deep cleft at an interface between subunits, such that residues from both subunits interact with the cofactor and are required for binding. The ThDP-binding motif identified by Hawkins et al. [21] and subsequently found in all known ThDP-dependent enzymes [22] is responsible for the binding of a metal ion that anchors ThDP through its phosphate groups. This motif is located in the C-terminal domains of pyruvate oxidase, pyruvate decarboxylase and benzoylformate decarboxylase but is in the N-terminal domain of transketolase. ThDP is bound in the 'V' conformation that brings the 4′-amino group of the pyrimidine ring very close to the reactive C2 atom of the thiazolium ring. In each case, there is a conserved glutamate residue that interacts with N1′ of the aminopyrimidine ring and is thought to be involved in the formation of the imino group at the 4′ position which in turn promotes the formation of the active ylid [15,23]. Substitution of this glutamate residue in various ThDP-dependent enzymes results in a substantial decrease of enzyme activity [24–26]. Because of the very poor homology shared among ThDP-dependent enzymes, the identification of the active site amino acid residues in E1 has proved to be difficult by protein sequence alignment.

In this paper, we suggest that Glu^{89} of the β-subunit is the catalytic glutamate of human pyruvate dehydrogenase, based on the results of protein sequence alignment with transketolase (EC 2.2.1.1), and between the E1β subunits of pyruvate dehydrogenase complex and related enzymes from various species. This hypothesis is strongly supported by the results of substitution of Glu^{89} with glutamine, aspartate and alanine.

*Corresponding author. Fax: (61) (7) 3365 4699.
E-mail: duggleby@biosci.uq.edu.au

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2. Materials and methods

2.1. Materials

Chemicals used were of the highest quality available commercially. T4 DNA polymerase, T4 DNA ligase, and restriction enzymes were from Progen (Brisbane, Australia). Sequencing and mutagenesis DNA oligonucleotides were purchased from Pacific Oligos (Lismore, Australia). DNA sequencing was performed using the Big Dye Terminator kit from Perkin-Elmer (Norwalk, CT, USA).

2.2. Sequences and sequence alignment

Sequences were obtained from the GenBank or SwissProt databases and aligned using ClustalW [27]. Residues are numbered from the initiating methionine and include the mitochondrial transit peptides of each E1 subunit.

2.3. Plasmids and Escherichia coli strains

The plasmid pQE-9-6HE1α/E1β that was used for expression of PDH in *E. coli* was kindly provided by Prof. M.S. Patel, Department of Biochemistry, SUNY, Buffalo, NY, USA. It is derived from pQE-9 (Qiagen, Valencia, CA, USA) and encodes the mature form (i.e. minus the mitochondrial transit peptide) of both the α- and β-subunits of human E1. The α-subunit has a hexahistidine tag attached to the N-terminus of the protein that does not affect the function of the enzyme [28]. Propagation of this plasmid was done in *E. coli* JM109, or in *E. coli* BMH 71-18 *mutS* during mutagenesis. *E. coli* M15 cotransformed with pREP4 (Qiagen) was used for expression. Transformation and other molecular biological techniques generally followed the methods described by Sambrook et al. [29].

2.4. Site-directed mutagenesis

Mutagenesis was performed by the 'unique site elimination' method described by Deng and Nickoloff [30]. Briefly, a control primer was designed to introduce a mutation in a unique restriction site, *Sma*I or *Nde*I. After the vector was denatured by alkali and purified by sodium acetate-ethanol precipitation, the control primer and the primer with the desired mutation were annealed to the same strand of the plasmid. The mutant strand was synthesized by T4 DNA polymerase and T4 ligase in the presence of dNTP and ATP. The mixture was transformed into *E. coli* BMH 71-18 *mutS* and then into *E. coli* JM109. Before each transformation, the mixture was digested by *Sma*I or *Nde*I to linearize the parent plasmid. To obtain the desired mutants, 5′-GACACTCCCATATCACATATGGGCTTG-3′ was used to change Glu^{89} to histidine and introduce a *Nde*I site to facilitate screening of mutants. The bases in each primer that differ from wild-type are underlined. 5′-ACTTTCACCCGTGGCCTTTCCGTC-3′ was used as the control primer to introduce a silent mutation at Arg^{127} of α-subunit gene and eliminate the unique *Sma*I site. In the second round of mutation, 5′-CTGAGAGTGCATGCTATGCGGTGTG-3′ was used as the control primer to eliminate an *Nde*I site outside the β-subunit gene, and the codon of His^{89} was replaced by that of glutamine (CAG), aspartate (GAT) or alanine (GCA). Mutant plasmids were screened by *Nde*I digestion and the whole length of α- and β-subunit genes was sequenced.

2.5. Expression and purification

The plasmid carrying either the wild-type or mutant PDH gene was transformed into *E. coli* M15 (pREP4) for expression. The method for expression and purification was adapted from that described by Korotchkina et al. [28]. When the OD_{600} of the culture reached 0.9–1.0, the cells were induced with 0.1 mM IPTG and incubated overnight at 25°C. The enzyme was purified [28] using 5 ml of His•Bind resin (Novagen, Madison, WI, USA). The purified enzyme was precipitated with 50% saturated ammonium sulfate in the presence of 0.2 mM DTT and 1 mM EDTA. Finally, it was desalted by gel filtration using a PD-10 column (Pharmacia-Biotech, Melbourne, Australia) and stored for several days at 4°C in 50 mM potassium-phosphate buffer (pH 7.0) containing 0.2 mM DTT and 1 mM EDTA. For long-term storage at −20°C, 20% glycerol was added.

2.6. Protein determination

The protein concentration was determined using Coomassie brilliant blue G-250 [31]. The OD_{595} was measured in a 96-well plate using a SPECTRAmax 250 Microplate Spectrophotometer, with bovine serum albumin as standard.

2.7. Enzyme activity assay

PDH samples were separated from DTT (which interferes in the assay) by gel filtration on a PD-10 column. The enzyme activity was determined by measuring the rate of production of hydroxyethyl-ThDP, which reduces DCPIP and results in a decrease of OD_{600} [32]. The final 200 μl reaction mix contained 2 mM $MgCl_2$, 0.2 mM ThDP, 0.2 mM pyruvate, 80 μM DCPIP, 50 mM potassium-phosphate buffer (pH 7.0) and enzyme. The reaction was started by adding pyruvate and the absorbance at 600 nm was monitored for 5 min using a SPECTRAmax 250 Microplate Spectrophotometer. To determine the K_m for pyruvate, the concentration of pyruvate was varied and the rate was measured as described above.

2.8. ThDP binding assay

Cofactor binding to the apoenzyme was determined by fluorescence quenching. In a cuvette, the apoenzyme was diluted to a final volume of 2 ml using 50 mM potassium-phosphate buffer (pH 7.0) containing 2 mM $MgCl_2$ and 1 mM DTT. The protein concentration was in the range 0.2–0.4 mg/ml. The concentration of ThDP was increased by adding small volumes (usually 2–5 μl) of a stock solution to the same cuvette. The sample was then left to stand at room temperature for 5 min to establish equilibrium, then the emission spectrum was recorded on a JASCO FP-770 Spectrofluorometer with an excitation wavelength of 300 nm (bandwidth 5 nm) and an emission wavelength over the range of 300–400 nm (bandwidth 5 nm). The excitation wavelength of 300 nm was chosen to minimize light absorption by ThDP.

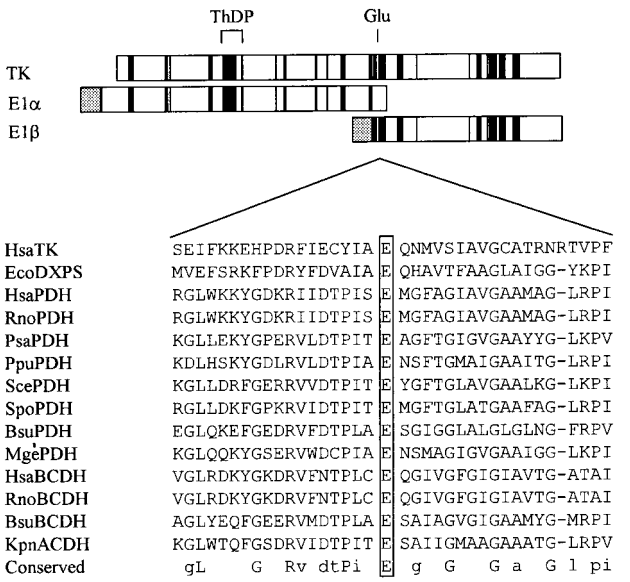

Fig. 1. Alignment of human transketolase with pyruvate dehydrogenase and related enzymes. The upper schematic shows an alignment between human transketolase (TK) and the α- and β-subunits of human E1. Dark bars indicate regions of high (>50%) sequence conservation, while the stippled regions represent the mitochondrial transit peptides of E1α and E1β. The positions of the ThDP-binding motif and the catalytic glutamate are also shown. The lower part of the diagram shows details of the alignment between human TK, *E. coli* DXP synthase (DXPS) and selected E1β subunits from pyruvate dehydrogenase (PDH), the branched-chain 2-ketoacid dehydrogenase (BCDH) and acetoin dehydrogenase (ACDH). The conserved amino acids are indicated in upper case if totally conserved in all the E1β sequences shown, and in lower case if conserved in at least two-thirds of those sequences. Abbreviations for the species are: Bsu, *Bacillus subtilis*; Eco, *E. coli*; Hsa, *Homo sapiens*; Kpn, *Klebsiella pneumoniae*; Mge, *Mycoplasma genitalium*; Ppu, *Porphyra purpurea*; Psa, *Pisum sativum*; Rno, *Rattus norvegicus*; Sce, *Saccharomyces cerevisiae* and Spo, *Schizosaccharomyces pombe*.

3. Results

3.1. Sequence alignment

Due to its distant evolutionary relationship to those ThDP-dependent enzymes with known crystal structures, together with its organization into α- and β-subunits, it has been difficult to identify the catalytic glutamate residue of the E1 of any 2-ketoacid dehydrogenase complex. Robinson and Chun [33] proposed an alignment in which Glu^{59} of the β-subunit (Glu^{89} in our numbering) of human E1 corresponded to the presumed catalytic glutamate of transketolase. However, our confidence in this identification was somewhat weakened by the fact that it failed to locate the catalytic glutamate of yeast PDC, and that no corresponding glutamate was aligned in *Bacillus stearothermophilus* or *E. coli* E1. More recently, Sprenger et al. [34] have alluded to an alignment between transketolases, PDH E1 and DXP synthases, and Dr. Sprenger has kindly provided an example of this alignment to us that we have adapted and extended (Fig. 1).

The overall similarity between human E1 and human transketolase is not high (32%) but contains regions with much higher similarity. One of these regions is near Glu^{89} of E1β which aligns with Glu^{366}, the catalytic glutamate of human transketolase [35]. Comparison with the E1β subunit of pyruvate dehydrogenase from other species and the E1β of related enzymes such as the branched-chain 2-ketoacid dehydrogenase complex and the acetoin dehydrogenase complex revealed that this glutamate was conserved without exception. We therefore propose that Glu^{89} of human E1β is the catalytic residue and to test this hypothesis we introduced various mutations at this position, expressed the enzymes and measured some of their catalytic properties.

3.2. Expression and purification

The design of pQE-9-6HE1α/E1β is such that it may express more E1β than E1α [28]. We observed that when the culture was induced at 37°C, or using a high IPTG concentration (e.g. 0.8 mM), E1β was found mostly in inclusion bodies and the soluble E1β was insufficient to saturate E1α (data not shown). When induced at 25°C using 0.1 mM IPTG, the soluble fraction of E1β was increased and was able to saturate E1α, as judged by the observation that the two bands corresponding to the 41 kDa α-subunit and 36 kDa β-subunit gave similar density in SDS-PAGE of the purified E1 (data not shown). Wild-type and mutant enzymes were obtained at better than 95% purity by the method described. The mutants were unstable in the absence of DTT.

3.3. Kinetic studies

The specific activity of the wild-type enzyme (31.7 mU/mg, Table 1) is very similar to that reported (26.9 mU/mg) previously [28] using the same assay. The catalytic efficiency of the mutant enzymes is much lower; the specific activity of E89D was the highest among the mutants but is only about one-seventh of wild type, while E89Q and E89A had very low activities of 5% and 1%, respectively (Table 1). The K_m for pyruvate of both wild-type and E89D were determined (Fig. 2 and Table 1) and it was found that the value was elevated by more than five-fold for this mutant.

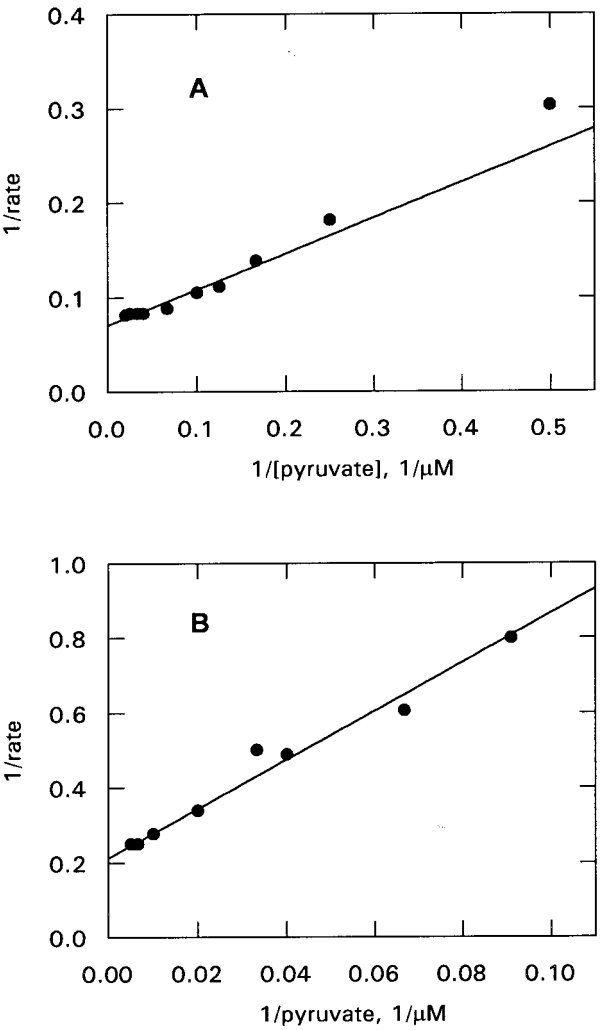

Fig. 2. Substrate saturation curve of human pyruvate dehydrogenase E1. A: Wild-type. B: The βE89D mutant.

In the process of enzyme purification, ThDP was not added and the purified products were predominantly apoenzyme; with no added ThDP the activity of wild-type is only about 0.6% of that observed in the presence of a saturating concentration of ThDP. The ThDP saturation curve yielded a K_m for this cofactor of 0.15 ± 0.02 μM. Because of the very low activity and poor stability in the absence of DTT of the mutants, further kinetic studies were not pursued. Instead, we measured ThDP binding more directly as outlined below.

Table 1
Characteristics of wild type and mutant of human pyruvate dehydrogenase E1

	Wild type	E89D	E89Q	E89A
Specific activity (mU/mg)	31.7	4.4	1.6	0.32
K_m for pyruvate (μM)	5.3 ± 0.6	29 ± 5	N.D.	N.D.
K_s for ThDP (μM)	0.72 ± 0.05	198 ± 37	37 ± 13	414 ± 63

N.D., not determined.

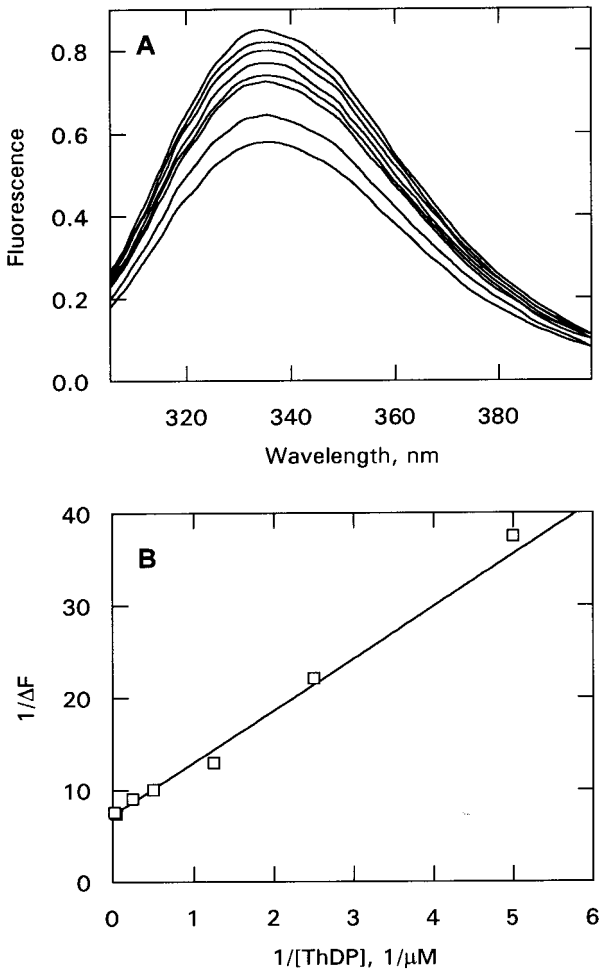

Fig. 3. Fluorescence quenching of human pyruvate dehydrogenase E1 by added ThDP. A: Tryptophan fluorescence was excited at 300 nm and the emission spectrum was measured over a range of added ThDP concentrations from 0 μM (top curve) to 40 μM (bottom curve). B: The decrease of fluorescence signal at 334 nm was measured as a function of the ThDP concentration.

3.4. ThDP binding

The binding of ThDP can be followed by its effect on tryptophan fluorescence. The advantage of this technique is that it is not necessary to remove DTT which is needed for stability of the mutants but which interferes in the activity assay. The fluorescence emission of PDH was excited at 300 nm and exhibited a maximum at 334 nm (Fig. 3A). As increasing amounts of ThDP were added to the solution of wild type enzyme, the fluorescence signal at 334 nm decreased. After correction for filters effect arising from light absorption by ThDP, the decrease of fluorescence followed the equation:

$$\Delta F = \theta[\mathrm{ThDP}]/(K_s + [\mathrm{ThDP}])$$

where ΔF is the decrease of the fluorescence signal, θ is a fluorescence constant of the protein and K_s is the dissociation constant of the ThDP-enzyme complex.

This equation gave a good fit to the data for wild-type (Fig. 3B) and mutants (data not shown). The value of K_s for mutants determined by this method (Table 1) shows that the binding of ThDP to E89Q is about 50 times weaker than wild type. The effects of the aspartate and alanine substitutions are even more pronounced, resulting in 275- and 575-fold weaker binding, respectively.

4. Discussion

ThDP-dependent enzymes utilize this cofactor to catalyze reactions involving the cleavage of a carbon-carbon bond adjacent to an oxo function. There are two common intermediates; the ylid form of ThDP, resulting from the deprotonation of the C2 atom of the thiazolium ring in the initial step of the catalytic cycle, and an α-carbanion which is formed after the nucleophilic attack of the ylid on the substrate (for review see [36]). In the crystal structures of transketolase, pyruvate oxidase, pyruvate decarboxylase and benzoylformate decarboxylase, there is no enzymatic base in a position suitable for proton abstraction from the C2 atom of the thiazolium ring of ThDP. Instead, a cofactor-assisted deprotonation mechanism has been proposed [15,23] where a conserved glutamate residue, in a position that allows it to form a hydrogen bond with N1′, catalyzes the conversion of the aminopyrimidine to its imino tautomeric form. The 4′-imino group, in turn, accepts a proton from C2, thereby forming the ylid. The participation of the 4′-amino group and N1′ are supported by the experiments showing that pyruvate decarboxylase reconstituted with ThDP analogs missing either of these functionalities is catalytically inactive [37,38].

In this paper, we deduced from protein sequence alignment that Glu^{89} of the β-subunit is the catalytic glutamate for the E1 component of human pyruvate dehydrogenase. The results of replacing Glu^{89} by mutagenesis suggest that hydrogen bonding is important for ThDP binding and that the acidic character of the carboxyl group is critical for catalysis. Thus, E89Q has the best ThDP-binding ability among mutants, while E89D has the highest activity, although both are much lower than wild-type. Small changes of the side chain cause significant decrease in ThDP binding (E89Q) and activity (E89D). Evidently, the E89D mutant enzyme undergoes a structural perturbation that permits some interaction of the carboxyl group of aspartate with N1′ to enable catalysis at the cost of a weakened binding of ThDP.

It might have been expected that E89Q and E89A would be totally inactive. However, a similar effect has been reported upon mutagenesis of the catalytic glutamate of yeast transketolase to glutamine or alanine [24] and that of *Zymomonas mobilis* pyruvate decarboxylase to glutamine [25]. Conversely, mutagenesis of the catalytic glutamate of yeast pyruvate decarboxylase to glutamine or alanine [26] abolishes almost all activity. Evidently the effect of mutagenesis of the catalytic glutamate varies between different ThDP-dependent enzymes and in this context it is of interest that Kern et al. [39] have proposed that the main role of this residue is to increase the rate, rather than the extent, of C2 deprotonation. Since E1 has an intrinsically low activity compared to other ThDP-dependent enzymes, it might be expected to be less susceptible to replacement of the catalytic glutamate.

In conclusion, we have identified Glu^{89} of the β-subunit of the E1 component of human pyruvate dehydrogenase complex as the catalytic glutamate. This is the first member of the enzyme family comprising the 2-ketoacid dehydrogenase complexes where this residue has been identified. This enzyme has a remote evolutionary relationship only to the ThDP-dependent enzymes for which 3D structures have been solved so

it will be of great interest if our proposal can be verified by structure determination of E1.

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