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

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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\textsuperscript{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\textsuperscript{89} of the \( \beta \)-subunit was identified as a possible catalytic residue by sequence alignment. Human E1 was expressed in Escherichia coli and purified. Mutating Glu\textsuperscript{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 (dihydrolipoil transferase, EC 2.3.1.12) and E3 (dihydrolipoil 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 \( \alpha \)- and two \( \beta \)-subunits. The activity of E1 is regulated by both nutritional status and hormones (for review see [4]). Mutations in the \( \alpha \)-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\textsuperscript{\textprime} 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\textsuperscript{\textprime} 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\textsuperscript{89} of the \( \beta \)-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 E1\( \beta \) subunits of pyruvate dehydrogenase complex and related enzymes from various species. This hypothesis is strongly supported by the results of substitution of Glu\textsuperscript{89} with glutamine, aspartate and alanine.
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-9-6HEinE1β 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 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. JM109, or in

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, Smal or NdeI. 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 Smal or NdeI to linearize the parent plasmid. To obtain the desired mutants, 5′-GACACTCCATACACATATGGGCTTG-3′ was used to change Glu47 to histidine and introduce a NdeI site to facilitate screening of mutants. The bases in each primer that differ from wild-type are underlined. 5′-ACTTTCACCCG

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 Koole et al. [31]. The 200 μl reaction mix contained 2 mM MgCl2, 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 Km for pyruvate, the concentration of pyruvate was varied and the rate was measured as described above.

2.6. Protein determination

The 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 hydroxymethyl-THDP, which reduces DCPIP and results in a decrease of OD600 [32]. The final 200 μl reaction mix contained 2 mM MgCl2, 0.2 mM ThDP, 0.2 mM pyruvate, 80 μM DCPIP, 50 mM potassium-phosphate buffer (pH 7.0) and enzyme. 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 MgCl2 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.

2.7. Enzyme activity assay

The final 200 μl reaction mix contained 2 mM MgCl2, 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 Km for pyruvate, the concentration of pyruvate was varied and the rate was measured as described above.

2.8. ThDP binding assay

Protein determination

The concentration of PDH 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.

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2.6. Protein determination

The protein concentration was determined using Coomassie brilliant blue G-250 [31]. The OD600 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 hydroxymethyl-THDP, which reduces DCPIP and results in a decrease of OD600 [32]. The final 200 μl reaction mix contained 2 mM MgCl2, 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 Km 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 MgCl2 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.
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 Glu59 of the β-subunit (Glu89 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 \textit{Bacillus stearothermophilus} or \textit{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 Glu89 of E1β which aligns with Glu366, 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 Glu89 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-9-6HE1/α/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.

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

N.D., not determined.
3B) and mutants (data not shown). The value of $\text{ured}$ as a function of the ThDP concentration.

B: The decrease of £uorescence signal at 334 nm was meas-

ured as a function of the ThDP concentration.


tophan £uorescence. The advantage of this technique is that it

cost of a weakened binding of ThDP.

3.4. ThDP binding

The binding of ThDP can be followed by its effect on trypt-

cation that permits some interaction of the carboxyl group with the

274 of the C2 atom of the thiazolium ring in the initial step of the
catalytic cycle, and an $\alpha$-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 oxid-

ease, pyruvate decarboxylase and benzoylformate decarboxy-
lase, 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 mechan-

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
the emission spectrum was measured over a range of added
ThDP concentrations from 0 $\mu$M (top curve) to 40 $\mu$M (bottom
curve). B: The decrease of fluorescence signal at 334 nm was meas-
ured as a function of the ThDP concentration.

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

where $\Delta F$ is the decrease of the fluorescence signal, $\theta$ 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 mu-
tants 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 substitu-
tions 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 ad-

tent relationship only to the ThDP-de-

In conclusion, we have identified Glu$^{89}$ of the $\beta$-subunit as 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 activ-
ity (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 trans-
ketolase to glutamine or alanine [24] and that of Zymomonas
mobilis pyruvate decarboxylase to glutamine [25]. Conversely,
mutagenesis of the catalytic glutamate of yeast pyruvate de-
carboxylase 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 $\beta$-subunit of
the E1 component of human pyruvate dehydrogenase com-
plex 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-de-
pendent 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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References
