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Biochemical characterization of two mutants of human pyruvate dehydrogenase, F205L and T231A of the E1α subunit

YONG-GE WU[1], WEN-YANG CHEN[1], ZI-WEI ZHANG[1], GUI-ZHENG YANG[2], WEI LI[1] and RONALD G. DUGGLEBY[3]*
[1]*Life Science College,* [2]*Basic Medical College, Jilin University, Changchun, Peoples Republic of China;* [3]*Department of Biochemistry and Molecular Biology, The University of Queensland, Brisbane, Queensland, Australia*

**Correspondence*: *Department of Biochemistry and Molecular Biology, The University of Queensland, Brisbane, QLD 4072, Australia.*
E-mail: Ronald.Duggleby@mailbox.uq.edu.au

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Summary: Mutations in the E1α subunit of the pyruvate dehydrogenase multienzyme complex may result in congenital lactic acidosis, but little is known about the consequences of these mutations at the enzymatic level. Here we characterize two mutants (F205L and T231A) of human pyruvate dehydrogenase *in vitro*, using the enzyme expressed in *Escherichia coli*. Wild-type and mutant proteins were purified successfully and their kinetic parameters were measured. F205L shows impaired binding of the thiamin diphosphate cofactor, which may explain why patients carrying this mutation respond to high-dose vitamin B_1 therapy. T231A has very low activity and a greatly elevated K_m for pyruvate, and this combination of effects would be expected to result in severe lactic acidosis. The results lead to a better understanding of the consequences of these mutations on the functional and structural properties of the enzyme, which may lead to improved therapies for patients carrying these mutations.

One of the best-studied multienzyme complexes is the pyruvate dehydrogenase complex (PDHC), which consists of multiple copies of three enzymes termed E1, E2 and E3 (Yeaman 1986). E1 is a pyruvate dehydrogenase (PDH, EC 1.2.4.1) that catalyses the oxidative decarboxylation of pyruvate, yielding an acetyl group that is covalently bound to the E1 cofactor, thiamin diphosphate (ThDP). E2 is a dihydrolipoamide acyltransferase (EC 2.3.1.12) containing a lipoyl group that acts as the oxidant for E1 and accepts the acetyl group that is subsequently transferred to coenzyme A. E3 is a dihydrolipoamide dehydrogenase (EC 1.8.1.4), which catalyses the oxidation by NAD^+ of the lipoyl group of E2, completing the catalytic cycle. The overall reaction constitutes an irreversible step downstream of glycolysis,

in which pyruvate is converted to acetyl-CoA, accompanied by the conversion of NAD^+ to NADH. A failure to express a fully functional PDHC will result in the failure of tissues to obtain enough ATP from sugar metabolism, while the pyruvate formed in glycolysis will be diverted to lactate by lactate dehydrogenase (LDH, EC 1.1.1.27). In humans, multiple cases of the lactic acidosis that results from congenital PDHC deficiency have been reported (Robinson 1993). The lactic acidosis is a symptom of an underlying enzymatic deficiency; the major impact of this deficiency is in brain tissue, which is almost totally reliant on aerobic glucose oxidation as an energy source. Therefore, PDHC deficiency is almost always accompanied by neurological defects.

PDH contains two types of subunit, α and β. So far, most of the reported PDHC mutations are located in the X-linked gene for the α subunit of the enzyme. Nearly 80 different E1α mutations have been described (Lissens et al 2000) and in this study we shall focus on two of these, F205L and T231A.

PDH is a member of a large family of enzymes that use ThDP and a divalent metal ion (e.g. Mg^{2+}) as cofactors. All members of this family contain a characteristic sequence motif (Hawkins et al 1989) of $GDGX_{24-27}NN$ that participates in the binding of Mg^{2+}, which itself anchors ThDP to these enzymes. In human PDH, this motif spans residues 195–225, so F205L is located within this motif and it may therefore play an important role in cofactor binding or PDH catalysis. T231 is just beyond the motif and is immediately adjacent to a serine that is reversibly phosphorylated by a PDHC-associated kinase and a phosphatase. It has been reported that infants with the T231A mutation can survive beyond birth for only a few days (Chun et al 1993), pointing to a critical role for T231 in the activity of the enzyme.

In this study we constructed by *in vitro* site-directed mutagenesis these two E1α mutants and coexpressed the proteins with E1β in *Escherichia coli*. The mutated reconstituted enzymes and the wild-type were purified and characterized with respect to their specific activities, the K_m for the substrate, and the affinity for the ThDP cofactor. By understanding the biochemical consequences of these mutations, we hope to elucidate the structural, functional and catalytic roles of these residues. This knowledge may then lead to new treatments for patients with lactic acidosis.

MATERIALS AND METHODS

Site-directed mutagenesis: We described in a previous report (Wu et al 2001) our pHsaE1αβ expression vector, which is similar to that developed elsewhere (Korotchkina et al 1995). The F205L and T231A mutations were introduced by the megaprimer method (Brøns-Poulson et al 1998) and were confirmed by DNA sequencing. To construct each mutation, forward and reverse complementary oligonucleotide primers were used. For F205L, the forward primer was 5′-GGC GAT GGC GCC GCT AAC CAG GGC CAG ATA *TTA* GAA GCT-3′, and the reverse primer was 5′-GTA AGC TTC *TAA* TAT CTG GCC CTG GTT AGC GGC GCC ATC GCC-3′. The italics show the required alteration, from TTC to TTA in the forward primer, to replace phenylalanine with leucine at position 205. The underlining spans two silent changes, from GGT GCT to GGC GCC in the

forward primer, that introduce a *Nar*I restriction endonuclease recognition site to facilitate screening for the mutation. For T231A, the forward and reverse primers were 5′-CGC TAT GGA ATG GGC *GCC* TCT GTT GAG AGA GCG GCA GCC-3′ and 5′-GGC TGC CGC TCT CTC AAC AGA *GGC* GCC CAT TCC ATA GCG-3′, respectively. Italics show the required alteration, from ACG to GCC in the forward primer, to replace threonine with alanine at position 231, while underlining indicates the position of a *Nar*I site that was introduced simultaneously. Protocols for DNA manipulation generally followed standard methods (Sambrook et al 1989).

PDH expression, purification and kinetic properties: Coexpression of E1α and E1β in *E. coli* and measurements of PDH activity followed the methods outlined previously (Fang et al 1998), except that cells were transformed with wild-type or mutated pHsaE1αβ. For determination of the kinetic properties with respect to pyruvate or ThDP, the concentration of each was varied over an appropriate range while maintaining the other components at standard concentrations.

RESULTS AND DISCUSSION

The expression and purification of wild-type and mutants of PDH were accomplished with purities similar to those in an earlier publication (Wu et al 2001). Substrate and ThDP saturation curves of wild-type and mutants were measured (data not shown). The specific activity, K_m for pyruvate and half-saturating cofactor concentration (K_c) for ThDP of wild-type and the mutants are summarized in Table 1. The results for F205L show that the specific activity is approximately 30% of the wild-type, while the K_m for pyruvate and the K_c for ThDP are elevated 1.9-fold and 4.7-fold, respectively. It appears that for this mutant the major effect is on ThDP binding and it has been reported (Naito et al 2002) that patients carrying this mutation respond to vitamin B_1 therapy. The increase in K_c is not large but it is apparently sufficient to reduce PDH activity in a way that can be partially reversed by high doses of vitamin B_1, the precursor of ThDP.

The specific activity of T231A is only 3% of the wild-type, suggesting that this amino acid is very important for enzyme function. The low activity is accompanied by a large increase (14-fold) in the K_m. The substitution in T231A is rather conservative, so it would be surprising to find such large differences in catalytic properties unless T231 is playing a role in substrate binding and/or catalysis. It appears unlikely that the mutation results in any large conformational change, because the K_c for ThDP is not affected to any large degree (2-fold).

Table 1 Comparison of the specific activities K_m values for pyruvate and K_c values for ThDP of PDH wild-type and mutants

Enzyme	*Specific activity* (mU/mg)	K_m (*pyruvate*) (μmol/L)	K_c (*ThDP*) (μmol/L)
Wild-type	34.7	6.8 ± 0.5	0.7 ± 0.03
F205L	10.7	12.7 ± 0.8	3.32 ± 0.41
T231A	1.1	95.3 ± 27.4	1.51 ± 0.18

The recent publication of the three-dimensional structure of the enzyme (Ciszak et al 2003) will aid in the interpretation of the effects of these and other mutations on PDH activity. The side-chain oxygen of T231 is 11.6 Å from the active site (carbon 2 of ThDP), making it unlikely that mutation to alanine would have any direct effect of on catalysis. However, this oxygen forms a hydrogen bond (2.8 Å) with the backbone nitrogen of G228, which helps to position the adjacent M229. This residue forms part of a hydrophobic pocket that encloses the cofactor. Thus, it appears that T231A mutation could cause a conformational adjustment around the cofactor that has little effect on ThDP binding but affects its interaction with the substrate and subsequent catalysis.

F205 is located in the $GDGX_{24–27}NN$ motif but is also quite distant (>12 Å) from the cofactor. Although a chain of hydrogen bonds can be traced from the backbone nitrogen to D196 at one end of the motif, it is of interest that F205 is close to its partner in the adjacent α subunit of the $\alpha_2\beta_2$ heterotetramer that constitutes E1. Ciszak and colleagues (2003) have emphasized the importance of intersubunit movements in the activity of the enzyme. Therefore, peturbation of the subunit interface may be an important contributor to the defective properties of the F205L mutant.

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