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BRIEF COMMUNICATION

Congenital Lactic Acidosis: Evaluation of the Properties of the A199T Natural Variant of Human Pyruvate Dehydrogenase E1α by *in Vitro* Mutation

One cause of congenital lactic acidosis is a mutation in the E1α-subunit of the pyruvate dehydrogenase multienzyme complex. Little is known about the consequences of these mutations at the enzymatic level. Here we study the A199T mutation by expressing the protein in *Escherichia coli.* The specific activity is 25% of normal and the K_m for pyruvate is elevated by 10-fold. Inhibitors of lactate dehydrogenase might be a useful therapy for patients with such mutations. © 2001 Academic Press

Key Words: **enzyme kinetics; *in vitro* mutagenesis; lactate dehydrogenase; lactic acidosis; pyruvate dehydrogenase; thiamin diphosphate.**

Congenital lactic acidosis is a rare but serious disease of newborn infants. The underlying cause is usually a decreased ability to convert pyruvate to acetyl-CoA; the unused pyruvate is converted to lactate. More importantly, ATP production by the citric acid cycle is impaired and tissues that rely on pyruvate as a source of acetyl-CoA, such as nervous tissue, are most affected, accounting for the mental retardation and ataxia that are often associated with this condition (1).

A reduced ability to convert pyruvate to acetyl-CoA has been traced in many patients to a defect in the mitochondrial multienzyme system (2), pyruvate dehydrogenase (PDH). The first step in catalysis is the decarboxylation of pyruvate catalyzed by the E1 component (EC 1.2.4.1), which requires thiamin diphosphate (ThDP) as an essential cofactor. In eukaryotes and gram-positive bacteria, E1 is an $\alpha_2\beta_2$ heterotetramer.

Two further enzymatic components, E2 (EC 2.3.1.12, dihydrolipoamide transacetylase) and E3 (EC 1.8.1.4, dihydrolipoamide dehydrogenase), complete the overall PDH reaction. The complex also contains "protein X", whose function is unclear, a specific protein kinase (EC 2.7.1.99), and a phosphoprotein phosphatase (EC 3.1.4.43). The latter two enzymes inhibit and reactivate, respectively, by phosphorylating three serine residues of the E1α-subunit.

In almost all instances where lactic acidosis has been shown by DNA sequencing to be due to a PDH defect, the mutation is in the E1α gene. Nearly 80 different such mutations have now been reported (3). Although these mutations might have effects at the RNA level, it is likely that many are manifested as changes to the properties of the E1α protein, including its interactions with other components of the PDH complex. These changes can include impairment of: (1) folding or stability of E1α; (2) transport of E1α to mitochondria and/or its correct processing in this organelle; (3) association of E1α with other components of the PDH complex; (4) catalysis or binding of substrate or cofactors by the E1 $\alpha_2\beta_2$ tetramer; or (5) regulation of E1 by phosphorylation and/or dephosphorylation.

For any given mutation, knowledge of which defects apply could be useful in designing appropriate therapies. For example, one of the standard treatments (4) is high doses of vitamin B_1 (5), the precursor of ThDP. This treatment is most likely to be effective if the main result of the mutation is impaired ThDP binding, or instability that can be overcome by ThDP binding.

Only in very few instances have the alterations in the functional properties of E1 been determined

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for a particular mutation. A notable exception is for the H44R mutation (nomenclature as in (6)). Using cultured fibroblasts from a patient it was shown clearly that E1 exhibits a large decrease in the affinity for ThDP, compared to controls (7).

The A199T mutation was first reported by Chun *et al.* (8) in a 5-year-old boy with mild mental retardation and ataxia. PDH activity in cultured fibroblasts was 17% of normal. No further information has been published about this patient and the mutation has not been reported in any other individual. Although the PDH activity of this individual was higher than that found in many patients, he later developed basal ganglia lesions and died (B. H. Robinson, personal communication).

Measurements of PDH activity in affected individuals are fraught with difficulties. We believe that measurement of the properties of purified E1 mutants expressed in *Escherichia coli* can yield data that are relevant to their properties in human patients. By identifying the nature of the defect in the enzyme, preferred or even new treatments can be chosen. Here we explore this approach using the A199T mutation as a model.

MATERIALS AND METHODS

Construction and Mutagenesis of the pHsaE1αβ Coexpression Vector

Protocols for bacterial growth and DNA manipulation generally followed the methods described by Sambrook *et al.* (9). The DNA used for constructing the expression vector for the human PDH E1 α- and β-subunits was two EST clones (Accession Number AA211750/AA213956 for E1α and T55314/T54050 for E1β) obtained from the Human Genome Mapping Project Resource Centre (Hinxton, England) and the plasmids pQE3 and pQE9 (QIAGEN). The E1α- and E1β-subunit cDNA was amplified by PCR and subcloned into a coexpression vector that is substantially the same as that described by Korotchkina *et al.* (10). Introduction of the E1α A199T mutation was based on the megaprimer method (11) and confirmed by DNA sequencing.

E1 Expression, Purification, and Kinetic Characterization

Coexpression of E1α and E1β in *E. coli* and measurements of E1 activity followed the methods

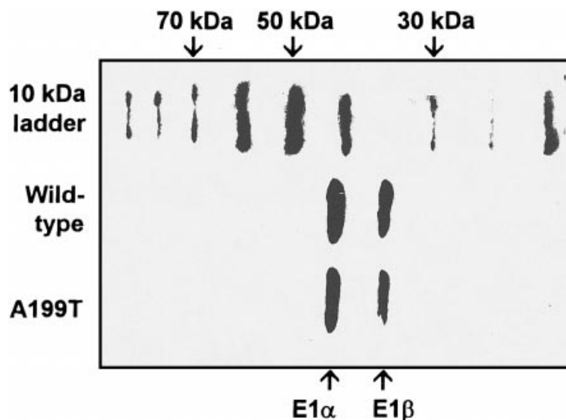

FIG. 1. SDS-PAGE analysis of purified wild-type E1 and the A199T E1α mutant. Each sample lane contained approximately 2 μg of protein. The proteins were stained by Coomassie blue.

outlined by Fang *et al.* (12) except that cells were transformed with 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 the standard concentrations (12).

RESULTS

Earlier we described work on human E1 (12) using the *E. coli* expression plasmid pQE-9-6HE1α/E1β developed by Korotchkina *et al.* (10). Previously granted permission to use this plasmid was not continued by Professor Patel (Department of Biochemistry, SUNY, Buffalo, NY) so we have constructed a very similar expression system. None of the DNA was derived from pQE-9-6HE1α/E1β. The amount of expression, as judged by SDS-PAGE analysis of cell-free extracts, appears to be similar in the A199T mutant and wild type (data not shown); however, the E1 activity in the mutant is substantially lower. The mutant enzyme was purified and has a specific activity of approximately 8.3 mU/mg, which is 25% of the wild-type value of 34.0 mU/mg. SDS-PAGE analysis of the purified enzymes (Fig. 1) illustrates two main features. First, while not completely pure, both preparations contain similar levels of impurities indicating that the 4-fold lower specific activity of the mutant is not due to the presence of 4-fold more impurities. Thus, the activity of A199T is intrinsically lower. Second, the relative amounts of the

α- and β-subunits appear similar, after correcting for the 50% higher content of basic amino acids in the α-subunit that makes it stain more intensely with Coomassie blue.

The purification relies on a hexahistidine tag that is present only on the α-subunit and copurification of both subunits clearly demonstrates that there is no defect in the association of the α- with the β-subunit. This implies that the α-subunit folds correctly. These conclusions then clearly focus attention on the idea that the $\alpha_2\beta_2$ tetramer is defective in catalysis or in binding of substrate or cofactor.

The low specific activity of A199T suggests that this mutant is impaired in catalysis; however, this low activity could be due to substantial increases in the K_m for pyruvate or for ThDP, so that the standard assay conditions (200 μM pyruvate and 200 μM ThDP) for the wild type are suboptimal for A199T. The K_m for ThDP of the mutant (0.43 $\pm$ 0.05 μM) and wild type (0.86 $\pm$ 0.08 μM) showed a small difference that is statistically significant ($P <$ 0.001) as judged by an F test (13). However, it is probably too small to have biological consequences and does not explain the low activity of A199T. The K_m for pyruvate is substantially elevated in the mutant (64.7 $\pm$ 6.1 μM) compared to wild type (6.41 $\pm$ 0.41 μM). This difference does not explain the low specific activity of A199T because the activity was measured at pyruvate concentrations (200 μM) that are close to saturating for both the wild type and A199T.

DISCUSSION

In this report we have described some properties of the human PDH E1α A199T mutant. The purified enzyme appears to be intact insofar as it contains both subunits and binds the cofactor ThDP with a K_m similar to that of wild type. The kinetic properties of A199T differ from those of wild type; the K_m with respect to pyruvate is elevated by 10-fold and the specific activity of the mutant is approximately 4-fold lower.

The alteration in the K_m for pyruvate suggests a potential therapy; the increase in K_m will have the greatest effect on PDH activity if the intracellular pyruvate concentration is relatively low. Typical values (14–17) are below 100 μM, so the elevated K_m of 65 μM found here for A199T could have substantial effects on PDH activity. If pyruvate concentrations are increased then this would partly counter the adverse K_m, but normally pyruvate accumulation is limited by conversion to lactate through the action of lactate dehydrogenase (EC 1.1.1.27). Therefore, treatment with inhibitors of this enzyme such as oxamate, in combination with other therapies (4), might be beneficial.

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