Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 225-230

PRODUCTION OF AN ENZYMATICALLY ACTIVE E1 COMPONENT OF HUMAN PYRUVATE DEHYDROGENASE COMPLEX IN *ESCHERICHIA COLI*: SUPPORTING ROLE OF E1β SUBUNIT IN E1 ACTIVITY

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Received June 20, 1994

A co-expression plasmid containing the coding sequence of both the human liver pyruvate dehydrogenase (PDH) E1α and E1β subunits was constructed. Functionally active PDH E1 protein was produced when this co-expression plasmid was introduced into the host *Escherichia coli* cell, BL21 (DE3)/plysS. In contrast, the production of E1α alone resulted in a catalytically inactive protein, suggesting an important role of the E1β subunit in constituting enzyme activity. The PDH E1 protein produced in *E. coli* was capable of being phosphorylated by PDH-specific kinase. This co-expression system will provide a useful tool for studying the biochemical properties of human PDH E1.

The pyruvate dehydrogenase complex (PDC) is one of the key enzymes involved in aerobic glucose metabolism. The regulation and molecular structure of the PDH complex have been studied extensively (for review see ref. 1 and 2). The mammalian PDC consists of six different subunits: three catalytic enzymes (E1, E2 and E3), two regulatory enzymes (PDH-specific kinase and PDH-specific phosphatase) and a less-characterized protein X. In mammalian systems, PDH E1 subunit is a thiamine pyrophosphate (TPP)-containing heterotetrameric enzyme consisting of two E1α subunits (monomer M_r 41,000) and two E1β subunits (monomer M_r 36,000). It catalyzes the decarboxylation of pyruvate with the generation of CO_2 followed by transfer of the acetyl group from hydroxyethylidene-TPP-E1 (HETPP-E1) to the lipoyl group of the E2 subunit. The overall activity of the PDC can be regulated by phosphorylation (resulting in inactivation) and dephosphorylation (re-activation) of the E1α subunit via PDH-kinase and PDH-phosphatase, respectively (3-5).

It is well known that deficiency in PDC is often associated with congenital lactic acidosis accompanied by abnormal growth and development in nervous systems (6-8). In most cases, the deficiencies are due to defects on the E1 subunit (9-13). However, the molecular mechanisms of the genetic defects on the PDH E1 subunit are still unclear.

PDH E1 from mitochondria has been successfully purified (14). However, the purification procedure is time consuming and the resulting protein often contains trace amount of other components of the PDC such as the two regulating enzymes, which complicate the interpretation of subsequent studies. In addition, it is difficult to obtain significant quantities of human tissues to study the biochemical characteristics of human enzymes.

0006-291X/94 $5.00

Although numerous reports have been available for the cDNA structures of E1 subunits of the human PDC (15-19), production of a functionally active enzyme has not been reported. In this manuscript, we describe a co-expression system which over-expresses the human liver PDH E1 component in *E. coli*. The resulting PDH E1 is enzymatically active and can be phosphorylated by PDH-kinase *in vitro*. Thus, this system could be a useful tool to study the biochemical properties of PDH E1 component of human origin.

MATERIALS AND METHODS

Construction of plasmids for production of human PDH E1 components in E. coli: Oligodeoxynucleotides were synthesized by a DNA synthesizer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol. Polymerase chain reactions (PCR) were performed using primer sets JJ38/JJ34, JJ39/JJ40, JJ42/JJ34, and JJ43/JJ40 (for the sequences of the primers, see Table 1) to introduce appropriate restriction sites into the 5' and 3' ends of the sequences coding for human liver PDH E1α (16), PDH E1β (19), mature PDH E1α or mature PDH E1β. The amplified DNA fragments were subcloned into the expression vector pT7-7 (United States Biochemicals, Cleveland, OH) and the resulting plasmids were used for the production of various PDH E1 components in *E. coli*.

Construction of plasmids for co-production of PDH E1α and PDH E1β in E. coli: A Fsp I/Pvu I fragment (3.7 kb) containing the coding region of mature human liver PDH E1α, the T7 promoter, and ribosome binding site of expression vector pT7-7 was obtained from plasmid mHuPDHE1a (Fig. 1, upper left diagram). Restriction digestion by Fsp I resulted in a blunt end and Pvu I resulted in a cohesive end. Plasmid mHuPDHE1b (Fig. 1, upper right diagram), which contains the coding region of mature human liver PDH E1β, was digested with Bgl II and treated with DNA polymerase large fragment (Klenow fragment) to make blunt end. This linearized mHuPDHE1b fragment was then digested with Pvu I. The resulting Pvu I/Bgl II* (* blunt ended) fragment (1.9 kb) contained the coding region of human liver PDH E1β, the T7 promoter and ribosome binding site from pT7-7. The Fsp I/Pvu I fragment (3.7 kb) from mHuPDHE1a and the Pvu I/Bgl II* fragment (1.9 kb) from mHuPDHE1b were ligated. The resulting co-expression vector (5.7 kb) was designated as HuPDHE1ma/mb. The co-expression plasmid for the precursor form of human liver PDH E1α and E1β was also constructed using the same procedure and the resulting plasmid was designated as HuPDHE1a/b.

Production of PDH E1 in E. coli: *E. coli* strain BL21 (DE3)/plysS (Novagen, Madison, WI) was used as the host for over-production. This *E. coli* strain carrying the desired plasmid was grown in LB medium at 37° C in the presence of ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml) until A_{600} reached 0.4. Isopropyl β-D-thiogalactopyranoside (IPTG) was added at the concentration of 0.4 mM to induce the synthesis of T7 polymerase and subsequently the synthesis of PDH E1 subunits for 4 h. Rifampicin (0.2 mg/ml) was added 1 h after induction with IPTG. Rifampicin is an inhibitor of bacterial RNA polymerase but not of T7 RNA polymerase and therefore specifically reduces the synthesis of host proteins. The cells were harvested by centrifugation at 2,500 x g for 10 min, suspended in PBS containing 0.1% Triton X-100 and disrupted by freezing and thawing three times and sonication. The cell lysates were centrifuged in a refrigerated microcentrifuge for 20 min and the supernatant fraction were used for SDS-PAGE analysis and measurement of enzymatic activity by the method (20) using [1-^{14}C]pyruvate as substrate.

In vitro phosphorylation of E. coli produced human PDH E1: The reaction buffer for phosphorylation by PDH-kinase contained 30 mM Tris (pH 7.4) with 10 mM $MgCl_2$, 1 mM NaF and 2 mM DTT. The PDH E1 (mα/mβ) produced in *E. coli* (25 μg total protein) were incubated with partially purified PDH-kinase prepared by the methods described previously. (14, 21). The reaction was initiated by adding 0.47 mM [γ-^{32}P]ATP (specific activity 2.94 mCi/mmol). After incubation at 30° C for indicated time periods, the kinase reaction were stopped by adding same volume of 2X SDS treatment buffer and boiled for 4 min. The proteins were subjected to SDS-PAGE followed by autoradiography at -80° C.

RESULTS AND DISCUSSION

Production of human PDH E1 in E. coli: The plasmids which allow the production of human liver PDH E1 (mα/mβ), its precursor form (α/β), and the mature PDH E1α (mα) were constructed using different sets of oligomers (listed in Table 1) and introduced into *E. coli* strain BL21 (DE3)/plysS (Fig. 1). At least half of the expression product was present in the soluble fraction and the soluble fraction was used for further analysis. The production of these proteins can be detected by SDS-PAGE stained with Coomassie blue. The yield of the PDH E1 production was estimated to be about 2 -3% of the soluble *E. coli* protein. The activity of the *E. coli* produced PDH E1 was estimated to be similar to that of the crude mitochondrial extract. The PDH E1α and E1β subunits co-produced in *E. coli* were found to co-elute from a DEAE-sepharose column (data not shown), suggesting a tight association between these two subunits. The identities of the subunits were confirmed by immunodetection using monospecific polyclonal antibodies against PDH E1α and PDH E1β, respectively (Fig. 2A and 2B). Immunoreactive bands corresponding to the correct sizes (precursor E1α: M_r 45 kD; precursor E1β: M_r 35 kD; mature E1α: M_r 42 kD; mature E1β: M_r 33 kD) were observed, confirming that the expression plasmids used in this study indeed produced the respective component of the PDH E1 subunits.

Properties of recombinant human PDH E1: The *E. coli* produced PDH E1 (mα/mβ) is enzymatically active (Table 2). On the other hand, when mature PDH E1α (mα) was produced alone, it was not active. It appeared that the presence of PDH E1β subunit is necessary to constitute an active enzyme, suggesting a supporting role of the E1β subunit for E1 activity. A similar role of the E1β subunit was recently reported for the activity of branched-chain α-ketoacid dehydrogenase E1 component (22).

The co-produced precursor form of PDH E1 (α/β) was also enzymatically inactive, suggesting that prior processing of the signal peptides was required for an assembly of the active enzyme. Our data differs from the results reported for other mitochondrial enzymes such as aspartate aminotransferase (23) and aldehyde dehydrogenase (24), where the removal of the putative mitochondrial leader sequences is not necessary for constituting their catalytic activities. It is possible that the activity of PDH E1 heterotetramer may be more sensitive to subtle change in the assembly of individual subunits.

TABLE 1 Primers used to perform PCR for construction of expression plasmids

primers	sequences (5' ----> 3' direction)	sites introduced	product (plasmid)
JJ38	CCTGTGCCATATGAGGAAGATGCTC	Nde I	α
JJ34	CCTTGTCGACCCCTTAACTGACTGA	Sal I	(HuPDHE1a)
JJ39	CCCATATGGCGGCGGTGTCTGGCTT	Nde I	β
JJ40	TCGGATCCAAACTAAATATTTAATG	BamH I	(HuPDHE1b)
JJ42	TCCCATATGTTTGCAAATGATGATGCTA	Nde I	mα
JJ34	Same as above	Sal I	(mHuPDHE1a)
JJ43	GCTCATATGCAGGTGACAGTTCGTG	Nde I	mβ
JJ40	Same as above	BamH I	(mHuPDHE1b)

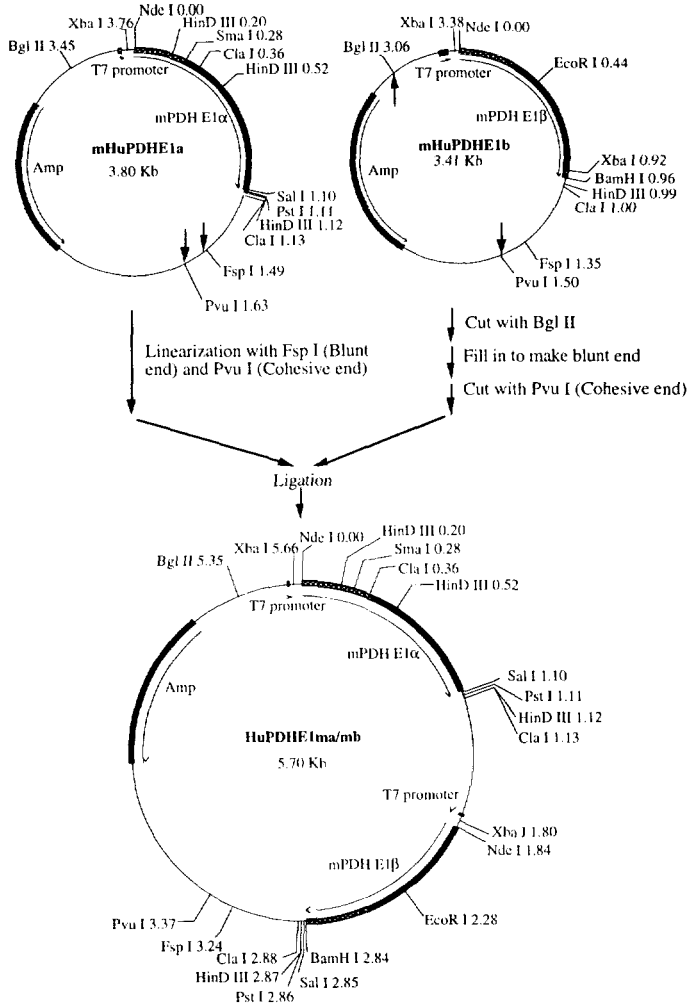

Figure 1. Construction of co-production plasmid, HuPDHE1ma/mb.
Plasmid HuPDHE1a (upper left) contained the coding region of human PDH E1α and plasmid HuPDH E1b (upper right) contained the coding region of human PDH E1β. The construction of co-production plasmid, HuPDH E1ma/mb (lower), was as described in Materials and Methods section.

The human liver PDH E1 produced in *E. coli* was phosphorylated with [γ-^{32}P]ATP by partially purified PDH-specific kinase (Fig. 2C). The data indicate that the over-produced human PDH E1 protein could be recognized and phosphorylated by PDH-specific kinase, similar to the authentic PDH E1 from *in vivo* animal tissues. However, the rate of phosphorylation of the *E. coli* produced human PDH E1 appeared relatively slower than the E1 in PDH complex, probably due to the absence of a core protein, PDH E2 subunit.

Future applications: One of the most powerful methods used to identify functional groups is site-directed mutagenesis. The assignment of functional groups such as catalytic domain and TPP-

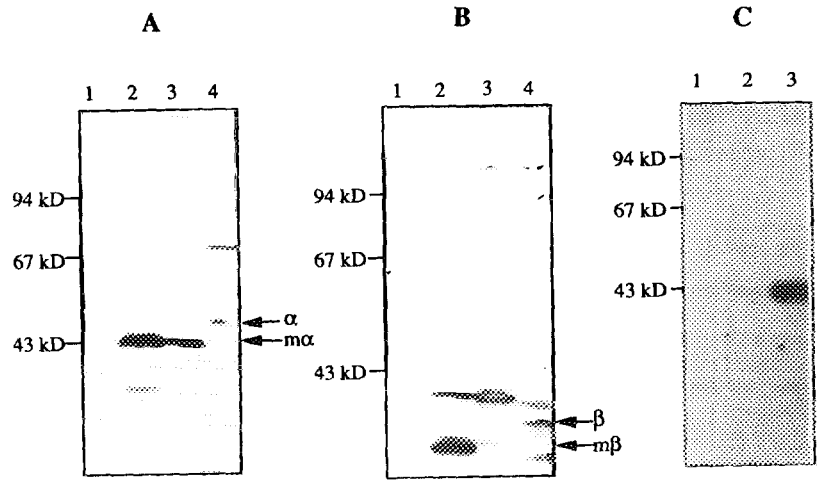

Figure 2. Production of human liver PDH E1 components in *E. coli.*
Bacterial cells were induced and lysed as described in Materials and Methods. The various soluble fractions were subjected to SDS-PAGE followed by immunoblot analyses using antibodies against PDH E1α and E1β subunits as described (19).
Panel A and Panel B: Immunostaining with antibodies against PDH E1α (A) and PDH E1β (B). Lane 1: control cells. Lane 2: production of PDH E1 (mα/mβ). Lane 3: production of PDH E1α alone (mα). Lane 4: production of PDH E1 precursor (α/β).
Panel C: *In vitro* phosphorylation of *E. coli* produced human PDH E1 by PDH-kinase. The *E. coli* produced human PDH E1 was *in vitro* phosphorylated by PDH-kinase as described in Materials and Methods for different incubation times. Lane 1: 2 min. Lane 2: 10 min. Lane 3: 30 min.

binding sites of PDH E1 is difficult since PDH E1 is a heterotetrameric enzyme. The co-production system which we present here can be used to study the properties of PDH E1 with altered amino acids in the putative functional groups. The newly synthesized protein produced in *E. coli* cells using this system can be specifically labeled by ^{35}S-methionine with low background (24) and therefore allows the distinction between a functional mutation and a mutation which

TABLE 2 Enzymatic activity of *E. coli* produced human liver PDH E1 components

Expression product	PDH E1 activity (cpm/μg total *E. coli* protein)
Control cell	52.5
human liver PDH E1 (mα/mβ)	279.0
human liver PDH E1a (mα)	42.4
human liver PDH E1 (α/β)	44.2

creates an unstable protein, as reported (25). The natural mutations occurring in the DNA sequence of human specimens as recently reported(9,10) can also be studied by using this system. For this purpose, the *E. coli* co-expression system will be much more desirable over direct purification from human tissues since it can provide large quantity of material as needed and also eliminate the potential risk of human pathogens transmission.

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