Disruption and Mutagenesis of the *Saccharomyces cerevisiae PDX1* Gene Encoding the Protein X Component of the Pyruvate Dehydrogenase Complex†

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ABSTRACT: Disruption of the *PDX1* gene encoding the protein X component of the mitochondrial pyruvate dehydrogenase (PDH) complex in *Saccharomyces cerevisiae* did not affect viability of the cells. However, extracts of mitochondria from the mutant, in contrast to extracts of wild-type mitochondria, did not catalyze a CoA- and NAD^+-linked oxidation of pyruvate. The PDH complex isolated from the mutant cells contained pyruvate dehydrogenase ($E_1\alpha + E_1\beta$) and dihydrolipoamide acetyltransferase (E_2) but lacked protein X and dihydrolipoamide dehydrogenase (E_3). Mutant cells transformed with the gene for protein X on a unit-copy plasmid produced a PDH complex that contained protein X and E_3, as well as $E_1\alpha$, $E_1\beta$, and E_2, and exhibited overall activity similar to that of the wild-type PDH complex. These observations indicate that protein X is not involved in assembly of the E_2 core nor is it an integral part of the E_2 core. Rather, protein X apparently plays a structural role in the PDH complex; i.e., it binds and positions E_3 to the E_2 core, and this specific binding is essential for a functional PDH complex. Additional evidence for this conclusion was obtained with deletion mutations. Deletion of most of the lipoyl domain (residues 6–80) of protein X had little effect on the overall activity of the PDH complex. This observation indicates that the lipoyl domain, and its covalently bound lipoyl moiety, is not essential for protein X function. However, deletion of the putative subunit binding domain (residues ~144–180) of protein X resulted in loss of high-affinity binding of E_3 and concomitant loss of overall activity of the PDH complex. This domain apparently plays an important role in binding E_3.

Mammalian (De Marcucci & Lindsay, 1985; Jilka et al., 1986) and *Saccharomyces cerevisiae* (Behal et al., 1989) pyruvate dehydrogenase (PDH)[1] complexes and, presumably, other eukaryotic PDH complexes as well contain small amounts of a protein designated protein X or component X. Protein X is tightly associated with the E_2 core, and, like the E_2 component, it can undergo reductive acetylation in the presence of pyruvate and E_1. In view of these observations, the possibility was considered that protein X is a proteolysis product of the E_2 subunit. However, immunological, peptide mapping, and limited proteolysis studies have provided evidence that protein X is a distinct polypeptide (De Marcucci & Lindsay, 1985; Jilka et al., 1986; Rahmatullah et al., 1989; Neagle et al., 1989). Furthermore, distinct genes encoding E_2 and protein X from *S. cerevisiae* have been cloned and sequenced (Niu et al., 1988; Behal et al., 1989). Comparison of the deduced amino acid sequences of the two proteins indicates that they evolved from a common ancestor. The amino-terminal half of protein X resembles E_2, but the remainder is quite different. The two proteins exhibit 50% sequence identity in the amino-terminal segment that corresponds to the putative lipoyl domain (residues 1–84) of E_2 and 33% sequence identity in the putative subunit binding domain (residues 146-181).

Evidence based on limited proteolysis and immunological studies indicates that protein X contributes to the binding and function of E_3 (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989). It should be noted in this connection that the 60-subunit E_2 core of the bovine kidney PDH complex contains about 6 molecules of tightly bound protein X (Jilka et al., 1986) and the E_2 core can bind only about six E_3 dimers (Wu & Reed, 1984), with a dissociation constant of about 3 nM.

Additional insight into the function of protein X has been obtained by molecular genetic studies with the protein X gene (*PDX1*) from *S. cerevisiae*. In this paper, we report that disruption of the *PDX1* gene results in loss of ability of the mutant PDH complex (E_1E_2) to bind E_3 and concomitant loss of CoA- and NAD^+-linked pyruvate oxidation activity. Deletion mutations show that the lipoyl domain (residues 1–80), and its covalently bound lipoyl moiety, is not essential for protein X function and that the putative subunit binding domain (residues 144–180) plays an important role in binding E_3. The data indicate that protein X plays a structural role in the PDH complex; i.e., it binds and positions E_3 to the E_2 core, and this specific binding is essential for a functional PDH complex.

Experimental Procedures

Materials. Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, and Promega Biotec. [α-^{32}P]-dCTP (3000 Ci/mmol) was obtained from New England Nuclear. Immobilon-P [poly(vinylidene difluoride)] membrane was purchased from Millipore. Yeast recombinant E_3, expressed in *Escherichia coli*, was provided by Dr. M. A. Yazdi. Other reagents and materials were of the highest grade available commercially.

Strains and Growth Media. S. cerevisiae strain W303 (*MAT*a/α *ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-*

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[1] Abbreviations: PDH complex, pyruvate dehydrogenase complex; E_1, pyruvate dehydrogenase; E_2, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfae; EDTA, ethylenediaminetetraacetic acid.

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100) and JLY61c (*MATα pdx1::LEU2 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*) were used. *E. coli* strains JM101 [*supE thi* Δ(*lac-proAB*)[F′*traD36 proAB lacI*^{q} *lacZΔM15*]] (Yanisch-Perron et al., 1985) and MC1066 (F′*lacX74 galU galK rspL hsdR trpC9830 leuB600 pyrF::Tn5*) (Casadaban & Cohen, 1980) were used for plasmid construction and amplification. The following plasmids were used: pGEM-7Zf(+) (Promega), YEp13(2μ *LEU2*) (Broach et al., 1979), pSEY8(2μ *URA3*), pSEYc63(*CEN4 ARS1 URA3*) (Emr et al., 1986), pVTU(102) (Vernet et al., 1987), and YEp352(2μ *URA3*) (Hill et al., 1986).

Yeast strains were grown on YP medium (1% Bacto-yeast extract/2% Bacto-peptone) with one of the following carbon sources: 2% dextrose, 2% ethanol, 2% lactate, 2% acetate, or 3% glycerol. YNB (1× yeast nitrogen base) plus the appropriate nutritional additives and carbon source was used for marker selection. For sporulation, diploid yeast strains were grown on presporulation medium (0.8% Bacto-yeast extract, 0.3% Bacto-peptone, and 10% dextrose) for 2 days at 30 °C and then transferred to sporulation medium (1% potassium acetate, 0.1% Bacto-yeast extract, and 0.05% dextrose). Dissected spores were germinated on YPD medium and tested for genetic markers on YNB medium plus supplements (Sherman et al., 1979).

Isolation of DNA and Southern Blots. Digestion with restriction endonucleases and ligations with T_4 DNA ligase were performed according to the supplier's instructions. Probes for Southern blots were labeled with [α-^{32}P]dCTP by random primer labeling (Feinberg & Vogelstein, 1983). Standard methods were used for isolation of yeast genomic DNA (Sherman et al., 1983) and for agarose gel electrophoresis (Maniatis et al., 1982). DNA was depurinated and blotted to nylon filters by alkaline transfer (Reed & Mann, 1985). Hybridizations were performed as described (Church & Gilbert, 1984) at 42 °C. The blots were washed in 2× SSC (1× SSC = 8.77 g of NaCl/4.41 g of sodium citrate, pH 7), 0.1% SDS, and 50 mM NaH_2PO_4, pH 7.2, at 42–55 °C (Maniatis et al., 1982). Autoradiography was performed with Kodak XAR-5 film.

Transformation and Gene Disruption. Standard methods were used for *E. coli* transformation (Cohen et al., 1971) and miniplasmid preparation from *E. coli* (Maniatis et al., 1982). Yeast were transformed by the lithium acetate/poly(ethylene glycol) procedure (Ito et al., 1983). Gene disruption was done as described (Rothstein, 1983).

Construction of Deletion Mutations. Deletion mutations were constructed by amplifying specific regions of the *PDX1* gene by PCR and sequentially subcloning these fragments into yeast vectors. The mutant constructs were sequenced completely to verify the mutations.

Polymerase Chain Reaction. PCR was performed according to the supplier's instructions with the GeneAmp DNA amplification kit from Perkin-Elmer/Cetus. The template for each reaction was 0.5–1.0 ng of the *PDX1* gene (coding region plus 1.6 kb of 5′-flanking and 705 bp of 3′-flanking DNA) subcloned in plasmid pSEYc63. A step program of 30 cycles was used: 94 °C, 5 min, followed by 29 cycles (94 °C, 1 min; 55 °C, 2 min; 72 °C, 3 min) and a 10-min extension step at 72 °C. Oligonucleotide primers were synthesized by using Applied Biosystems Model 381A DNA synthesizer.

DNA Sequencing. Single- and double-stranded DNA was sequenced with Sequenase Version 2.0, as described by the supplier (United States Biochemical).

Preparation of Mitochondria and Mitochondrial Extracts. Small-scale preparations of mitochondria were made by mechanical breakage of yeast cells with glass beads (Deters & Ewing, 1985). Larger-scale mitochondrial preparations were made by a modification of the procedure of Daum et al. (1982). Mitochondria were lysed by incubating a suspension containing 20–25 mg of protein/mL in 250 mM sucrose, 5 mM potassium phosphate buffer, pH 7.0, and 1 mM EDTA with 10 mg/mL Triton X-100 for 30 min on ice. The mixture was clarified by centrifugation for 15 min at 10000 rpm in an Eppendorf microcentrifuge.

Preparation of Rabbit Antibodies to Protein X. Highly purified preparations of *S. cerevisiae* PDH complex (Uhlinger et al., 1986) were subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and the proteins were transferred electrophoretically onto nitrocellulose. Following staining with Fast Green, the protein X band was excised, dried thoroughly, and dispersed in a minimal volume of dimethyl sulfoxide (Knudsen, 1985). An equal volume of complete or incomplete Freund's adjuvant was then added, and the mixture, which contained 60–70 μg of protein, was injected subcutaneously into a rabbit at multiple sites at 2-week intervals. After a total of four injections, the rabbit was bled from the ear, and the serum was tested for antibody response.

Immunoblotting. Proteins were separated by SDS–polyacrylamide gel (12.5% acrylamide) electrophoresis and then transferred electrophoretically onto a Immobilon-P membrane (Matsudaira, 1987). Immunoblot analysis was performed with rabbit anti-protein X serum and goat anti-rabbit IgG conjugated to alkaline phosphatase as described by the supplier (Bethesda Research Labs).

Enzyme Assays. Assay of the overall activity of the PDH complex is based on the initial rate of the CoA- and NAD^+-dependent oxidation of pyruvate (Uhlinger et al., 1986). One unit is defined as the amount of enzyme complex that produces 1 μmol of NADH per minute. Some mutant PDH complexes were assayed by measuring E_2 activity, which is based on the initial rate of transfer of radioactive acetyl groups from [1-^{14}C]acetyl-CoA to dihydrolipoamide (Butterworth et al., 1975). Units are expressed as nanomoles of acetyl groups transferred per minute. Protein was determined as described by Bradford (1976).

Purification of PDH Complexes. Wild-type and mutant cells were grown on 12 L of YNB dextrose medium plus supplements (Sherman et al., 1979) for 48 h at 30 °C in a New Brunswick Model SF-116 MicroGen fermentor. *All buffers* contained 1 mM 2-mercaptoethanol, 1 mM EDTA, 0.01 mM thiamin diphosphate, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and 0.01 mM benzyloxycarbonyl-Phe-Ala diazomethyl ketone, a thiol protease inhibitor (Watanabe et al., 1979). All operations were carried out at about 4 °C, except as noted. About 70 g (wet weight) of cells was resuspended in 75 mL of 100 mM potassium phosphate buffer, pH 7.0. The suspension was passed 3 times through a French press at 16000 psi. The pH was adjusted to 6.2 with 10% acetic acid, and the cell debris was removed by centrifugation at 14000 rpm for 25 min in a Beckman JA-14 rotor. The extract was filtered through four layers of cheesecloth, and the protein concentration was adjusted to about 10 mg/mL. The solution was brought to 25 °C, and the PDH complex was precipitated by dropwise addition, with stirring, of 0.035 volume of 50% (w/w) poly(ethylene glycol) 8000. After 15 min the precipitate was collected by centrifugation at 14000 rpm for 15 min at 25 °C. The pellets were resuspended in 50–100 mL of ice-cold 100 mM phosphate buffer, pH 7.0. The suspension was stirred for 1 h and then centrifuged at 18000 rpm for 15 min. To the supernatant fluid was added dropwise,

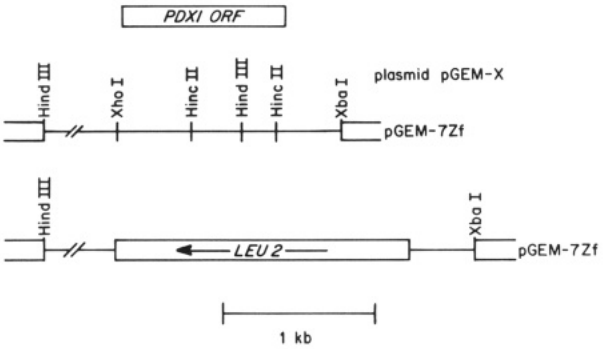

FIGURE 1: Disruption of the *PDX1* gene. All but 75 bp at the 3′ end of the *PDX1* coding region was removed from plasmid pGEM-X by digestion with *Xho*I and *Hin*cII. The deleted 1.17-kb fragment was replaced by a 2.2-kb fragment containg the yeast *LEU2* gene. The 3.6-kb *Hin*dIII–*Xba*I fragment was used to transform the diploid yeast strain W303a/α. The position of the *PDX1* open reading frame (ORF) is indicated by the bar above the restriction map. The distance from the upstream *Hin*dIII site to the *Xho*I site is ~1.6 kb.

with stirring, 0.025 volume of 2% (w/v) protamine sulfate. After 15 min, the precipitate was removed by centrifugation at 18 000 rpm for 30 min. The supernatant fluid was centrifuged over a 5-mL layer of 15% sucrose (w/w) in 26-mL centrifuge tubes for 90 min at 50 000 rpm in a Beckman 50.2 Ti rotor. The pellets were dissolved in a minimal volume of 20 mM phosphate buffer, pH 7.0. Aliquots (0.2–0.4 mL) of the solution were carefully layered over a 15–40% (w/w) linear sucrose gradient in 5-mL centrifuge tubes, and the tubes were centrifuged for 2 h at 50 000 rpm in a Beckman SW55 Ti rotor. Fractions were collected with an Isco Model 640 density gradient fractionator. The fractions were analyzed for overall activity and/or E_2 activity to locate the PDH complex. With some preparations, chromatography on hydroxylapatite (Uhlinger et al., 1986) was used instead of the sucrose density gradient step. The yield of highly purified PDH complex was 0.5–3 mg.

Results and Discussion

Disruption of the PDX1 Gene. A plasmid containing the entire coding sequence of *PDX1* plus 1.6 kb of 5′-flanking DNA and 0.6 kb of 3′-flanking DNA was constructed in the vector pGEM-7Zf by sequentially subcloning a 1.6-kb *Hin*dIII–*Xho*I fragment and a 2.1-kb *Xho*I–*Xba*I fragment (Figure 1). This construct (pGEM-X) was cut at a *Xho*I site 19 bp upstream of the ATG start codon and at an internal *Hin*cII site 75 bp upstream of the termination codon. The 1.17-kb *Xho*I–*Hin*cII fragment, containing most of the *PDX1* coding sequence, was replaced with a 2.2-kb fragment carrying the yeast *LEU2* gene. The disrupted gene was excised from the plasmid by digestion with *Hin*dIII and *Xba*I and was used to transform the diploid yeast strain W303a/α. Stable transformants were selected on YNBD plates lacking leucine.

Twelve of the diploids were sporulated. All spores were viable. DNA was prepared from representative tetrads and subjected to Southern analysis (Figure 2). The filter was probed with a 2.5-kb *Hin*dIII fragment which contained 5′-flanking DNA and about 70% of the *PDX1* coding region (Figure 1). The blot shows that, as expected, the meiotic segregation of *PDX1* to *pdx1*::*LEU2* is 2:2 in each tetrad, indicating one chromosomal copy of *PDX1* was replaced by *pdx1*::*LEU2*.

That the disrupted gene had indeed integrated into the *PDX1* locus was confirmed by genetic and immunological analyses of the tetrads. Two spores of each tetrad were Leu^+, and two were Leu^-. The Leu^+ spores contained the larger

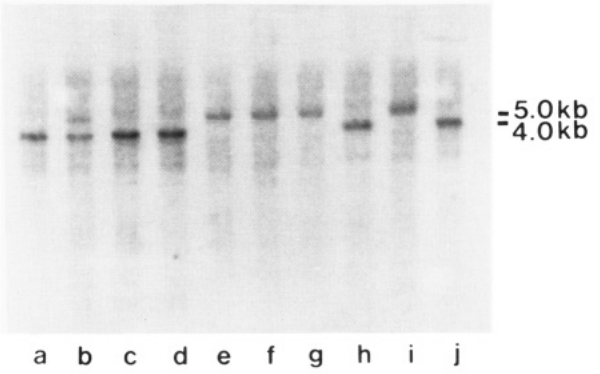

FIGURE 2: Southern blot analysis of *PDX1* disruption. Genomic DNA isolated from representative tetrads was digested with *Xba*I. The digest was electrophoresed on a 1% agarose gel, and the fragments were transferred onto a Zeta-Probe blotting membrane (Bio-Rad). The blot was probed with a radiolabeled 2.5-kb *Hin*dIII fragment containing *PDX1* and its 5′-flanking DNA (Figure 1). The probe hybridized to both wild-type *PDX1* (4.0 kb) and *pdx1*::*LEU2* (5.0 kb). Lane a, DNA from the wild-type diploid; lane b, DNA from the disrupted diploid containing one copy each of *PDX1* and *pdx1*::*LEU2*; lanes c–f; DNA from one tetrad; lanes g–j, DNA from a second tetrad.

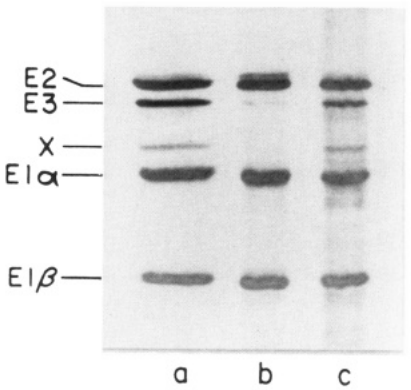

FIGURE 3: SDS–PAGE patterns of highly purified PDH complexes from wild-type cells (lane a), *pdx1* null mutant (lane b), and null mutant transformed with the gene for protein X on a unit-copy plasmid (lane c). Protein bands were detected by silver staining (Oakley et al., 1980).

hybridizing DNA fragment arising from the disrupted gene, and the Leu^- spores contained the wild-type fragment (Figure 2). Immunoblot analysis showed that Leu^- spores contained protein X but Leu^+ spores did not (data not shown).

No significant difference in the growth characteristics of the wild-type and *pdx1* mutant strains was observed in solid or liquid, rich YP, or minimal YNB media, with dextrose, glycerol, lactate, ethanol, or acetate as the carbon source (data not shown). These results indicate that a functional *PDX1* gene is not required for yeast cell growth under the conditions tested.

Comparison of PDH Complexes from Wild-Type and PDX1-Disrupted Strains. Isolated mitochondria from wild-type and mutant cells were lysed with Triton X-100. Extracts of the mutant mitochondria, in contrast to extracts of wild-type mitochondria, did not catalyze the CoA- and NAD^+-dependent oxidation of pyruvate (0 versus 0.008 unit/mg of protein). This observation indicated that extracts of the mutant mitochondria lacked a functional PDH complex. The wild-type and mutant extracts exhibited similar dihydrolipoamide acetyltransferase (E_2) activity (~0.4 unit/mg of protein).

The wild-type and mutant PDH complexes were purified to near-homogeneity as described under Experimental Procedures. The SDS–PAGE pattern showed that the mutant PDH complex contained $E_1\alpha$, $E_1\beta$, and E_2, but lacked protein

Table I: Enzymatic Activity of Wild-Type and Mutant PDH Complexes[a]

PDH complex	specific activity[b]
wild type	12.6
JLY61c	0
JLY61c/pSEYc63-X	11.8
JLY61c/pX80	8.4
JLY61c/pX180	0
JLY61c/pX217	0
JLY61c/pX180-2	0

[a] The yeast strain harboring the disrupted *PDX1* gene (JLY61c) was transfrmed with a unit-copy plasmid carrying the *PDX1* gene (pSEYc63-X) and with plasmids harboring deletion mutations in the *PDX1* gene as defined in Figure 4. [b] Units per milligram of protein.

X and E_3 (Figure 3). Mixtures containing highly purified mutant E_1E_2 subcomplex and a relatively high concentration ($\sim 10^{-6}$ M) of yeast recombinant E_3 (expressed in *E. coli*) showed slight pyruvate oxidation activity. At a molar ratio of E_1E_2 to E_3 of $\sim$1:1000, the mixture exhibited about 5% of the pyruvate oxidation activity of the wild-type PDH complex. This activity was not affected by anti-protein X serum, indicating lack of contamination by protein X. These observations suggest a possible explanation of the apparent lack of a growth phenotype for the *pdx1* null mutant. The relatively high concentration of E_3 and other enzymes in the mitochondrial matrix, about 10^{-5} M (Srere, 1972), may permit an effective interaction between the mutant E_1E_2 complex and loosely bound E_3. Another possibility is that flux through the pyruvate dehydrogenase complex is not required for growth of *S. cerevisiae* under the conditions tested. Current studies on disruption of the *LAT1* gene encoding the E_2 component of the complex should provide further insight on this question.

The sedimentation coefficients ($s_{20,w}$) of the mutant PDH complex and the wild-type PDH complex were 73.3 and 82.6 S, respectively. These values are consistent with the absence from the mutant PDH complex (E_1E_2) of about six E_3 dimers (calculated M_r 104 668) and about six protein X molecules (calculated M_r 42 052). These observations indicate that protein X is not involved in assembly of the E_2 core nor is it an integral part of the E_2 core. Rather, protein X is apparently involved in high-affinity binding of E_3 to the E_2 core. Support for this conclusion was obtained by transforming the *pdx1* null mutant (JLY61c) with a unit-copy plasmid harboring the *PDX1* gene (pSEYc63-X). PDH complex isolated from the transformed cells contained protein X and E_3, as well as $E_1\alpha$, $E_1\beta$, and E_2 (Figure 3), and the complex exhibited overall activity similar to that of wild-type PDH complex (Table I). These findings are consistent with the observation that the catalytic inner core domain of E_2 from *S. cerevisiae*, when expressed in *E. coli*, undergoes self-assembly (in the absence of protein X) to a pentagonal dodecahedron-like structure (60-mer) that is characteristic of the wild-type E_2 (Niu et al., 1990).

Deletion Mutations in the PDX1 Gene. Comparison of the deduced amino acid sequences of *S. cerevisiae* E_2 (Niu et al., 1988) and protein X (Behal et al., 1989) indicates that protein X possesses the segmented structure shown in Figure 4. The putative lipoyl domain encompasses residues $\sim$1–80, and the putative subunit binding domain encompasses residues $\sim$144–180. On the basis of sequence homologies among dihydrolipoamide acyltransferases, the lipoyl moiety in protein X is thought to be covalently attached to Lys-43.

Roche has proposed (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989) that protein X plays a functional, as well as a structural, role in the bovine kidney PDH complex, perhaps by transferring electrons via its lipoyl moiety to the

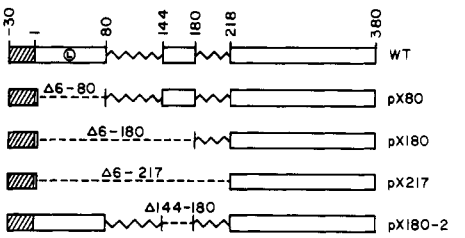

FIGURE 4: Diagrammatic representation of the structural domains of *S. cerevisiae* protein X (WT) and deletion mutations. The domains are connected by hinge regions (squiggly lines). The limits of these domains are approximate and are indicated with amino acid residue numbers. The hatched rectangles correspond to the presequence of the protein X precursor, and L denotes the putative lipoyl domain.

Table II: Oligonucleotide Primers for PCR[a]

primer	sequence	location
1	SalI GTTCGTCGACTCTCAATCTAAAAATATG	5 (F)
2	XbaI GGTTTCTAGAAATGTCTTTACAGCAAGTAA	456 (R)
3	XbaI TATCTCTAGATGATGATTTAGCTACTATA	681 (F)
4	EcoRI ACAAGAATTCACCCAGACACCTACGATG	1783 (R)
5	SmaI GATACCCGGGTAATAGCGTCTGTTCAAG	866 (R)
6	SmaI TTGCCCGGGAAAATACCACAAGATTCG	982 (F)
7	XbaI TATCTCTAGAGCAAGCTCAAACAAAAGCT	1092 (F)
8	XbaI TATCTCTAGAAGGGAAAATACCACAAGAT	981 (F)

[a] Sequences are listed 5′–3′. The first four nucleotides and portions of the underlined restriction sites were added for cloning purposes and do not correspond to *PDX1* sequences. Location refers to the specific nucleotide of *PDX1* at which hybridization to the primer begins and continues in the forward (F) or reverse (R) direction.

E_3 component. To evaluate the importance of the lipoyl moiety in the function of protein X and to investigate the requirements for binding E_3 to protein X, a set of deletions in the *PDX1* gene was constructed. Construction of the deletion mutations was simplified by employing PCR to amplify specific regions of the *PDX1* gene. For example, in the construction of pX80 (Figure 4), primers 1 and 2 (Table II) were used to direct a PCR yielding a *Sal*I–*Xba*I DNA fragment extending from nucleotides 5 through 456. Similarly, primers 3 and 4 were used to generate an *Xba*I–*Eco*RI fragment extending from nucleotides 681 through 1783. The *Sal*I–*Xba*I fragment was cloned into plasmid YEp352 (multiple cloning site: *Hin*dIII, *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI) followed by the subcloning of the *Xba*I–*Eco*RI fragment into the adjacent position, resulting in a reconstructed *PDX1* gene in which nucleotides 457 through 680 were deleted. Further deletions were constructed with the following primers: pX180, primers 1,2 and 8,4; pX217, primers 1,2 and 7,4; pX180-2, primers 1,5 and 6,4. The addition of the restriction sites (*Xba*I or *Sma*I) between the fragments resulted in the addition of one or two amino acids immediately following amino acid 5. These additions are as follows: pX80, leucine; pX 217 and pX180, leucine and glutamate. The deleted forms of *PDX1* were moved into pSEYc63 (unit-copy plasmid) on a *Sal*I–*Eco*I fragment, and the resulting plasmids were transformed into yeast strain JLY61c (*pdx1*::*LEU2*).

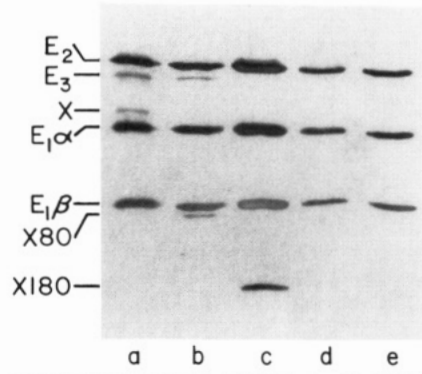

FIGURE 5: SDS–PAGE patterns of highly purified PDH complexes from deletion mutants. Lane a, wild-type; lanes b–e, pX80-, pX180-, pX180-2-, and pX217-encoded complexes, respectively. Protein bands were detected by silver staining.

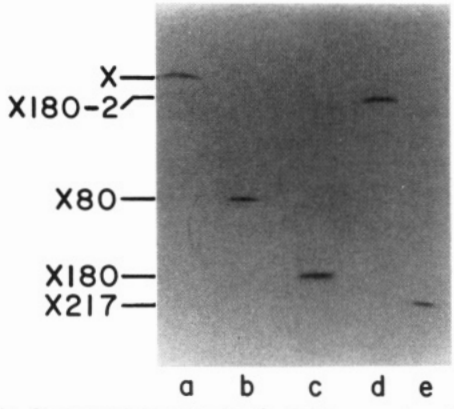

FIGURE 6: Immunoblot analysis of wild-type and mutant PDH complexes with anti-X serum. Lanes a–e correspond to the lanes in Figure 5. The membrane was probed with a 1:30000 dilution of rabbit anti-X serum.

Characterization of PDH Complexes from Mutant Strains. PDH complexes were isolated from the mutant cells and characterized by enzymatic assays, SDS–PAGE, and immunoblotting. Although the pX80-encoded PDH complex lacks most of the lipoyl domain (residues 6–80) of protein X, including the covalently bound lipoyl moiety, this mutant complex exhibited 60–70% of the overall activity of the wild-type PDH complex (Table I), and the complex contained E_3 (Figure 5). These observations demonstrate that the lipoyl domain (and its covalently bound lipoyl moiety) is not essential for protein X function in *S. cerevisiae*. Deletion of the putative subunit binding domain of protein X resulted in complete loss of overall activity of the corresponding PDH complexes (pX180-, pX180-2-, and pX217-encoded complexes, Table I). SDS–PAGE patterns (Figure 5) and immunoblot analysis with anti-E_3 serum (data not shown) confirmed the absence of E_3 in these mutant PDH complexes. The presence of truncated forms of protein X in the PDH complexes from the mutant strains was confirmed by immunoblot analysis (Figure 6). Comparison of Figures 5 and 6 shows that X180-2 migrated with $E_1\alpha$. X217 was detected by immunoblotting but not by SDS–PAGE. These observations indicate that the subunit binding domain of protein X plays an important role in E_3 binding.

Expression of Protein X. The 3.6-kb DNA fragment containing *PDX1* was moved from pGEM-X (Figure 1) to plasmids YEp352 and pVTU(102) on a *Bam*HI–*Xba*I fragment, and from YEp352-X to plasmid pSEYc63 on a *Bam*HI–*Sal*I fragment to construct multiple-copy (YEp352-X) and unit-copy (pSEYc63-X) plasmids, and to place *PDX1* under the control of an alcohol dehydrogenase promoter in the expression vector pVTU-X. The recombinant plasmids were used to transform yeast strain JLY61c, in which *PDX1* is disrupted. Transformed cells were selected on YNBD(−uracil) plates. Immunoblots of mitochondrial proteins from the transformed cells are shown in Figure 7. The amount of protein X produced by pSEYc63-X was comparable with that produced by the wild-type strain. Appreciably more protein X was produced by YEp352-X and even more by pVTU-X. A higher molecular weight form of protein X, presumably the precursor of protein X, also accumulated.

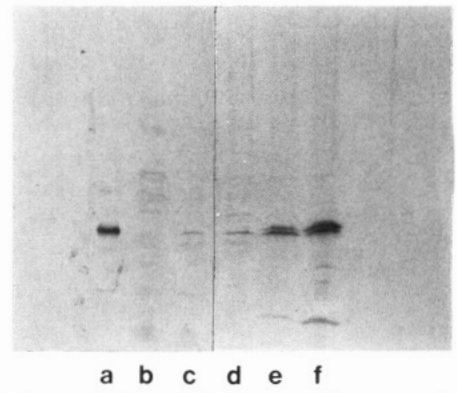

FIGURE 7: Immunoblot analysis for recombinant protein X in mitochondrial extracts. Isolated mitochondria were heated for 3–5 min at 95 °C with denaturing buffer (Laemmli, 1970). The proteins were separated by SDS–PAGE and transferred electrophoretically to Immobilon-P, and the membrane was probed with a 1:30000 dilution of anti-X serum. Lane a, wild-type PDH complex; lane b, JLY61c(*pdx1*::*LEU2*) mutant; lane c, wild-type yeast; lanes d–f, JLY61c transformed with pSEYc63-X (lane d), YEp352-X (lane e), and pVTU-X (lane f). Approximately 40 μg of protein was applied to lane b and 30 μg of protein to lanes c–f.

Table III: Reconstitution of PDH Complex Activity[a]

E_1E_2 (μg)	E_3 (μg)	mitochondrial extract (μL)	specific activity[b]
1.0	0.35	20	1.67
1.0	0.35	33	2.59
1.0	0.35	50	1.94
0.5	3.5	20	1.17
3.1	1.0	90	2.05
5.5	0	0	0
0	35.0	0	0
0	0	50	0
10.5	3.5	0	0

[a] Mixtures of E_1E_2 subcomplex, recombinant E_3, and partially purified protein X (7.8 mg of protein/mL) were kept at room temperature for 5 min and then assayed for overall PDH complex activity. [b] Units per milligram of E_1E_2 complex.

Reconstitution of PDH Complex Activity with Recombinant Protein X. Extracts of mitochondria from yeast strain JLY61c transformed with pVTU-X (in which *PDX1* is overexpressed) were used as a source of protein X. The extracts were centrifuged for 90 min at 50000 rpm in a Beckman SW55 Ti rotor to sediment the PDH complex. The presence of protein X in the supernatant fluid was confirmed by immunoblotting. Mixtures containing varying amounts of E_1E_2 subcomplex (isolated from the *pdx1* null mutant), yeast recombinant E_3, and partially purified protein X exhibited a maximum pyruvate oxidation activity of about 20% of that of wild-type PDH complex (Table III). Pretreatment of the protein X preparation with anti-X serum eliminated the pyruvate oxidation activity; preimmune serum had no effect (data not shown). These observations suggest that protein X may combine with the preformed E_2 core. However, it will be necessary to confirm these results with highly purified protein X. Toward this end, we are attempting to construct expression vectors that

produce more protein X than does pVTU-X.

The findings that protein X is required to bind E_3 to the E_2 core and that there are only about six molecules of protein X associated with the E_2 core in the bovine PDH complex (Jilka et al., 1986) provide an explanation of the observation that the bovine kidney E_2 core can bind only about six E_3 dimers (Wu & Reed, 1984). Although it is uncertain where or how protein X is bound to the E_2 inner core, it appears that there is a relatively small, fixed number of binding sites for protein X, probably six. Thus, when purified PDH complexes from *S. cerevisiae* cells transformed with the unit-copy plasmid (pSEYc63-X) and the multicopy plasmid (pVTU-X) harboring the *PDX1* gene were compared by SDS-PAGE, no significant difference was detected in the amount of protein X present in the two PDH complexes (data not shown).

Thus far, protein X has been detected only in eukaryotic PDH complexes and not in any other α-keto acid dehydrogenase complexes. In the latter complexes, E_3 binds directly to the E_2 core, and this binding apparently involves a domain, the subunit binding domain, which is apparently conserved among all members of the dihydrolipoamide acyltransferase family. It should be noted that an analogous domain in protein X (Figure 4, residues ~144–180) is apparently involved in binding E_3 in the *S. cerevisiae* PDH complex. The molecular basis of these differences in E_3 binding remains to be determined. There does not appear to be a correlation between the design of the E_2 core, i.e., whether octahedral or icosahedral, and the presence or absence of protein X. Thus, dihydrolipoamide acetyltransferase from *Bacillus stearothermophilus* has an icosahedral design but apparently does not contain protein X (Perham & Packman, 1989).

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