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Functional Analysis of the Domains of Dihydrolipoamide Acetyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: The *LAT1* gene encoding the dihydrolipoamide acetyltransferase component (E_2) of the pyruvate dehydrogenase (PDH) complex from *Saccharomyces cerevisiae* was disrupted, and the *lat1* null mutant was used to analyze the structure and function of the domains of E_2. Disruption of *LAT1* did not affect the viability of the cells. Apparently, flux through the PDH complex is not required for growth of *S. cerevisiae* under the conditions tested. The wild-type and mutant PDH complexes were purified to near-homogeneity and were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblotting, and enzyme assays. Mutant cells transformed with *LAT1* on a unit-copy plasmid produced a PDH complex very similar to that of the wild-type PDH complex. Deletion of most of the putative lipoyl domain (residues 8–84) resulted in loss of about 85% of the overall activity, but did not affect the acetyltransferase activity of E_2 or the binding of pyruvate dehydrogenase (E_1), dihydrolipoamide dehydrogenase (E_3), and protein X to the truncated E_2. Similar results were obtained by deleting the lipoyl domain plus the first hinge region (residues 8–145) and by replacing lysine-47, the putative site of covalent attachment of the lipoyl moiety, by arginine. Although the lipoyl domain of E_2 and/or its covalently bound lipoyl moiety were removed, the mutant complexes retained 12–15% of the overall activity of the wild-type PDH complex. Replacement of both lysine-47 in E_2 and the equivalent lysine-43 in protein X by arginine resulted in complete loss of overall activity of the mutant PDH complex. These observations indicate that the lipoyl domain of protein X can substitute, at least in part, for the lipoyl domain of E_2 and that the lipoyl domain of protein X can couple with the catalytic domain of the truncated E_2. Deletion of residues 8–181, encompassing the lipoyl domain, first hinge region, and the subunit binding domain, resulted in loss of ability of the truncated E_2 to bind E_1. Similar results were obtained by deleting the putative subunit binding domain itself (residues 145–181). This domain apparently plays an important role in binding E_1.

Mammalian and *Saccharomyces cerevisiae* pyruvate dehydrogenase (PDH)[1] complexes are organized about a 60-subunit E_2 core, to which multiple copies of E_1, E_3, and protein X are bound by noncovalent bonds (Reed & Hackert, 1990). Protein X binds and positions E_3 to the E_2 core, and this specific binding is essential for a functional PDH complex (Powers-Greenwood et al., 1989; Gopalakrishnan et al., 1989; Lawson et al., 1991).

Studies involving limited proteolysis, molecular genetics, and ^{1}H NMR spectroscopy have provided evidence that dihydrolipoamide acyltransferases possess a unique multidomain structure (Reed & Hackert, 1990; Guest et al., 1989; Perham & Packman, 1989). The amino-terminal lipoyl domain (or domains) is followed by a putative E_3 and/or E_1 binding domain and then by the carboxyl-terminal catalytic domain. The domains are connected by conformationally flexible segments (hinge regions).

An intriguing aspect of structure–function relationships in dihydrolipoamide acyltransferases is the nature and function of the putative E_3 and/or E_1 binding domain. This conserved domain is apparently involved in binding E_3 in the *Escherichia*

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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); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid.

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coli dihydrolipoamide acetyltransferase and succinyltransferase (Packman & Perham, 1986), E_1 in the mammalian acetyltransferase (Rahmatullah et al., 1989), and E_3 and E_1 in the acetyltransferase from *Acetobacter vinelandii* (Hanemaaijer et al., 1988) and *Bacillus stearothermophilus* (Packman et al., 1988). A similar domain in protein X is involved in binding E_3.

To gain further insight into the nature and function of the domains in *S. cerevisiae* dihydrolipoamide acetyltransferase, we have disrupted the *LAT1* gene, and we have constructed deletion and site-specific mutations in this gene. The effects of these mutations on the subunit composition and activities of the PDH complex were determined.

Experimental Procedures

Materials. Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. [α-^{32}P]dCTP (3000 Ci/mmol) and [1-^{14}C]acetyl-CoA were obtained from New England Nuclear. Immobilon-P [poly(vinylidene difluoride)] membrane was purchased from Millipore, and Zeta-Probe nylon membrane was purchased from Bio-Rad. Random DNA primers were purchased from Boehringer Mannheim. 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 can1-100*) and JLY21c (*MAT*α *lat1::HIS3 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*) were used. Yeast strain JLY62-2a(*pdx1::LEU2 lat1::HIS3 ade2-1 leu2-3,112 his3-11,15 trp1-1 ura3-1 can1-100*) was constructed by mating JLY21c (this paper) and JLY61c (Lawson et al., 1991). The desired genotype was isolated by random-spore analysis (Rose et al., 1990). *E. coli* strain JM101 [*supE thi* Δ(*lac-proAB*) [F′*traD36 proAB lacI*^{q} *lacZ*Δ*M15*]] (Yanisch-Perron et al., 1985) was used for plasmid construction. The following plasmids were used: pGEM-7Zf(+) (Promega) and pSEYc63 (*CEN4 ARS1 URA3*) (Emr 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 1X (0.67 g/100 mL of yeast nitrogen base without amino acids) plus the appropriate nutritional additives and carbon source was used for marker selection. For sporulation, diploid yeast strains were grown on YP dextrose for 2 days at 30 °C, and then in liquid sporulation medium (1% potassium acetate, 0.1% Bacto-yeast extract, and 0.05% dextrose) for 3–4 days at 30 °C (Sherman et al., 1979).

Isolation of DNA and Southern Blots. DNA isolation and manipulations, blotting, and washing were as described (Lawson et al., 1991).

Transformation and Gene Disruption. Standard methods were used for *E. coli* transformation (Cohen et al., 1971). Plasmid DNA was prepared as described (Zhou et al., 1990). Yeast was transformed using lithium acetate (Rose et al., 1990). One-step gene disruption was done as described by Rothstein (1983).

Construction of Deletion Mutations. Deletion mutations were constructed by amplifying specific regions of the *LAT1* gene by PCR and sequentially subcloning these fragments into yeast/*E. coli* shuttle vectors. PCR was done as described (Lawson et al., 1991). Wild-type *LAT1* on a 2.8-kb *Xba*I–*Bam*HI fragment in plasmid pSEYc63 was used as a template. The mutant constructs were sequenced to verify the mutations.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed as described (Zoller & Smith, 1984). To replace Lys-47 by Arg in the E_2 lipoyl domain, nucleotides 1422 through 2634 of *LAT1* (Niu et al., 1988) were subcloned into mp18 (*Bam*HI–*Xba*I sites). A mutagenic oligonucleotide was synthesized that was complementary to bases 2215 through

				47			
	Glu	Thr	Asp	Lys	Ala	Gln	Met	
5′	GAA	ACA	GAC	AAG	GCT	CAA	ATG	3′
3′	CTT	TGT	CTG	TCT	CGA	GTT	TAC	5′
				Arg				

2236, except that bases 2225 and 2226 (italics) were changed to produce an arginine codon in the sense strand. The nucleotide substitutions also created a new *Sac*I site to facilitate screening for mutants. Positive plaques were selected by differential hybridization to the radiolabeled mutagenic oligonucleotide and by the appearance of the new *Sac*I site. The mutation was confirmed by DNA sequencing. The mutagenized *lat1* was then reconstructed in pSEYc63 for analysis.

To replace Lys-43 by Arg in the lipoyl domain of protein X, a 2.0-kb *Xho*I–*Xba*I fragment of *PDX1* (strand complementary to the published sequence) was subcloned into mp19 (Lawson et al., 1991). A mutagenic oligonucleotide was synthesized that was complementary to nucleotides 559

				43			
	Glu	Thr	Asp	Lys	Ser	Gln	Ile	
5′	GAA	ACA	GAT	AAA	TCT	CAA	ATT	3′
3′	GAA	ACA	GAC	AGA	TCT	CAA	ATT	5′
				Arg				

through 580, except at 567 and 569 (italics). These nucleotide substitutions changed the amino acid specified by codon 43 from lysine to arginine and created a *Bgl*II site. Mutagenized plaques were selected by differential hybridization to the mutagenic oligonucleotide and by the presence of the new *Bgl*II site. The mutagenized *pdx1* was reconstructed in pSEYc63 for analysis.

To construct a double mutant, the mutagenized *lat1* and *pdx1* were subcloned into the same plasmid (pSEYc63) and expressed simultaneously in the yeast strain in which *LAT1* and *PDX1* were disrupted (JLY62-2a).

Preparation of Mitochondria. Small-scale preparations of mitochondria were made by mechanical breakage of yeast cells with glass beads (Deters & Ewing, 1985).

Immunoblotting. Proteins were separated by SDS–polyacrylamide gel (10% or 12.5% acrylamide) electrophoresis and then transferred electrophoretically onto an Immobilon-P membrane (Matsudaira, 1987). Immunoblot analysis was performed with rabbit anti-E_2 serum and goat anti-rabbit IgG conjugated to alkaline phosphatase or peroxidase as described by the supplier.

Enzyme Assays. Activities of E_1, E_2, and E_3 were measured as described, respectively, by Lau et al. (1990), Niu et al. (1990), and Reed et al. (1966). Units of E_1, E_2, and E_3 are expressed, respectively, as micromoles per minute of 2,6-dichlorophenolindophenol reduced, acetyl groups transferred from [1-^{14}C]acetyl-CoA to dihydrolipoamide, and NADH oxidized by lipoamide. The molar extinction coefficient of 2,6-dichlorophenolindophenol was taken as 21 000 M^{-1} cm^{-1} (Steyn-Parve & Beinert, 1958). The overall activity of the PDH complex was assayed as described by Uhlinger et al. (1986). One unit is defined as the amount of enzyme complex that produces 1 μmol of NADH per minute. Protein was determined as described by Bradford (1976).

Purification of PDH Complexes. Wild-type and mutant cells were grown in 12 L of YNB dextrose medium plus supplements (Sherman et al., 1979) for 36 h at 30 °C in a New Brunswick Model SF-116 MicroGen fermentor. Cells were harvested when the absorbance at 600 nm was about 1.7.

Approximately 50 g (wet weight) of cells was resuspended in 150 mL of ice-cold buffer P-100 [100 mM potassium phosphate, pH 7.3, 0.01 mM thiamin diphosphate, 1 mM dithiothreitol, 1 mM EDTA, 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. The cells were broken by passing the suspension twice through a French press at 16000 psi, and the cell debris was removed by centrifugation at 20000 rpm for 30 min in a Beckman JA-20 rotor. The cell extract was filtered through four layers of cheesecloth. The pH and protein concentration of the extract were adjusted to 6.2 and about 15 mg/mL, respectively. To the extract was added dropwise, with stirring, 0.025 volume of 2% (w/v) protamine sulfate. After 10 min, the precipitate was removed by centrifugation at 20000 rpm for 30 min in a JA-20 rotor. The supernatant fluid was fractionated with poly(ethylene glycol) 6000. A 0.02-volume aliquot of 50% (w/v) poly(ethylene glycol) was added dropwise, with stirring. After 15 min, the precipitate was collected by centrifugation, and the supernatant fluid was assayed for E_2 activity. Addition of the poly(ethylene glycol) was continued, in 0.02-volume increments, until the E_2 activity of the supernatant fluid was reduced to less than 5% of the initial activity. The amount of 50% poly(ethylene glycol) required for precipitation of the wild-type and mutant complexes varied between 0.04 and 0.06 volume. The pellet containing E_2 activity was dissolved in a total of about 20 mL of buffer P-100. Isoelectric precipitation of the PDH complex was carried out by stepwise addition, with stirring, of 10% acetic acid. Precipitates were collected by centrifugation, and the supernatant fluids were assayed for overall activity or E_2 activity. The PDH complexes precipitated at about pH 6.0. The pellet was dissolved in a minimal volume of buffer P-100. A 0.2-mL sample was subjected to fast protein liquid chromatography on a Superose 6 column equilibrated and developed with buffer P-100 at room temperature and a flow rate of 6 mL/h. With some preparations, 0.1-mL samples were layered over a 15–40% (w/w) linear sucrose gradient in 5-mL centrifuge tubes, and the tubes were centrifuged for 2 h at 50000 rpm in a Beckman SW55 Ti rotor. Fractions were collected with an Isco Model 640 density gradient fractionator. The fractions were assayed for E_1, E_2, E_3, and overall PDH complex activity. The active fractions were analyzed by Tricine–SDS–PAGE (Schägger & von Jagow, 1987). The yield of highly purified wild-type and mutant complexes was 0.3–2 mg.

Results and Discussion

Disruption of the LAT1 Gene. The coding region of *LAT1* plus 869 bp of 5′-flanking DNA and 489 bp of 3′-flanking DNA was inserted into plasmid pGEM-7Zf between the *Xba*I and *Bam*HI sites. A 1360 bp segment of the coding region and 283 bp of 5′-flanking DNA were removed by digestion with *Bcl*I (Figure 1), leaving 94 bp of coding region (at the 3′ end) and all of the 3′-flanking DNA. The yeast *HIS3* gene on a 1.77 bp *Bam*HI fragment was inserted into the *Bcl*I site to replace the excised *LAT1* DNA.

The disrupted gene was excised from the plasmid by digestion at the upstream *Xba*I site and at a *Sac*I site (just downstream from the *Bam*HI site) in pGEM-7Zf. This 2.9-kb fragment was used to transform the diploid yeast strain W303a/α. Stable transformants were selected on minimal media (YNB dextrose) lacking histidine.

The transformed diploids were sporulated, and all spores were viable. DNA was prepared from representative tetrads and analyzed by Southern blotting (Figure 2). The genomic DNA was digested with *Xba*I, which cuts within the portion of the *LAT1* coding region that was removed in the disruption. The blot was probed with 2.5-kb *Bam*HI DNA fragment containing the entire *LAT1* coding region plus 579 bp of 5′-flanking DNA and 489 bp of 3′-flanking DNA (Figure 1). Spores which contained the wild-type *LAT1* (nondisrupted) yielded two *Xba*I fragments, 1.50 and 1.58 kb, which hybridized to the probe. The disrupted *LAT1* locus lacked the internal *Xba*I site; therefore, the probe recognized a larger (3.0 kb) *Xba*I fragment. As expected, *LAT1* and *lat1*::*HIS3* segregated 2:2 in each tetrad, indicating that one chromosomal copy of *LAT1* was disrupted by *HIS3*.

HIS3 integration was confirmed genetically and immunologically. Those spores which contained the disrupted gene (the larger hybridizing fragment) were found to be HIS$^+$ (able to grow without supplemental histidine) while those spores which contained only wild-type *LAT1* were HIS$^-$. On immunoblots (Figure 3), the E_2 protein was detected in mito-

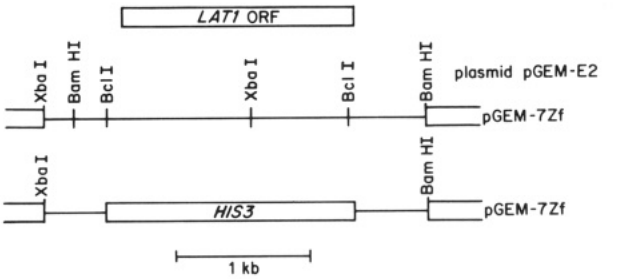

FIGURE 1: Disruption of the *LAT1* gene. Plasmid pGEM-E2 was digested with *Bcl*I to remove all but 94 bp of the *LAT1* coding region. The deleted 1.64-kb fragment was replaced by a 1.77-kb fragment containing the yeast *HIS3* gene. A 2.9-kb *Xba*I–*Sac*I fragment (*Sac*I site of pGEM) was used to transform yeast strain W303a/α. The bar above the restriction map indicates the position of the *LAT1* open reading frame (ORF).

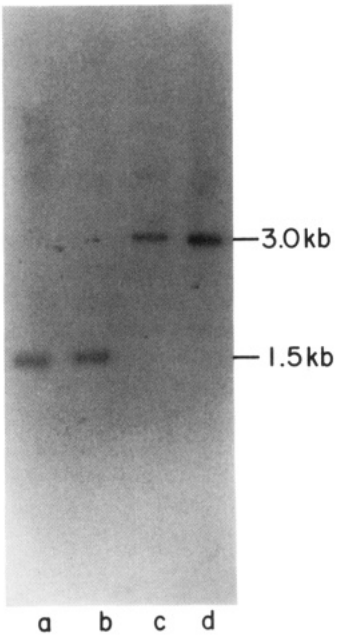

FIGURE 2: Southern blot analysis of *LAT1* disruption. Genomic DNA from representative tetrads was digested with *Xba*I. The digest was electrophoresed on a 1% agarose gel, and the fragments were transferred onto a Zeta-Probe membrane. The blot was probed with a radiolabeled 2.5-kb *Bam*HI fragment containing *LAT1* (Figure 1). The probe hybridized to 1.50- and 1.58-kb fragments of the wild-type *LAT1* gene (lanes a and b) and to *lat1*::*HIS3* (3.0 kb; lanes c and d). *LAT1* but not the disrupted form of the gene contains an internal *Xba*I site.

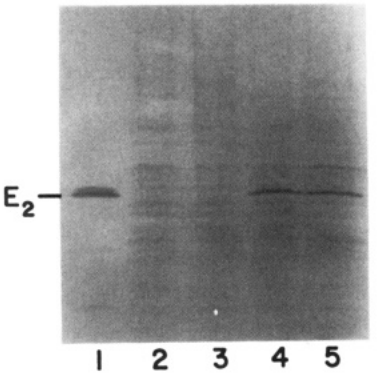

FIGURE 3: Immunoblot analysis of disruption of *LAT1*. Mitochondria from a representative tetrad were heated in denaturing buffer (Laemmli, 1970) for 3 min at 95 °C, and the extracts were subjected to SDS–PAGE (12.5% acrylamide). The proteins were transferred electrophoretically onto a Immobilon-P membrane, and the membrane was probed with a 1:20 000 dilution of rabbit anti-E_2 serum. Lane 1, wild-type PDH complex; lanes 2 and 3, mitochondrial proteins from *lat1*::*HIS3* spores; lanes 4 and 5, mitochondrial proteins from spores wild-type for *LAT1*. Approximately 30 μg of protein was applied to lanes 2–5.

chondria from the wild-type, HIS^- spores (lanes 4 and 5) but not in mitochondria from the disrupted, HIS^+ spores (lanes 2 and 3).

Growth rates of the wild-type and disrupted (null mutant) spores were compared on solid and in liquid, rich and minimal media, with dextrose, glycerol, lactate, acetate, or ethanol as the carbon source. No significant difference in the growth characteristics of the wild-type and *lat1* mutant strains was seen under any of the above conditions. Apparently, *S. cerevisiae* does not require a functional *LAT1* gene under these vegetative growth conditions.

In a previous investigation (Lawson et al., 1991), we found that a functional *PDX1* gene, encoding protein X, is also not required for yeast cell growth. Disruption of both the *LAT1* and *PDX1* genes in *S. cerevisiae* (strain JLY62-2a) did not affect the viability of the cells under these growth conditions. Apparently, flux through the pyruvate dehydrogenase complex is not required for growth of *S. cerevisiae* under the conditions tested. By contrast, disruption of the genes encoding the α-ketoglutarate dehydrogenase (E_1) or the dihydrolipoamide succinyltransferase (E_2) components of the α-ketoglutarate dehydrogenase complex resulted in inability of *S. cerevisiae* to utilize the nonfermentable substrate glycerol as the carbon source (Repetto & Tzagoloff, 1989, 1990). α-Ketoglutarate, in contrast to pyruvate, is a precursor of essential cell components.

Deletion Mutations in the LAT1 Gene. The proposed segmented structure of yeast dihydrolipoamide acetyltransferase (Niu et al., 1990; Lawson et al., 1991) is shown in Figure 4. Amino acid residues ~1–84 comprise the putative lipoyl domain, residues ~146–181 comprise the putative E_1 binding domain, and residues ~221–454 comprise the catalytic inner core domain. To gain further understanding of the nature and function of these domains, a series of deletion mutations in *LAT1* was constructed (Figure 4). Defined regions of *LAT1* were amplified by PCR, and the mutant genes were reconstructed in a yeast/*E. coli* shuttle vector. For example, using primers 1 and 2 (Table I), a fragment coding for residues −28 to 7 was synthesized. Using primers 3 and 4, a fragment which encodes residues 85–454 was synthesized. These two fragments were sequentially subcloned into pSEYc63 (*Bam*HI–*Hin*dIII sites) to produce plasmid pE84 in which nucleotides 2106–2336 of *LAT1* were deleted. This deletion produces a protein missing residues 8–84, i.e., most

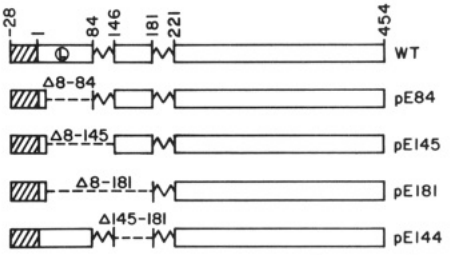

FIGURE 4: Diagrammatic representation of the putative structural domains of *S. cerevisiae* E_2 (WT) and deletion mutations. The domains are connected by hinge regions (squiggly lines). The limits of these domains are approximate and are indicated with amino acid residue numbers. The hatched areas correspond to the presequence of the E_2 precursor, and L denotes the putative lipoyl domain.

Table I: Oligonucleotide Primers for PCR[a]

primer	sequence	location
	BamHI	
1	GTTCGGATCCCTATCAAGTGGGAAGAAC	1513 (F)
	SalI	
2	GGTTGTCGACGTGTGCTCTGGGTACGATGC	2105 (R)
	SalI	
3	GTTCGTCGACCAAAGCTGATGTGCCAGC	2337 (F)
	HindIII	
4	GGTTAAGCTTCTCATGGTTTATTTG	3849 (R)
	SalI	
5	GTTCGTCGACGATTTTTGCCTCTCCAC	2520 (F)
	SalI	
6	GTTCGTCGACGTCATATCTAGAAAAGTCG	2628 (F)
	SalI	
7	GGTTGTCGACCCTTGAGGAGCAGCAAC	2516 (R)

[a] Sequences are listed 5′–3′. The first four nucleotides and portions of the underlined restriction sites were added to facilitate cloning and do not correspond to *LAT1* sequences (Niu et al., 1988; Niu, 1989). Location refers to the nucleotide of *LAT1* at which hybridization to the primer begins and continues in the forward (F) or reverse (R) direction.

of the putative lipoyl domain. The primers for PCR contained *Sal*I recognition sites to facilitate cloning of the DNA fragments. This resulted in the addition of two amino acids, serine and threonine, at the junction sites in each mutant construct. Primers 1,2 and 5,4 were used to construct pE145, primers 1,2 and 6,4 were used for pE181, and primers 1,7 and 6,4 were used for pE144. The mutant genes in pSEYc63 (unit-copy plasmid) were transformed into yeast strain JLY21c (*lat1*::*HIS3*) for further analysis.

Isolation and Characterization of PDH Complexes. PDH complexes were purified to near-homogeneity from wild-type and mutant cells as described under Experimental Procedures. Mild procedures were used to minimize dissociation of subunits. The purified complexes were characterized by enzymatic assays (Table II), SDS–PAGE (Figures 5 and 6), and immunoblotting (Figure 7).

E_2 was not detectable in extracts of the *lat1* null mutant (JLY21c) by immunoblot analysis with anti-E_2 serum (data not shown). However, when this mutant was transformed with a unit-copy plasmid harboring the *LAT1* gene (pSEYc63-E2), the transformed cells produced a PDH complex that was very similar to the wild-type PDH complex in enzymatic activities (Table II) and subunit composition, as revealed by SDS–PAGE (Figure 5).

Deletion of most of the lipoyl domain (residues 8–84) resulted in loss of about 85% of the overall activity but did not affect the acetyltransferase activity of E_2 (Table II) or the

Table II: Enzymatic Activities of Wild-Type and Mutant PDH Complexes[a]

PDH complex	specific activity[b]			
	NAD^+ reduction	E_1	E_2	E_3
wild type	86.2	0.34	1.9	36.2
JLY21c/pSEYc63-E2	81.9	0.29	2.0	32.8
JLY21c/pE84	11.9	0.37	1.7	36.4
JLY21c/pE145	13.6	0.37	1.8	33.6
JLY21c/pE181	0	0	1.8	22.8
JLY21c/pE144	0	0	1.8	37.3
JLY21c/pER1	10.5	0.26	1.9	31.3
JLY62-2a/pR1K6	0	0.30	1.9	29.9

[a] The yeast strain harboring the disrupted *LAT1* gene (JLY21c) was transformed with a unit-copy plasmid carrying *LAT1* (pSEYc63-E2), with plasmids harboring deletion mutations in *LAT1* as defined in Figure 2, and with a plasmid harboring a Lys-47 → Arg substitution in *LAT1* (pER1). Yeast strain JLY62-2a (disrupted *LAT1* and *PDX1*) was transformed with a unit-copy plasmid carrying both a Lys-43 → Arg substitution in *PDX1* and a Lys-47 → Arg substitution in *LAT1* (pR1K6). [b] Units per nanomole of complex.

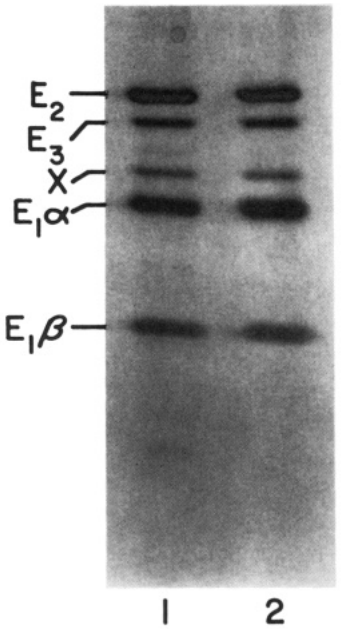

FIGURE 5: SDS–PAGE patterns of highly purified PDH complexes from wild-type cells (lane 1) and *lat1* null mutant transformed with the wild-type gene for E_2 on a unit-copy plasmid (lane 2). Approximately 1 μg of protein was applied to each lane. Protein bands were detected by silver staining (Oakley et al., 1980).

binding of E_1, protein X, and E_3 to the truncated E_2 (Figure 6, lane 2). Similar results were obtained by deleting the lipoyl domain plus the first hinge region, comprising residues 85–145. The presence of truncated forms of E_2 in the PDH complexes from the mutant strains was confirmed by immunoblot analysis (Figure 7). Deletion of residues 145–181 or residues 8–181 resulted in loss of ability of the truncated E_2 to bind E_1 (Figure 6 and Table II). These observations indicate that the putative subunit binding domain (residues ~146–181) plays an important role in binding E_1. A similar domain in bovine kidney dihydrolipoamide acetyltransferase has been shown by limited proteolysis and immunoblotting studies to play an important role in binding bovine kidney E_1 (Rahmatullah et al., 1989). The finding that the truncated yeast E_2(Δ8–181) retained activity in the acetyltransfer reaction comparable to that of wild-type E_2 confirms and extends previous observations that the active site of E_2 resides in the inner core domain, comprising residues ~221–454 (Niu et al., 1990). Expression of residues 221–454 in *E. coli* produced a large oligomer which exhibited acetyltransferase activity very similar to that of

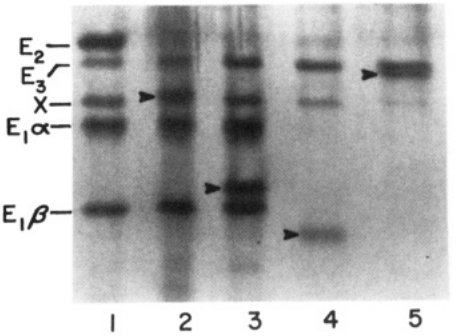

FIGURE 6: SDS–PAGE patterns of highly purified PDH complexes from deletion mutants. Lane 1, wild-type PDH complex (1 μg); lanes 2–5, mutant PDH complexes (1 μg) containing truncated forms of E_2—(Δ8–84), (Δ8–145), (Δ8–181), and (Δ145–181), respectively. The arrows indicate the location of the bands corresponding to these truncated E_2's. Protein bands were detected by silver staining.

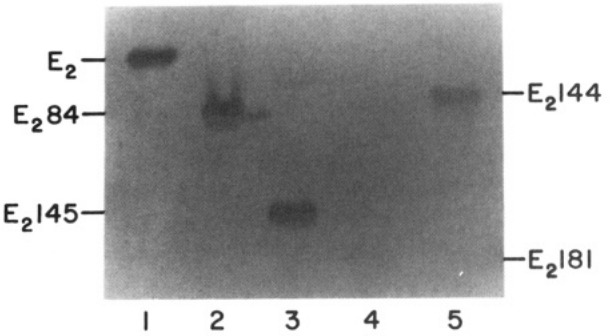

FIGURE 7: Immunoblot analysis of wild-type and mutant PDH complexes with anti-E_2 serum. Lanes 1–5 correspond to the lanes in Figure 6. The membrane was probed with a 1:20 000 dilution of rabbit anti-E_2 serum. The truncated E_2 in lane 4 (Δ8–181) reacted less effectively with the antiserum, probably due to the presence of fewer epitopes.

wild-type E_2. The appearance of the truncated and wild-type E_2 was also very similar, as observed by negative-stain electron microscopy, namely, a pentagonal dodecahedron.

Although the PDH complexes containing E_2(Δ8–84) and E_2(Δ8–145) lack most of the lipoyl domain of E_2, including the covalently bound lipoyl moiety, these mutant complexes exhibited about 15% of the overall activity of the wild-type PDH complex (Table II). Yeast protein X contains a lipoyl domain (Behal et al., 1989; Lawson et al., 1991), but it does not exhibit acetyltransferase activity (M. A. Yazdi, X.-D. Niu, J. E. Lawson, and L. J. Reed, unpublished observations). These observations indicate that the lipoyl domain of protein X and its covalently bound lipoyl moiety can substitute, at least in part, for the lipoyl domain of E_2 and that the lipoyl domain of protein X can couple with the catalytic domain of E_2. A similar proposal was made by Rahmatullah et al. (1990) for a protease-modified bovine kidney PDH complex. These investigators reported that *Clostridium histolyticum* collagenase selectively removed the lipoyl domains from the E_2 core of the bovine PDH complex without affecting the lipoyl domain of the E_2-bound protein X. After separation of the lipoyl domain from the truncated E_2–protein X subcomplex, E_1 and E_3 were added to reconstitute the PDH complex. The protease-modified PDH complex retained about 10% of the overall activity of the untreated PDH complex. The authors concluded that the lipoyl domain of protein X supported the overall reaction of the PDH complex.

Site-Directed Mutagenesis. Comparison of deduced amino acid sequences of *S. cerevisiae*, *E. coli*, and rat liver dihydrolipoamide acetyltransferases (Niu et al., 1988) suggests that the lipoyl moiety in the lipoyl domain of the yeast ace-

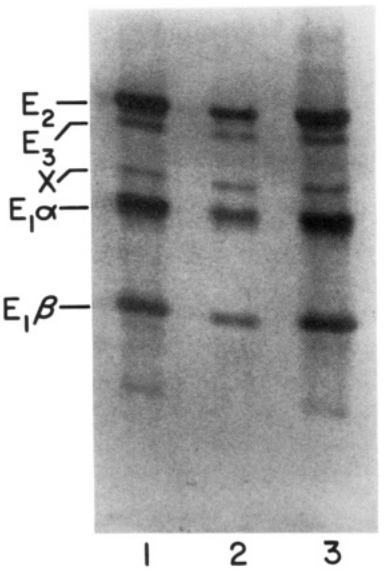

FIGURE 8: SDS–PAGE patterns of wild-type PDH complex (lane 1), a mutant PDH complex harboring a Lys-47 → Arg substitution in E_2 (lane 2), and a mutant PDH complex harboring both a Lys-47 → Arg substitution in E_2 and a Lys-43 → Arg substitution in protein X. Approximately 1.5 μg of protein was applied to lanes 1 and 3, and about 0.8 μg was applied to lane 2. Protein bands were detected by silver staining.

tyltransferase is bound to Lys-47. To confirm this deduction, Lys-47 was replaced by Arg by site-directed mutagenesis. The mutant gene was transformed into strain JLY21c on a unit-copy plasmid (pER1). The Lys-47 → Arg substitution resulted in a 88% reduction in the overall activity of the PDH complex, without affecting the E_1, E_2, and E_3 activities in the model reactions (Table II). The fact that about 12% of the overall activity was retained by the mutant PDH complex provides additional support for the proposal that the lipoyl domain of protein X can substitute, at least in part, for the lipoyl domain of E_2. Additional evidence for this proposal was obtained by constructing a double mutant in which both Lys-47 in E_2 and the corresponding Lys-43 in protein X were replaced by Arg. The mutant PDH complex (JLY62-2a/pR1K6) showed no detectable activity in the overall reaction (Table II) due, presumably, to the absence of protein-bound lipoyl moieties. The E_1, E_2, and E_3 activities of the complex in the model reactions were unaffected (Table II). The SDS–PAGE patterns of the two mutant PDH complexes were very similar to that of the wild-type PDH complex (Figure 8). To gain further insight into functional relationships of the structural domains of E_2 and protein X, we are constructing chimeric proteins in which these domains are interchanged.

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Registry No. E_1, 9001-04-1; E_2, 9032-29-5; Lys, 56-87-1; PDH, 9014-20-4.

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