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Overexpression and Mutagenesis of the Catalytic Domain of Dihydrolipoamide Acetyltransferase from *Saccharomyces cerevisiae*†

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ABSTRACT: The inner core domain (residues ~221-454) of the dihydrolipoamide acetyltransferase component (E_2p) of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae* has been overexpressed in *Escherichia coli* strain JM105 via the expression vector pKK233-2. The truncated E_2p was purified to apparent homogeneity. It exhibited catalytic activity (acetyl transfer from [1-^{14}C]acetyl-CoA to dihydrolipoamide) very similar to that of wild-type E_2p. The appearance of the truncated and wild-type E_2p was also very similar, as observed by negative-stain electron microscopy, namely, a pentagonal dodecahedron. These findings demonstrate that the active site of E_2p from *S. cerevisiae* resides in the inner core domain, i.e., catalytic domain, and that this domain alone can undergo self-assembly. The purified truncated E_2p showed a tendency to aggregate. Aggregation was prevented by genetically engineered attachment of the interdomain linker segment (residues ~181-220) to the catalytic domain. All dihydrolipoamide acyltransferases contain the sequence His-Xaa-Xaa-Xaa-Asp-Gly near their carboxyl termini. By analogy with chloramphenicol acetyltransferase, the highly conserved His and Asp residues were postulated to be involved in the catalytic mechanism [Guest, J. R. (1987) *FEMS Microbiol. Lett. 44*, 417-422]. Substitution of the sole His residue in the *S. cerevisiae* truncated E_2p, His-427, by Asn or Ala by site-directed mutagenesis did not have a significant effect on the k_{cat} or K_m values of the truncated E_2p. However, the Asp-431 → Asn, Ala, or Glu substitutions resulted in a 16-, 24-, and 3.7-fold reduction, respectively, in k_{cat}, with little change in K_m values. These findings indicate that a His residue is not involved in the catalytic mechanism of E_2p from *S. cerevisiae* but that Asp-431 plays an important role. Whether this role is structural or catalytic remains to be established.

All dihydrolipoamide acyltransferases possess a unique multidomain structure (Reed & Hackert, 1990; Guest et al., 1989; Perham & Packman, 1989). The amino-terminal part of the polypeptide chain contains 1, 2, or 3 highly similar lipoyl domains, each of about 80 amino acid residues, in tandem array. The lipoyl domain (or domains) is followed by another structurally distinct segment that is involved in binding dihydrolipoamide dehydrogenase (E_3)[1] or the α-keto acid dehydrogenase (E_1), or both. These domains are linked to each other and to the carboxyl-terminal part of the polypeptide chain (inner core domain) by conformationally flexible seg-

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[1] Abbreviations: E_1, α-keto acid dehydrogenase or pyruvate dehydrogenase; E_2p, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; PCR, polymerase chain reaction; r, recombinant; IPTG, isopropyl β-thiogalactoside; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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ments that are rich in the conservatively substituted residues alanine, proline, serine, and threonine and in charged amino acid residues. Limited proteolysis studies (Bleile et al., 1979; Packman & Perham, 1986) and genetically engineered deletions in the *Escherichia coli* dihydrolipoamide acetyltransferase (E_2p; Angier et al., 1987) have demonstrated that the active site of E_2p resides in the inner core domain.

Comparison of amino acid sequences of the *E. coli* dihydrolipoamide acetyltransferase and succinyltransferase with those of chloramphenicol acetyltransferases revealed well-aligned similarities (Guest, 1987; Guest et al., 1989). His-195 in the sequence His-Xaa-Xaa-Xaa-Asp-Gly near the carboxyl terminus has been implicated in the catalytic mechanism of chloramphenicol acetyltransferase (Kleanthous et al., 1985; Leslie et al., 1988). Asp-199 was thought to position the correct tautomer of His-195 which apparently acts as a general base in the acetyl-transfer reaction. However, establishment of the three-dimensional structure of chloramphenicol acetyltransferase (Leslie et al., 1988) and site-directed mutagenesis (Lewendon et al., 1988) have revealed that Asp-199 plays a structural rather than a catalytic role. The presence of this conserved sequence near the carboxyl termini of dihydrolipoamide acyltransferases led Guest to suggest that acyl transfer in dihydrolipoamide acyltransferases is mediated by a base-catalyzed mechanism analogous to that proposed for chloramphenicol acetyltransferase (Guest, 1987).

In this paper, we report overproduction in *E. coli* of the inner core of E_2p from *S. cerevisiae*, and we evaluate by directed mutagenesis the importance of the His and Asp residues in the putative active-site sequence.

Experimental Procedures

Materials

Rabbit antibody to *S. cerevisiae* E_2p was prepared as described (Niu et al., 1988). E_2p containing small amounts of E_3 and protein X was prepared by treatment of highly purified pyruvate dehydrogenase complex (Uhlinger et al., 1986) with 2 M NaCl at pH 7.0, followed by FPLC on a Superose 12 column (Cook & Yeaman, 1988). Restriction endonucleases and DNA-modifying enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, and Promega Biotec (Madison, WI). Yeast genomic DNA was obtained from Clontech Laboratories. The DNA sequencing reagents were purchased from United States Biochemical. Horseradish peroxidase conjugated goat anti-rabbit IgG was obtained from Kirkegaard and Perry (Gaithersburg, MD). [1-^{14}C]Acetyl-CoA and α-[^{35}S]thio-dATP were purchased from New England Nuclear. Immobilon [poly(vinylidene difluoride)] membrane was obtained from Millipore. Nitrocellulose membrane was obtained from Schleicher & Schuell. *E. coli* expression vector pKK233-2 and *E. coli* host strain JM105 [*thi*, *rps*L, *end*A, *sbc*B15, *hsa*R4, Δ(*lacpro*AB/F'*tra*D36, *pro*AB, *lac*I$^q\Delta$M15] were purchased from Pharmacia. Culture media were purchased from Difco (Detroit, MI). Protein molecular weight markers, ampicillin, streptomycin, DEAE-Sepharose, and heparin–agarose were obtained from Sigma. Other reagents and materials were of the highest grade available commercially.

Methods

Preparation of Oligonucleotides. Oligonucleotide primers for DNA amplification and sequencing were synthesized on an Applied Biosystems Model 381A DNA synthesizer. The oligonucleotide primers were eluted from the cartridge with 1 mL of 30% ammonium hydroxide, incubated at 50 °C for 5 h, dried in a Speed-Vac concentrator (Savant), and dissolved in 0.5 mL of H_2O. The primers were used without further purification.

DNA Sequencing. The double-stranded plasmid DNA was sequenced with Sequenase (United States Biochemical) following the manufacturer's instructions, except that the plasmid was first denatured in 0.2 M NaOH, precipitated with ethanol, and hybridized to an oligonucleotide primer for 15 min at 37 °C.

Design of Oligonucleotide Primers. Design of oligonucleotide primers for DNA amplification was based on the nucleotide sequence of the *S. cerevisiae* gene encoding E_2p (*LAT1*; Niu et al., 1988). Each primer (sense or antisense) contained a restriction enzyme site (e.g., *Hin*dIII, *Nco*I, or *Nar*I) at its 5' end, followed by a nucleotide sequence, 10–20 bases in length, which matched a particular sequence of the *LAT1* gene. In order to amplify the *LAT1* gene or part of the gene by PCR, a set of two primers, a sense primer and an antisense primer, was synthesized. These two primers specifically hybridize to the sequences of the genomic DNA which correspond to the 5' ends of the complementary strand of the DNA fragment to be amplified.

Polymerase Chain Reaction. PCR was performed with the GeneAmp DNA amplification reagent kit with AmpliTaq recombinant *Taq* DNA polymerase according to the manufacturer's instructions (Perkin-Elmer/Cetus). Each reaction mixture included a set of two primers (50 ng of each) and 1 μg of yeast genomic DNA as template. A step program of 30 cycles was set for a DNA thermal cycler: 94 °C, 5 min; then 29 cycles (94 °C, 1 min; 42 °C, 2 min; 72 °C, 3 min), followed by a 10-min extension step at 72 °C. The amplified DNA fragments had a built-in restriction enzyme site at both ends, introduced by the specific primers.

Construction of Expression Vectors. DNA fragments generated by PCR were precipitated with ethanol and then dissolved in 50 μL of H_2O. A 10-μL aliquot ($\sim$2 μg of DNA) was treated with a combination of two restriction endonucleases, specific for the built-in restriction sites on the fragment. The fragment was then purified by electrophoresis on a 1% low-melting agarose gel and ligated into plasmid pKK233-2, which had been treated with the same restriction enzymes to expose compatible ends.

Host Cell Transformation. The ligation mixture containing the recombinant plasmid was used to transform *E. coli* strain JM105 (Miller, 1987). Plasmids containing inserts were identified by digestion with appropriate restriction enzymes, followed by agarose gel electrophoresis. Nucleotide sequences were confirmed by direct DNA sequencing as described above. Transformed *E. coli* JM105 cells were inoculated on an M9 media plate (Maniatis et al., 1982) containing 0.05 mg/mL ampicillin and streptomycin.

Expression of Recombinant Proteins. Single colonies of *E. coli* strain JM105 harboring the recombinant plasmid were inoculated into 2 mL of LB medium containing 0.05 mg/mL ampicillin, and the cultures were grown overnight at 37 °C. To 5 mL of LB medium was added 0.1 mL of the overnight culture. After growth at 37 °C for approximately 3 h, the culture ($\sim 10^6$ cells/mL) was brought to 4 mM IPTG. After incubation for 2 h, the cells were harvested by centrifugation and lysed in 0.1 mL of denaturing buffer (65 mM Tris-HCl, 100 mM dithiothreitol, 1% SDS, and 0.004% bromophenol blue, pH 6.8). For preparation of cell extract under nondenaturing conditions, the harvested cells were washed and resuspended in 1.5 mL of ice-cold buffer containing 50 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, and

5% glycerol, pH 7.3. Cell disruption was performed with a microsonicator (40-W model; Sonics and Materials, Danbury, CT). The cold cell suspension was subjected to a 30-s sonication and then kept on ice for 1 min. This procedure was repeated 3 times. The soluble fraction was separated from cell debris by centrifugation at 20000*g* for 30 min at 4 °C. The cell debris was suspended in 0.1 mL of denaturing buffer.

Identification of Expressed Proteins. Aliquots of the *E. coli* cell lysates or extracts (10 μL) and the solubilized *E. coli* cell debris (5 μL) were analyzed by SDS–PAGE (12.5% acrylamide) mini-gel electrophoresis as described (Laemmli, 1970), except that TBEN (89 mM Tris–borate, 89 mM boric acid, 2 mM EDTA, and 0.1% SDS, pH 8.0) was used as electrophoresis buffer. The samples were heated for 5 min at 95 °C prior to electrophoresis. Proteins separated by gel electrophoresis were transferred electrophoretically onto a nitrocellulose membrane for 2 h at 10 °C and a voltage gradient of 7 V/cm in a transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol, pH 10.5. The membranes were stained with a solution containing 0.1% Fast Green, 10% acetic acid, and 45% methanol, and a parallel set was subjected to immunoblot analysis with rabbit anti-E2 serum and peroxidase-conjugated goat anti-rabbit IgG. 4-Chloro-1-naphthol was used as the chromogen in conjunction with H_2O_2.

Assay of E_2p Activity. Assay of E_2p activity is based on the initial rate of transfer of radioactive acetyl groups from [1-^{14}C]acetyl-CoA to dihydrolipoamide (Butterworth et al., 1975):

$$CH_3CO\text{–}SCoA + Lip(SH_2) \rightleftarrows CH_3CO\text{–}S\text{–}Lip\text{–}SH + CoA\text{–}SH$$

The reaction mixture contained 0.125 mL of 0.025 M potassium phosphate–0.005 M cysteine buffer, pH 7.4, 0.05 mL of 2.5 mM dihydrolipoamide in 25% ethanol, 0.05 mL of 2.5 mM [1-^{14}C]acetyl-CoA (8×10^5 cpm/μmol), and 0.025 mL of sample. The reaction was started by the addition of [1-^{14}C]acetyl-CoA, and the mixture was incubated at 25 °C for 2 min. Benzene (0.5 mL) was added to stop the reaction, and the mixture was shaken for 5–10 s on a Vortex mixer to extract the radioactive *S*-acetyldihydrolipoamide. A 0.25-mL aliquot of the benzene layer was withdrawn, mixed with 1 mL of scintillation fluid (Amersham), and counted. One unit of E_2p activity is defined as the amount of enzyme that transfers 1 nmol of acetyl groups per minute. Protein concentration was determined as described by Bradford (1976). Kinetic parameters were calculated from linear slope and intercept replots.

Purification of Truncated E_2p. Cells harvested from three 1-L IPTG-induced cultures (about 15 g wet weight) were resuspended in 60 mL of ice-cold buffer A [50 mM potassium phosphate, pH 7.3, 5% (v/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, and 1 mM benzamidine]. The suspension was passed twice through a French press at 16000 psi and then was centrifuged at 16000 rpm for 20 min in a Beckman JA-20 rotor. The pH value and protein concentration of the extract were adjusted to 6.6 and 7 mg/mL, respectively. To the extract was added dropwise, with stirring, 0.025 volume of 2% (w/v) protamine sulfate. After 15 min, the precipitate was removed by centrifugation at 20000 rpm for 30 min in a JA-20 rotor. The supernatant fluid was applied to a DEAE-Sepharose column (7 × 6 cm) that had been equilibrated with buffer A. The column was washed with 300 mL of buffer A, and the active protein was eluted with buffer A containing 0.1 M KCl. This fraction was applied slowly onto a heparin–agarose column (3 × 9 cm) equilibrated with buffer A. The column was washed with 100 mL of buffer A containing 0.1 M KCl and was developed with a 400-mL linear gradient from 0.1 to 0.5 M NaCl in buffer A. Active fractions were pooled, diluted 3-fold with buffer A to reduce the ionic strength, and concentrated by applying to a small column (~2 mL) of heparin–agarose, followed by elution with a small volume of buffer A containing 0.25 M KCl.

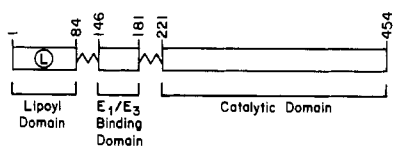

FIGURE 1: Diagrammatic representation of the structural domains of *S. cerevisiae* E_2p. The domains are connected by hinge regions (〰). The limits of these domains are approximate.

Amino-Terminal Sequence Analysis. The purified truncated E_2p was subjected to SDS–PAGE in a mini-slab gel apparatus, and the protein was transferred electrophoretically onto a poly(vinylidine difluoride) membrane (Matsudaira, 1987). The membrane was stained with Coomassie brilliant blue in 45% methanol, destained with 45% methanol, washed with deionized H_2O, and air-dried. The protein band corresponding to the truncated E_2p subunit was cut out with a razor blade and subjected to automated sequence analysis with an Applied Biosystems Model 470A gas-phase sequenator equipped with a Model 120A on-line phenylthiohydantoin amino acid analyzer.

Electron Microscopy. Protein samples (~0.2 mg/mL) were diluted to 10–20 μg/mL with 0.25% methylamine tungstate and immediately sprayed on Butvar-coated grids (Oliver, 1973). The wild-type E_2p was treated with 0.025% (v/v) glutaraldehyde for 2 min at room temperature before being applied to the grid film, as described above. The negatively stained specimens were examined in a JEOL 1200 electron microscope operated at 100 kV. The micrographs were recorded at magnification of 100000× and at an underfocus of ~0.1 μ.

RESULTS

Construction of Expression Vectors for Truncated E_2p. Comparison of the deduced amino acid sequences of *S. cerevisiae* E_2p and other dihydrolipoamide acetyltransferases (Niu et al., 1988) indicated that the yeast E_2p possesses the multidomain structure depicted in Figure 1. The putative inner core catalytic domain encompasses residues ~221–454. The strategy for construction of the expression vector for the catalytic domain is illustrated in Figure 2. The procedure was simplified by employing PCR to amplify, from yeast genomic DNA, that part of the *LAT1* gene encoding the E_2p(221–454) domain. On the basis of the nucleotide sequence of the *LAT1* gene (Niu et al., 1988), a pair of sense and antisense primers (1a and 2, respectively) was designed (Figure 2 and Table I). Both primers have a unique "built-in" restriction enzyme sequence at their 5′ ends for subcloning. The DNA fragment (subgene) generated by PCR was identified by agarose gel electrophoresis and by sequencing. The subgene had an *Nco*I restriction site at its 5′ end and a *Hin*dIII restriction site at the 3′ end. The restriction sites guaranteed ligation of the insert into the *E. coli* expression vector pKK233-2 in the correct orientation. The sequence ATG within the *Nco*I restriction site follows the IPTG-inducible *trc* promoter and the *lacZ* ribosome binding site (Amann & Brosius, 1985). The recombinant plasmid was designated $pYE2c_1$. The recombinant plasmid $pYE2c_2$ contained an insert

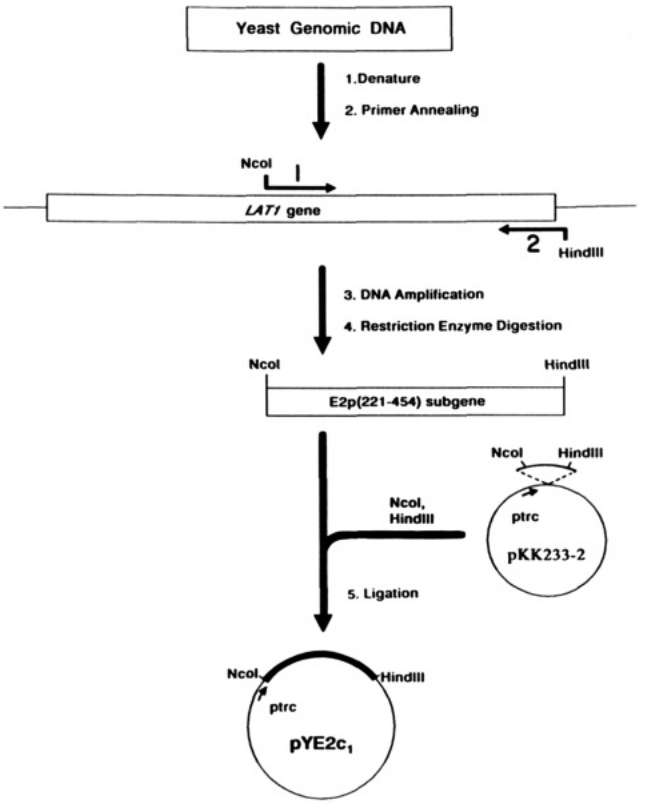

FIGURE 2: Construction of expression vector for the catalytic domain of E_2p. PCR was used to amplify, from yeast genomic DNA, that part of the *LAT1* gene (i.e., subgene) encoding the catalytic domain, $E_2p(221–454)$. The DNA fragment had a built-in *Nco*I restriction site at its 5′ end and a *Hin*dIII restriction site at its 3′ end. The DNA fragment was ligated into the *E. coli* expression vector pKK233-2. The *Nco*I restriction site follows the IPTG-inducible *trc* promoter (ptrc).

Table I: Design of Oligonucleotide Primers for PCR

primer	nucleotide sequence	location[a]
	*Nco*I[c]	
1a	CCTTCTTGCATGGCATCATATGAGGATGTTC	2764
	*Nco*I	
1b	GGCTGCCATGGAGTCATATCTAGA	2639
	*Hin*dIII	
2	CGAATTTAAGCTTCATTCTAACCTCACAATAGC	3441
	*Nar*I	
3	TGATGGCGCCAAAGGTGCAGA	3396
	*Nar*I	
4a (His427→Ala)[b]	CTTTGGCGCCATCAATGGTTCTGGCATCAAAGG	3366
4b (His427→Asn)[b]	CTTTGGCGCCATCAATGGTTCTATTATC	3361
4c (Asp431→Asn)[b]	CTTTGGCGCCATTAATGGTT	3353
4d (Asp431→Ala)[b]	CTTTGGCGCCTGCAATGGTTCTATG	3358
4e (Asp431→Glu)[b]	CTTTGGCGCCTTCAATGGTTCTATG	3358

[a] The number is assigned to the nucleotide residue at the 3′ end of each primer, corresponding to the numbering of the *LAT1* gene. [b] These are the antisense primers that introduce mutations. Replaced nucleotide residues are underlined. [c] Restriction sites are in bold type.

that encoded the $E_2p(181–454)$ sequence. This insert was generated by PCR with the primers 1b and 2 (Table I).

Expression, Purification, and Properties of Truncated E_2p. The recombinant plasmid $pYE2c_1$ was used to transform *E. coli* strain JM105. The concentration of IPTG and the induction time period for optimal production of $rE_2p(221–454)$ were determined to be about 4 mM and 2 h, respectively. Immunoblot analysis indicated that most of the recombinant protein was in the soluble fraction obtained from the disrupted cells. Analysis of the cell extract by FPLC with a Superose 6 column indicated that the recombinant protein eluted before thyroglobulin (M_r = 640 000) and after Blue Dextran (M_r = 2 000 000) (data not shown). This observation indicated that $rE_2p(221–454)$ was a large oligomer, consistent with a calculated molecular weight of 1 531 560 for the 60-subunit E_2p inner core.

Table II: Purification of $rE_2p(221–454)$[a]

	volume (mL)	protein (mg)	sp act.[b]	recovery (%)
extract	80	2204	11[c]	100
DEAE-Sepharose	155	182	57	40
heparin–agarose	4	4.9	1150	22

[a] From about 15 g of *E. coli* cells (wet weight). [b] Units of acetyltransferase activity per milligram of protein. [c] Corrected for endogenous activity of the *E. coli* pyruvate dehydrogenase complex (~2.3 units/mg of protein).

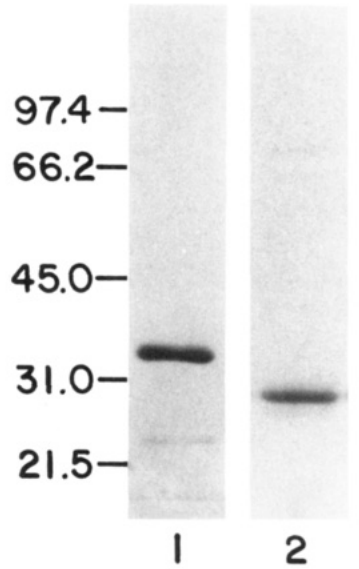

FIGURE 3: SDS–PAGE patterns of highly purified $rE_2p(181–454)$ (lane 1) and $rE_2p(221–454)$ (lane 2). Approximately 3 μg of protein was applied to each lane. The gel was stained with Coomassie Blue. The protein standards (in $M_r \times 10^{-3}$) are, from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor.

$rE_2p(221–454)$ was purified to apparent homogeneity by a two-step procedure involving chromatography on DEAE-Sepharose and heparin–agarose (Table II). The specific activity of the purified protein was similar to that of the E_2p component of the *S. cerevisiae* pyruvate dehydrogenase complex (1750 units/mg of protein, assuming that E_2p comprises about one-third the mass of the complex). When analyzed by SDS–PAGE, the purified protein showed a single band with apparent M_r ~28 000 (Figure 3). The calculated molecular weight is 25 526.

The amino-terminal sequence of the purified $rE_2p(221–454)$ was determined to be ASYEDVPI. This is the expected sequence, based on the nucleotide sequence of the *LAT1* gene (Niu et al., 1988). The amino-terminal Met residue, specified by the initiation codon ATG in the PCR primer 1a (Table I), apparently was removed during posttranslational processing.

The appearance of the oligomeric $rE_2p(221–454)$ as observed by negative-stain electron microscopy was very similar to that of the wild-type E_2p, namely, a pentagonal dodecahedron (Figure 4). It should be noted that in negative-stain electron microscopy it is difficult to detect the extended lipoyl domain and interdomain linker segments (Bleile et al., 1979).

Solutions of the highly purified $rE_2p(221–454)$ containing more than about 0.5 mg of protein per milliliter became cloudy

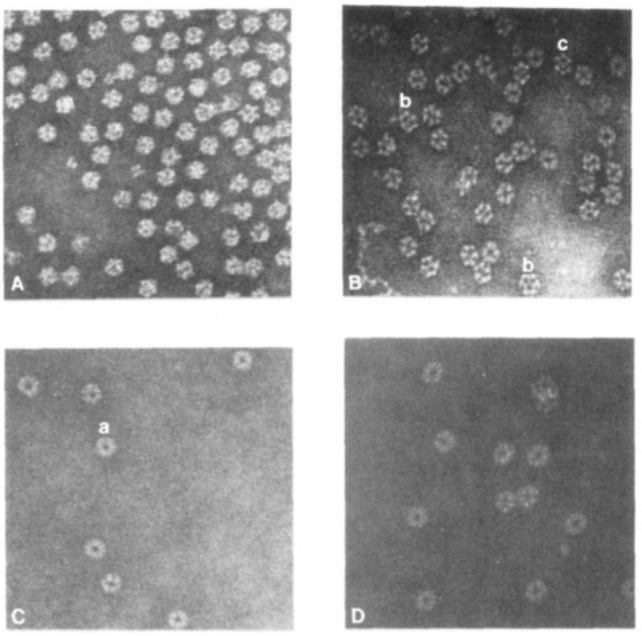

FIGURE 4: Electron micrographs of wild-type E_2p (A), $rE_2p(221–454)$ (B), $rE_2p(181–454)$ (C), and $rE_2p(181–454)D431N$ (D). The images exhibit 5-fold (a), 3-fold (b), and 2-fold (c) axes of symmetry that are characteristic of a pentagonal dodecahedron structure.

within 1 h at 4 °C. Solutions containing 0.1–0.5 mg of protein/mL became cloudy over a period of 24–48 h. This phenomenon was due to aggregation of the E_2p inner core, as revealed by electron microscopy (data not shown). Attempts to prevent aggregation by adjusting the pH, ionic strength, etc. were unsuccessful.

To prevent aggregation of the E_2p inner core, the interdomain linker segment (residues 181–220) was attached to the inner core domain (residues 221–454). The linker segment is rich in hydrophilic and charged amino acid residues (Niu et al., 1988). Construction of the expression vector $pYE2c_2$ was similar to that described for $pYE2c_1$. The $rE_2p(181–454)$ was overexpressed in *E. coli* strain JM105 and purified to apparent homogeneity as described for $rE_2p(221–454)$. The specific activity of $rE_2p(181–454)$ was higher than that of $rE_2p(221–454)$ (1563 vs 1150 units/mg of protein), and 2–3 times as much of the former recombinant protein was expressed. The highly purified $rE_2p(181–454)$ showed a single band on SDS–PAGE (Figure 3), with apparent $M_r \sim 33\,000$. The calculated molecular weight is 29 271. In contrast to $rE_2p(221–454)$, $rE_2p(181–454)$ did not aggregate when stored in a refrigerator for 1 week. The apparent molecular weight of $rE_2p(181–454)$ estimated by FPLC on Superose 6 was about 1 500 000 (data not shown). The appearance of $rE_2p(181–454)$ by negative-stain electron microscopy was very similar to that of $rE_2p(221–454)$ (Figure 4).

Site-Directed Mutagenesis of His-427 and Asp-431 in Truncated E_2p. A *Nar*I restriction site is adjacent to the nucleotide sequence encoding the consensus sequence HXXXDG near the carboxyl terminus of E_2p. This restriction site, in conjunction with PCR, was used to produce the desired mutations in the truncated E_2p. The strategy is presented in Figure 5. In addition to the sense and antisense primers (1a or 1b and 2, respectively, Table I) that were designed to amplify the subgene encoding the truncated E_2p, two internal primers, 3 and 4(a–e), were designed (Table I). Both internal primers contained a *Nar*I site sequence near their 5′ ends, and the antisense primer 4(a–e) was designed to introduce the mutation. The two combinations of sense and antisense primers, 1 and 4(a–e) and 2 and 3, respectively, were used to

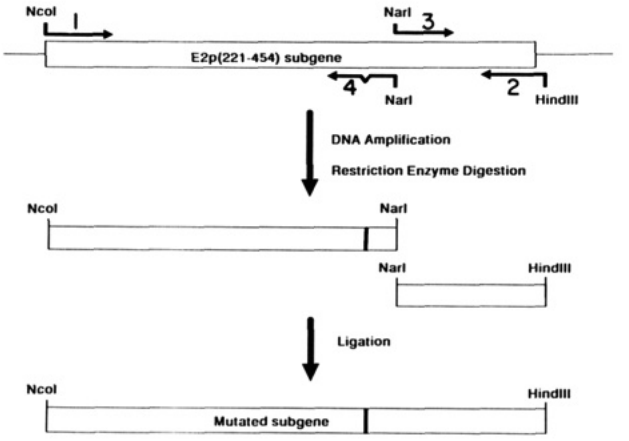

FIGURE 5: Site-directed mutagenesis of the E_2p catalytic domain. The subgene of *LAT1* encoding $E_2p(221–454)$ has an *Nar*I restriction site adjacent to the nucleotide sequence encoding the consensus sequence HXXXDG. PCR was used in conjunction with specific primers (Table I) to introduce the mutations. The DNA fragment bearing the desired mutation was ligated into pKK233-2.

Table III: Kinetic Parameters for Wild-Type and Mutant E_2p Inner Cores[a]

	k_{cat} (s^{-1})	K_m (mM)	
		dihydrolipoamide	acetyl-CoA
$rE_2p(181–454)$	7.8	4.0	0.15
$rE_2p(221–454)H427N$	3.5	3.8	0.45
$rE_2p(221–454)H427A$	5.6	4.3	0.23
$rE_2p(181–454)D431N$	0.50	6.9	0.11
$rE_2p(181–454)D431A$	0.33	4.5	0.11
$rE_2p(181–454)D431E$	2.1	5.6	0.49

[a] Kinetic parameters are the mean of two to four determinations.

amplify, from yeast genomic DNA, the desired DNA fragments. The DNA fragments were identified by agarose gel electrophoresis after treatment with specific restriction enzymes, and the DNA fragments were ligated into pKK233-2. The inserts were sequenced to confirm the mutations. The recombinant plasmids were used to transform *E. coli* strain JM105, and the mutant proteins were purified as described above.

The truncated E_2p, $rE_2p(221–454)$, contains only one His residue, His-427, and this residue is in the putative active-site sequence. The kinetic data presented in Table III show that the His-427 → Asn or Ala substitutions did not significantly affect the k_{cat} or K_m values of the truncated E_2p. The apparent molecular weights of the two mutant proteins, $rE_2p(221–454)H427N$ or A, were very similar to that of the unaltered protein $rE_2p(221–454)$ as estimated by FPLC on a calibrated Superose 6 column (data not shown).

Because of the tendency of $rE_2p(221–454)$ but not $rE_2p(181–454)$ to aggregate, subsequent mutations were made in $rE_2p(181–454)$. The Asp-431 → Asn, Ala, or Glu substitutions resulted in a 95, 96, and 77% decrease, respectively, in specific activity. The k_{cat} values decreased about 16-, 24-, and 3.7-fold, respectively (Table II). The K_m values showed little change. The apparent molecular weights of the mutant proteins were about 1 500 000, as estimated by FPLC on Superose 6 (data not shown). As observed by negative-stain electron microscopy, the purified $rE_2p(181–454)D431N$ or A consisted of pentagonal dodecahedron-like particles (Figure 4).

Discussion

One of the objectives of this investigation was to overexpress the inner core of E_2p from *S. cerevisiae* in a form suitable for crystallization and subsequent X-ray crystallographic analysis.

In previous investigations, the inner cores of the *E. coli* and bovine kidney E_2p and the *E. coli* dihydrolipoamide succinyltransferase were crystallized, and preliminary X-ray diffraction data were obtained (DeRosier et al., 1971; Fuller et al., 1979). These inner cores were produced by limited proteolysis of the acyltransferases. However, the crystals tended to be small, due probably to molecular heterogeneity of the inner cores resulting from proteolysis and multistep purification procedures. Overexpression of the yeast E_2p inner core and its rapid purification should eliminate these sources of microheterogeneity.

As described in this report, we amplified by PCR yeast genomic DNA encoding the inner core domain (residues 221–454) of yeast E_2p and then expressed this DNA fragment in *E. coli* strain JM105 via the expression vector pKK233-2. The recombinant protein $rE_2p(221–454)$ comprised 1–1.5% of the soluble protein of the *E. coli* cells. It was purified to apparent homogeneity by a two-step procedure. The purified protein exhibited an apparent $M_r \sim 1\,500\,000$, as estimated by gel exclusion chromatography on a calibrated Superose 6 column, and its appearance in negative-stain electron microscopy was very similar to that of the wild-type E_2p and bovine kidney E_2p (Oliver & Reed, 1982), namely, a pentagonal dodecahedron. The specific activity of $rE_2p(221–454)$ was similar to that of the wild-type E_2p. These findings demonstrate that the active site of *S. cerevisiae* E_2p resides in its inner core domain and that this domain is capable of self-assembly.

The purified $rE_2p(221–454)$ showed a tendency to aggregate. The inner core of *E. coli* E_2p, prepared by limited tryptic digestion (Bleile et al., 1979) and by recombinant DNA techniques (Angier et al., 1987), showed an even more pronounced tendency to aggregate. The recombinant *E. coli* protein was reported to be present largely in the insoluble fraction of the *E. coli* host cells. Attempts to prevent the purified yeast $rE_2p(221–454)$ from aggregating by varying the pH, ionic strength, etc. were unsuccessful. Because the interdomain linker segment comprising amino acid residues ~181–220 (Figure 1) is rich in hydrophilic and charged amino acid residues (Niu et al., 1988), we decided to attach this segment to the catalytic domain. PCR was used to amplify yeast genomic DNA encoding amino acid residues 181–454, and this DNA fragment was expressed in *E. coli* strain JM105. The specific activity of the purified $rE_2p(181–454)$ was about 36% higher than that of $rE_2p(221–454)$, and 2–3 times as much of the former recombinant protein was expressed (about 3% of the soluble *E. coli* protein). Solutions of $rE_2p(181–454)$, in contrast to $rE_2p(221–454)$, did not become cloudy when stored in a refrigerator for 1 week.

The catalytic domain of yeast E_2p contains only one His residue, His-427, and this residue is in the consensus sequence, His-Xaa-Xaa-Xaa-Asp-Gly, near the carboxyl terminus, which is a characteristic feature of dihydrolipoamide acyltransferases (Guest, 1987; Guest et al., 1989). Therefore, the yeast rE_2p catalytic domain provided a unique opportunity to test the hypothesis that this highly conserved His residue participates as a general base in the acyl-transfer reaction catalyzed by dihydrolipoamide acyltransferases. This His-427 → Asn or Ala substitutions did not significantly affect the k_{cat} or the K_m values of the truncated E_2p. These observations indicate that a His residue is not involved in the catalytic mechanism of *S. cerevisiae* E_2p and, by implication, of other dihydrolipoamide acyltransferases.

The conserved Asp-431 in $rE_2p(181–454)$ was replaced by Asn, Ala, or Glu. These mutations resulted in a 16-, 24-, and 3.7-fold reduction, respectively, in k_{cat}, with little change in K_m values. These observations indicate that Asp-431 plays an important role. Whether this role is structural or catalytic remains to be determined.

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