I. INTRODUCTION

Multienzyme systems that catalyze a lipoic acid-mediated oxidative decarboxylation of \(\alpha\)-keto acids have been isolated from prokaryotic and eukaryotic sources as functional units with molecular weights in the millions [1]. Three classes of these multienzyme complexes have been characterized, one specific for pyruvate (PDH complex), a second specific for \(\alpha\)-ketoglutarate (KGDH complex), and a third specific for the branched chain \(\alpha\)-keto acids derived from the amino acids valine, leucine and isoleucine (BCKDH complex). Each complex is composed of multiple copies of three enzymes: a substrate-specific decarboxylase-dehydrogenase (E1), a dihydrolipoamide acyltransferase (E2) specific for each type of complex, and dihydrolipoamide dehydrogenase (E3), a flavoprotein that is a common component of the three types of multienzyme complexes. The mammalian and yeast PDH complexes also contain small amounts of protein X [2-4], which is involved in specific binding of E3 to the E2 core. E1, E2, and E3, acting in sequence, catalyze the reactions shown in Fig. 1. E1 catalyzes both the decarboxylation of the \(\alpha\)-keto acid (Reaction 1) and the subsequent reductive acylation of the lipooyl moiety (Reaction 2), which is covalently bound to E2. E2 catalyzes the

![Diagram of reaction sequence in \(\alpha\)-keto acid oxidation](image)

Fig. 1. Reaction sequence in \(\alpha\)-keto acid oxidation. Abbreviations: TPP, thiamin diphosphate; LipS2 and Lip(SH)2, lipooyl moiety and its reduced form.
acyl transfer step (Reaction 3), and E₂ catalyzes the reoxidation of the dihydrolipoyl moiety with NAD⁺ as the ultimate electron acceptor (Reactions 4 and 5). Eukaryotic PDH and BCKDH complexes contain small amounts of two regulatory enzymes, PDH kinase and PDH phosphatase, BCKDH kinase and BCKDH phosphatase, respectively, which modulate the activity of E₁ by phosphorylation and dephosphorylation [5, 6]. In eukaryotic cells, the α-keto acid dehydrogenase complexes are located in mitochondria within the inner membrane-matrix compartment.

II. STRUCTURE-FUNCTION RELATIONSHIPS IN DIHYDROLIPOAMIDE ACYLTRANSFERASES

Each of the three types of α-keto acid dehydrogenase complexes is organized about a core consisting of the oligomeric E₂, to which multiple copies of E₁ and E₃ are bound by noncovalent bonds. Two distinct polyhedral forms of E₂ have been observed in the electron microscope, the cube and the pentagonal dodecahedron [7] (Fig. 2). The cubic design consists of 24 subunits arranged with octahedral (432) symmetry. The other design consists of 60 subunits arranged with icosahedral (532) symmetry.

All dihydrolipoamide acyltransferases possess a unique multidomain structure [8-10] (Fig. 3). The amino-terminal part of the E₂ subunit 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 E₃ or E₄, or both. These domains are linked to each other and to the carboxyl-terminal part of the E₂ subunit (inner core domain) by conformationally flexible segments (hinge regions) that are rich in the conservatively substituted residues alanine, proline, serine and threonine and in charged amino acid residues. The active site of E₂ resides in the inner core domain.

Genes and cDNAs encoding E₂ have been cloned and sequenced from bacteria, yeast, and mammalian cells [11]. The availability of the structural gene or cDNA for E₂ has facilitated investigation into self-assembly and structure-function relationships of this oligomeric enzyme. The inner core domain (Fig. 3, residues 221-454) of the dihydrolipoamide acetyltransferase component (E₂p) of the PDH complex from S. cerevisiae has been overexpressed in Escherichia coli strain JM105 via the expression vector pKK233-2 [12]. The purified truncated E₂p exhibited catalytic activity (acetyl transfer from [1-14C]-acetyl-CoA to dihydrolipoamide) very similar to that of wild-type E₂p. The appearance of the truncated and wild-type E₂p was also very similar, as observed by negative-stain electron
microscopy, namely, a pentagonal dodecahedron. These findings demonstrate that the active site of E₂p from *S. cerevisiae* resides in its inner core domain, i.e., catalytic domain, and that this domain alone can undergo self assembly.

Comparison of amino acid sequences of the *E. coli* dihydrolipoamide acetyltransferase and succinyltransferase with those of chloramphenicol acetyltransferases (CAT) revealed remote, but significant, similarities [8]. His-195 in the sequence His-Xaa-Xaa-Xaa-Asp-Gly near the carboxyl terminus has been implicated in the catalytic mechanism of CAT and Asp-199 apparently plays a structural role. The presence of this conserved sequence near the carboxyl termini of dihydrolipoamide acetyltransferases led Guest [8] to suggest that acyl transfer in dihydrolipoamide acetyltransferases is mediated by a base-catalyzed mechanism analogous to that proposed for CAT. Consistent with this proposal is the finding that substitution of the conserved His-602 by Cys in the *E. coli* E₂p resulted in loss of catalytic activity [13]. At variance with this proposal are the findings with *S. cerevisiae* E₂p [12]. The catalytic domain of yeast E₂p contains only one His residue, His-427, and this residue is present in the putative active site sequence. Substitution of His-427 by Asn or Ala by site-directed mutagenesis did not have a significant effect on the *k*ₘₐₜ or *K*ₘ values of the truncated E₂p. Replacement of the conserved Asp-431 in the truncated E₂p by Asn, Ala, or Glu resulted in a 16-, 24-, and 3.7-fold reduction, respectively, in *k*ₘₐₜ with little change in *K*ₘ values.

To gain further insight into the structure and function of the domains in *S. cerevisiae* E₂p, the LATI gene was disrupted and deletion and site-specific mutations in LATI were constructed [14]. Disruption of LATI did not affect 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 LATI on a unit-copy plasmid produced a PDH complex that was very similar to the wild-type PDH complex in enzymatic activities and subunit composition. 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 E₂p or the binding of E₁, protein X and E₃ to the truncated E₂p. Similar results were obtained by deleting the lipoyl domain plus the first
hinge region (residues 8-145), and by replacing Lys-47, the putative site of attachment of the lipoyl moiety, by Arg. Although the lipoyl domain of E2p 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. Yeast protein X contains a lipoyl domain, but it does not exhibit acetyltransferase activity. Replacement of both the lipoylatable Lys-47 in E2p and Lys-43 in protein X by Arg 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 E2p, and that the lipoyl domain of protein X can couple with the catalytic domain of the truncated E2p. A similar proposal was made for the bovine kidney complex [15]. A protease-modified PDH complex in which the lipoyl domains of E2p, but not of protein X, were removed by proteolysis retained about 10% of the overall activity of the untreated PDH complex.

Deletion of the putative subunit binding domain (residues 145-181) in yeast E2p resulted in loss of ability of the truncated E2p to bind E3 and concomitant loss of overall activity of the mutant PDH complex. This domain apparently plays an important role in binding E3. A similar domain in bovine kidney E2p has been shown to play an important role in binding bovine kidney E3 [16]. It is intriguing that a similar domain in protein X is involved in binding E3 (see below). This apparently conserved domain is involved in binding E3 in the E. coli dihydrolipoamide acetyltransferase and succinyltransferase, and E3 and E1 in the acetyltransferase from Acetobacter vinelandii and Bacillus stearothermophilus [8,10]. To gain further insight into functional relationships of the structural domains of yeast E2p and protein X, chimeric proteins are being constructed in which these domains are interchanged.

III. NATURE AND FUNCTION OF PROTEIN X

Mammalian and S. cerevisiae PDH complexes and, presumably, other eukaryotic PDH complexes as well, contain small amounts of a protein designated protein X or component X, with Mr ~50,000 [2,3]. Protein X is tightly associated with the E2p core and, like E2p, protein X contains a lipoyl moiety that can undergo reductive acetylation (Fig. 1, Reactions 1,2). Distinct genes encoding E2p and protein X from S. cerevisiae have been cloned and sequenced. Comparison of the deduced amino acid sequences of the two proteins indicates that they evolved from a common ancestor [17]. The amino-terminal half of protein X resembles E2p, but the remainder is quite different. The two proteins exhibit 50% sequence identity in the amino-terminal segment that corresponds to the lipoyl domain (residues ~1-84) and about 33% sequence identity in the E3/E1 binding domain (residues ~146-181, Fig.3).

Studies with the bovine kidney PDH complex indicate that protein X contributes to the binding and function of E3[2]. It is interesting to note in this connection that the 60-subunit E2p core of the highly purified bovine kidney PDH complex contains about 6 molecules of tightly bound protein X and binds only about 6 E3 dimers, 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 [4]. Disruption of PDX1 did not affect viability of the cells. 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 E1, E2p, and E3, but lacked protein X and E2p. The sedimentation coefficients (S20,w) of the mutant PDH complex and the wild-type PDH complex were 73.3 S and 82.6 S, respectively. These values are consistent with the absence from the mutant PDH complex of about 6 E3 dimers and about 6 protein X molecules. In the absence of protein X, the mutant PDH complex exhibited very little ability to couple with E3 to oxidize pyruvate. At a molar ratio of E1/E2p subcomplex to yeast recombinant E3 of ~1:1000, the mixture exhibited about 5% of the pyruvate oxidation activity of the wild-type PDH complex. Mutant cells transformed with the gene for protein X on a unit-copy plasmid produced a PDH complex that contained protein X and E2p, as well as E1, E3, and E3, and the complex exhibited overall activity similar to that of wild-type PDH complex. These observations indicate that protein X is not involved in assembly of the E2p core, nor is it an integral part of the E2p core. Rather, protein X apparently plays a structural role in the PDH complex, i.e., it is an E1 binding protein which binds and positions E3 to the E2p core and this specific binding is essential for a functional PDH complex.
Fig. 4. Reconstitution of PDH complex activity with recombinant protein X. Mixtures of E₁E₂ₚ subcomplex (0.33 μg) and a purified fraction containing recombinant E₃ and protein X were incubated at 30°C for 5 min and then assayed for overall PDH complex activity. The results were not affected by varying the time of incubation between 1 sec and 30 min and the temperature between 4 and 37°C. Activity is expressed as micromoles of NADH produced per minute per milligram of E₁E₂ₚ subcomplex.

That protein X combines with the preformed E₂ₚ core is indicated by reconstitution experiments. Mixtures containing E₁E₂ₚ subcomplex, recombinant E₃ and increasing amounts of recombinant protein X generated a dose response curve, with a maximum pyruvate oxidation activity similar to that of wild-type PDH complex (Fig. 4). The active reconstituted complex was isolated by fast protein liquid chromatography on a Superose 6 column. Although it is uncertain where or how protein X is bound to E₂ₚ, it appears that there is a small, fixed number of sites on E₂ₚ where protein X can bind.

To investigate structure-function relationships in protein X, a set of deletions in PDX1 was constructed. 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 E₃ and concomitant loss of overall activity of the PDH complex. This domain apparently plays an important role in binding E₃.

SUMMARY

The α-keto acid dehydrogenase multienzyme complexes play central roles in metabolism, are major sites of regulation, and are clinically important. Genes and cDNAs encoding the components of these complexes have been cloned and sequenced. Protein engineering and molecular cloning experiments are providing new insight into organization, structure-function relationships, and the molecular basis of genetic defects in these multienzyme complexes [11].
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