Subunit Interactions in the Mammalian α-Ketoglutarate Dehydrogenase Complex

EVIDENCE FOR DIRECT ASSOCIATION OF THE α-KETOGLUTARATE DEHYDROGENASE AND DIHYDROLIPOAMIDE DEHYDROGENASE COMPONENTS

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Selective tryptic proteolysis of the mammalian α-keto glutarate dehydrogenase complex (OGDC) leads to its rapid inactivation as a result of a single cleavage within the N-terminal region of its α-ketoglutarate dehydrogenase (E1) component, which promotes the dissociation of the dihydrolipoamide dehydrogenase (E3) enzyme and also a fully active E1 fragment. Similarities between the N-terminal region of E1 and the dihydrolipoamide acetyltransferase (E2) and E3-binding components (E3BP) of the pyruvate dehydrogenase complex are highlighted by the specific cross-reactivities of subunit-specific antisera. Analysis of the pattern of release of E1 and E1 polypeptides from the OGDC during tryptic inactivation suggests that both polypeptide chains of individual E1 homodimers must be cleaved to permit the dissociation of the E1 and E3 components. A new protocol has been devised that promotes E1 dissociation from the oligomeric dihydrolipoamide succinyltransferase (E2) core in an active state. Significant levels of overall OGDC reconstitution could also be achieved by re-mixing the constituent enzymes in stoichiometric amounts. Moreover, a high affinity interaction has been demonstrated between the homodimeric E1 and E3 components, which form a stable subcomplex comprising single copies of these two enzymes.

The α-ketoglutarate dehydrogenase (OGDC), along with the pyruvate dehydrogenase (PDC) and branched chain α-ketoacid dehydrogenase complex (BCOADC) constitute the family of α-ketoacid dehydrogenase multienzyme complexes, important in controlling carbon flux from carbohydrate precursors and a select group of amino acids into and around the tricarboxylic acid cycle. Located in the mitochondrial matrix, the mammalian complexes catalyze the irreversible oxidative decarboxylation of their respective α-ketoacid substrates yielding the appropriate acyl CoA derivatives, NADH and CO₂. Central to this catalysis is the consecutive action of three catalytic components, each present in multiple copies: a substrate-specific α-ketoacid dehydrogenase (E1), a distinct dihydrolipoamide acetyltransferase (E2), and a common dihydrolipoamide dehydrogenase (E3) (see Refs. 1 and 2 for reviews).

α-Ketoadid dehydrogenase complexes are assembled in a tight but noncovalent fashion. Their multi-component, multi-copy nature results in the formation of massive complexes with M₉ values of 5–10 × 10⁶ that are organized around 24-meric or 60-meric E2 cores, exhibiting octahedral and icosahedral symmetry, respectively. In prokaryotes, in combination with its catalytic role, the oligomeric E2 core is responsible for tethering and orientating both the E1 and E3 enzymes within the complexes via compact, peripheral subunit-binding domains (3–5). In higher eukaryotes the identification of an additional component of PDC, E3-binding protein, (E3BP, formerly protein X) with similarities in domain structure to E2 suggested a possible devolution of subunit function (6, 7). Subsequent studies of E3BP have shown that it is responsible for mediating the high affinity interaction of the E3 component to this multienzyme complex in eukaryotes (8–12).

Studies on mammalian OGDC in this laboratory, employing specific proteolysis and N-terminal sequence analysis, have identified a “lipoyl-like” region at the extreme N terminus of the E1 component with significant sequence similarity to the E3BP and E2 components of mammalian PDC (13). These similarities suggested that E1 of OGDC may perform some functions normally devolved to E2 in PDC and BCOADC. Further indirect evidence, in support of putative additional roles for E1 of OGDC came when the genes for rat and human E2-OGDC were cloned (14, 15). Analysis of their predicted primary structures failed to locate any E1- or E3-binding motifs, although such sequences were readily located in E2-OGDC genes from other organisms, notably Escherichia coli (16) and Azotobacter vinelandii (17).

Previous studies of OGDC have successfully purified the intact multienzyme complex to homogeneity (18, 19). However, no method for the dissociation of OGDC into functionally active E2 and E1/E3 fractions has been reported to date because of the high affinity of the E1 enzyme for the core assembly, a property it shares with the E3BP of PDC (20).

In this paper, we report the development of a method for the successful dissociation of mammalian OGDC into its E2 and
E1/E3 fractions. Subsequent reconstitution studies with the E2 core recovered appreciable levels (30%) of overall complex activity on addition of the isolated E1 and E3 components. Gel permeation of the E1/E3 fraction of OGDC, under associative conditions, demonstrated that these two enzymes interact with high affinity to form a stable subcomplex with an apparent Mr, consistent with a 1:1 stoichiometry. These studies provide the first direct biochemical evidence that uniquely, in mammalian OGDC, its constituent E1 enzyme is responsible for binding the E3 component to the multienzyme complex.

EXPERIMENTAL PROCEDURES

**Gel Permeation Analysis of Tryptic Digestion Products of Mammalian OGDC**—Following OGDC purification from bovine heart as detailed previously (19), samples were digested with 0.01% (w/v) trypsin at 30 °C in 50 mM potassium phosphate buffer, pH 7.6, 3 mM NADH, 2 mM MgCl2, 0.2 mM thiamine diphosphate until the complex was 50 or 90% inactivated. A control OGDC sample was incubated similarly in the absence of trypsin. Digestion was stopped by addition of soybean trypsin inhibitor, and the OGDC was immediately loaded on a Sephacryl S 300 gel permeation column (120 ml) equilibrated in 50 mM potassium phosphate buffer, pH 7.6, 10 mM NaCl. The column was eluted at a flow rate of 25 ml h–1, and fractions were assayed for OGDC activity (21), E3 activity (22), and E1 activity (23).

Preparative Dissociation of Mammalian OGDC—OGDC was dissociated into its E2 core and E1/E3 fractions as follows: OGDC (30 mg ml–1) was incubated at a 1:1 (v/v) ratio with dissociation buffer (50 mM MOPS/KOH, 7.6, 2 mM MgCl2, 20 mM dithiothreitol, 0.1% (v/v) Triton X-100) for 1.5 h on ice. The sample was spun at 10,000 × g in a bench top centrifuge for 15 min to remove any particulate material. The E2 core of OGDC was separated from the E1/E3 fraction on an FPLC system (Amersham Pharmacia Biotech) using a Superose 6 column (1.6 × 50 cm) equilibrated with 50 mM MOPS/KOH, pH 7.6, 1 mM MgCl2, 0.1% (v/v) Triton X-100. A pre-injection of the dissociation buffer (2 ml) was performed prior to OGDC sample loading (2 ml) and elution at a flow rate of 1 ml min–1. The eluant was monitored on-line at 280 nm. Pooled fractions were dialyzed extensively, at 4 °C against multiple changes of buffer into 20 mM MOPS/KOH, pH 7.6, 0.1% (v/v) Triton X-100.

Reconstitution of OGDC Activity from E2 Core and E1/E3 Fractions—The method used to reconstitute OGDC activity was derived from the one optimized previously for PDC (24). Reconstitution was started by adding quantities of E1/E3 (10–30 μg) to cuvettes containing E2 (10 μg) and the appropriate assay solutions. Following a 15-min incubation at 20 °C, the production of NADH was initiated by the addition of 14 μl of 100 mM α-ketoglutarate. NADH production was monitored at 340 nm, and activities are expressed either as μmol min–1 or as a percentage relative to the specific activity of undissociated OGDC.

**Association State Analysis of E1 and E3**—Gel permeation analysis of E1/E3 quaternary structure was performed on a Superose 12 column (1 × 30 cm) attached to an FPLC system. The column was calibrated with molecular mass markers (Sigma) for the construction of a calibration curve of V/Vo versus log Mr, after initial equilibration, at 0.3 ml min–1, in 25 mM MOPS/KOH, pH 7.0, 10 mM KCl, 0.1% (v/v) Triton X-100. E1/E3 samples were clarified for 15 min at 10,000 × g in a benchtop centrifuge prior to loading onto the column (500-μl sample). Elution profiles obtained were from on-line protein detection at 280 nm and from E3 activity assays on the eluted fractions.

**Immunological Analysis**—Denaturing gel electrophoresis and Western blot analysis were performed as detailed previously (20), except that immunocomplexes were detected with Amersham Pharmacia Biotech ECL detection reagents according to the manufacturer's instructions.

RESULTS

**Immunological Analysis of Similarities between the E1 Component of OGDC and the E2 and E3BP Subunits of PDC**—Previous biochemical studies on mammalian OGDC detected significant sequence similarities between the N terminus of its E1 component and the E2 and E3BP subunits of eukaryotic PDCs corresponding to the N-terminal regions of their peripherally located lipoyl domains (13). Similar investigation of the N terminus of the E1′ fragment indicated that these similarities extended into the interior of E1 and that the tryptic cleavage site was situated at a putative subunit-binding domain. In this respect, it resembles E3BP of PDC, which contains a susceptible protease arg C cleavage site on the N-terminal boundary of its E3-binding domain (24).

These similarities are confirmed in Fig. 1, which demonstrates significant immunological cross-reaction between these three components. Anti-E1 (OGDC) serum elicits a clear cross-reaction with E2 of mammalian PDC (lane 1) plus a strong and specific response (lane 2) to E1 of OGDC. In addition, anti-E3BP (PDC) serum (lane 3) also recognizes E1 of OGDC (lane 4). Two minor degradation products of E3BP are also highlighted by the anti-E3BP serum (lane 3). As indicated, sequence similarities between E1 of OGDC and the E2 and E3BP subunits of PDC have been documented previously (13); this immunological evidence confirms that these polypeptides possess shared antigenic determinants and are likely to contain equivalent domains with common or overlapping functions.

In Fig. 2 (inset) the specificity of tryptic digestion of intact OGDC is illustrated by SDS-PAGE following treatment resulting in 0% (lane 1), 50% (lane 2), and 90% (lane 3) loss of overall complex function. It is apparent that there is a close correlation between the extent of OGDC inactivation and the percentage of conversion of the 110,000 Mr, E1 subunit to a stable 100,000 Mr, E1′ fragment with no detectable degradation of the E2 and E3 enzymes. Resolution of the partially inactivated complex (50%) by Sephacryl S 300 gel filtration (Fig. 2) shows that its E1 and E3 activities are distributed approximately equally between two peaks corresponding to their complex-associated and free forms. These elute at the void volume along with residual OGDC activity and as individual homodimers with Mr values of 200,000 and 110,000, respectively. Similar profile analysis of intact OGDC indicates that all the constituent enzymes elute at the void volume, whereas after 50% tryptic inactivation, the vast majority of the E1′ and E3 activities are no longer associated with the complex (not shown). Moreover, there is no evidence for any interaction between the E1′ and E3 species, which are partially separated as expected on the basis of their differing Mr values.

Fig. 3A compares the percentage of OGDC inactivation by trypsin with time to the extent of E1 conversion to E1′ and the corresponding amount of E3 release from the complex. During the early stages of trypsin treatment, the rate of E1 proteolysis
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Fig. 2. Gel permeation analysis of OGDC partially inactivated by limited treatment with trypsin. OGDC (15 mg/ml) was digested with 0.01% (w/v) trypsin at 30 °C in 50 mM potassium phosphate buffer, pH 7.6. 2 mM MgCl₂, 0.2 mM thiamine diphosphate until it retained 50% of its original activity. Following termination of digestion by addition of a 10-fold molar excess of soybean trypsin inhibitor, the OGDC was loaded onto a Sephacryl S 300 gel permeation column (bed volume, 120 ml) equilibrated in 50 mM potassium phosphate buffer, pH 7.6, 10 mM NaCl at a flow rate of 25 ml/h. Fractions (1 ml) were collected and assayed for OGDC (●), E1 (○), and E3 (▲) activity. The inset depicts the specificity of trypsinic digestion of OGDC illustrating the extent of E1 degradation to E1' as detected by 10% (w/v) SDS-PAGE and Coomassie Blue staining. Lane 1, control OGDC (no trypsin); lane 2, after 50% inactivation with trypsin; lane 3, after 90% inactivation; lane M, marker proteins.

Exceeds the rate of complex inactivation or the extent of E3 dissociation. A possible explanation is that both subunits of E1 must be cleaved prior to complex dissociation and that the heterodimeric E1/E1' species, which will be produced preferentially in the initial stages of the digestion (assuming a random cleavage mechanism), can maintain the structural integrity and full catalytic activity of the complex. This idea is supported by assessment of the distribution of E1 and E1' species in the complex-bound and free forms of the enzyme (Fig. 3B) isolated by gel filtration after 50% inactivation of OGDC (Fig. 2). It is clear from SDS-PAGE analysis that the complex-associated E1 enzyme contains a mixture of E1 and E1' polypeptides (lane 3), whereas only E1' fragments are located in the peak of free E1 activity (lane 4). In contrast, if only a single cleavage were required to affect E1 dissociation, it would be expected that the complex-bound E1 should contain exclusively intact E1 polypeptide chains, whereas the peak of free E1 activity should contain a mixture of E1 subunits and E1' fragments. Lanes 1 and 2 of Fig. 3B are controls showing the subunit profile of OGDC before and after approximately 50% inactivation by trypsin prior to gel filtration.

Dissociation of Bovine Heart OGDC—The complete dissociation of OGDC into its individual E2 and E3 enzymes and an active, stable E1' species, induced by a single proteolytic cleavage event within the N-terminal region of E1, underlines the importance of this region in maintaining the structural integrity of the complex. Earlier research has also suggested that this proteolytically sensitive site may lie within a putative subunit-binding domain (13), implying the potential for direct interaction between the E1 and E3 components in this complex. It has not been possible to test this hypothesis to date because of the tight association of E1 with the oligomeric E2 core as no methods were available for releasing the E1 enzyme from the E1/E2 subcomplex in active form. Preliminary studies indicated that high concentrations of divalent cations, in particular Mg²⁺, were capable of disrupting the tight binding between the E1 and E2 enzymes while also promoting release of E3 under mildly alkaline conditions.

Fig. 4 demonstrates the ability of bovine heart OGDC to be reconstituted successfully after pre-treatment with 1 M MgCl₂ at a variety of pH values. The data suggest that high Mg²⁺ concentrations have a minimal effect on overall complex activity at pH 6.5 and 7.0. In contrast, treatment of OGDC in alkaline conditions (pH 8.0 or above) caused rapid initial inactivation with a further decrease over time, leading to almost complete disappearance of OGDC activity after 5 h. Incubation with MgCl₂ at pH 7.6 (mildly alkaline conditions) caused a slow inactivation of the complex, although 50–80% of the original OGDC activity could be restored after a 1–2-h incubation. Further control studies (not shown) indicated (a) that the intact complex was stable to pH treatment alone in the range 6.5–9.0, indicating that the marked pH sensitivity was induced by the dissociating conditions provided by the presence of Mg²⁺ and (b) that the increased stability to 1 M MgCl₂ at acidic or neutral pH partly reflected incomplete dissociation of E1. Accordingly, it was considered that pH 7.6 was optimal for OGDC disassembly, consistent with maintaining maximum stability of its E1 component.

Preparative dissociation of OGDC was performed by Superose 6 gel permeation chromatography (see “Experimental Procedures”). The elution profile suggested that dissociation of the high M₉ OGDC had occurred with two peaks of UV absorbance eluting from the column (Fig. 5A). The initial peak, corresponding to the high M₉ E2 core, eluted near the void volume (Ve, 30–32 ml). The second peak, containing the E1 and E3 enzymes, eluted in the later column fractions (Ve, 60–70 ml). The nature and extent of the dissociation was evident from SDS-PAGE analysis of column fractions (Fig. 5B). Clearly, MgCl₂ treatment and gel filtration had been successful in removing E1 and E3 from the E2 core of OGDC. Lanes corresponding to the E2 core (column fractions 15–17) show no significant contamination with associated E1 or E3 enzymes, although a small amount of E1 could be detected at the void volume (fractions 13 and 14), which may have contained some aggregated material. The elution profile is consistent with appearance of the individual enzymes on the basis of size: the 24-meric E2 core elutes

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2 V. Bunik, unpublished observations.
first, near the void volume, followed by the homodimeric E1 and E3 components. The E1 and E3 subunits appear as a single unresolved peak in the absorbance trace; however, SDS-PAGE reveals that in 1 M MgCl₂ elution buffer, E1 (220,000 M₀) elutes before E3 (110,000 M₀), although there is a degree of overlap as expected; however, under these conditions, the E1 and E3 components are clearly not associated as a complex.

Reconstitution Studies with Bovine OGDC—Although this protocol was successful in dissociating the E1 and E3 components of OGDC from its E2 core, its effects on the constituent enzyme activities remained unclear. For PDC, it is possible to examine all three enzymes in concert by mixing stoichiometric enzyme activities remained unclear. For PDC, it is possible to examine all three enzymes in concert by mixing stoichiometric quantities of E1/E3. In the case of OGDC reconstitution, maximal levels of complex activity are expected in this range because the ratio of E2:E1/E3 in the native complex is approximately 1:2, which is as predicted for the stoichiometry of the native complex, assuming six copies of the E1 and E3 enzymes present per E2 core.

Optimal reconstitution of mammalian PDC was previously obtained employing substoichiometric levels of E1/E3 (24). Whereas this may also be true for OGDC, the reason for this remains unclear but may reflect the situation in the native complex where full occupancy of all E1- and E3-binding sites is not essential for maximal rates of catalysis. Alternatively, it was noted that the recovery of the E1 component in the isolated E1/E3 fraction was only 30–50% of that achieved for E3, presumably reflecting its inherent instability and/or enhanced recovery of E3 relative to E1 resulting from the choice of fractions pooled for reconstitution studies.

Association State Analysis of the E1 and E3 Components of Mammalian OGDC—The measured levels of OGDC reconstitution were considered sufficient to permit detection of a direct interaction between the E1 and E3 components by gel permeation. After dissociation from the E2 core, the E1/E3 pool was interaction between the E1 and E3 components by gel permeation chromatography as shown in Fig. 2. Lane 1, intact OGDC (15 μg); lane 2, OGDC (15 μg) after 50% inactivation with trypsin.

purified E2 core nor E1/E3 fractions were able to support OGDC activity alone, indicative of good fraction resolution during the dissociation (Fig. 6B), while also providing definitive proof that the recovery of OGDC activity resulted from genuine complex formation. Maximal reconstitution was obtained with E2:E1/E3 ratios of 1:2 on a weight for weight basis. No increase in OGDC activity was obtained by adding extra quantities of E1/E3. In the case of OGDC reconstitution, maximal levels of complex activity are expected in this range because the ratio of E2:E1/E3 in the native complex is approximately 1:2, which is as predicted for the stoichiometry of the native complex, assuming six copies of the E1 and E3 enzymes present per E2 core.

Fig. 3. Analysis of rate of decline of OGDC activity as compared with E1' appearance and E3 release during trypsin-induced inactivation of intact OGDC. OGDC was degraded with trypsin as described in Fig. 2, and at the times indicated samples were taken for assay of residual overall complex activity (○), densitometric determination of E1 to E1' conversion (□) following 10% (w/v) SDS-PAGE (13) and for assay of E3 release (●) after ultracentrifugation of the complex at 130,000 × g for 5 h to pellet intact OGDC (A). All results are expressed as a percentage of initial activity (OGDC) remaining or percentage of E1' and E3 release into the supernatant fraction. B shows a similar SDS-PAGE analysis of the distribution of E1 and E1' species between the complex-bound (lane 3, 5 μg) and free states (lane 4, 5 μg) harvested from partially inactivated OGDC (50%), which had been separated by FPLC-based gel permeation chromatography as shown in Fig. 2. Lane 1, intact OGDC (15 μg); lane 2, OGDC (15 μg) after 50% inactivation with trypsin.

Fig. 4. Reactivation profiles for bovine heart OGDC following treatment with 1 M MgCl₂ at a variety of pH values. OGDC (2.5 mg·ml⁻¹) was incubated on ice for various times in 50 mM MOPS/KOH buffer, 20 mM dithiothreitol, 0.1% (v/v) Triton X-100 buffer at the stated pH values in the presence of 1 M MgCl₂. During the time course, samples (10–30 μg) were removed, and reactivation/renaturation was initiated by a rapid dilution into the appropriate assay buffer. The final protein concentration in the renaturation mixture was 10–30 μg/ml, and residual MgCl₂ was reduced to below 30 mM to prevent enzyme inhibition. Samples were assayed for OGDC activity after a 3-min pre-incubation. All activities are expressed relative to control samples of enzyme incubated in the absence of MgCl₂, ○, pH 6.5; □, pH 7.0; ▲, pH 7.6; △, pH 8.2.
present in the higher \( M_r \) peak, whereas only free E3 was detected in the lower \( M_r \) peak.

Estimation of the \( M_r \) values of the two resolved peaks of E3 activity was performed after column calibration with standard marker proteins. Because free E3 is a homodimer with a subunit \( M_r \) of 55,000, the average value of 126,000 (61,000) for the lower \( M_r \) peak is in close agreement with the expected value of 110,000. For the higher \( M_r \) peak, its measured value of 303,000 (91,000) correlates well with the expected \( M_r \) for an E1/E3 subcomplex comprising one E3 homodimer (110,000) bound specifically to one E1 homodimer (220,000). The presence of this distinctive subcomplex represents the first direct biochemical evidence that the E1 and E3 components of mammalian OGDC interact physically and functionally. The presence of a separate peak of free E3 representing 60–70% of total eluted E3 activity is to be expected in view of the reduced recoveries of E1 relative to E3 during the isolation procedure.

**DISCUSSION**

**Selective Proteolysis of OGDC**—Previous limited proteolysis and N-terminal sequencing studies have identified a lipoyl-like domain at the N terminus of the E1 component of mammalian OGDC. Selective tryptic degradation of E1 results in a single cleavage, producing an N-terminal fragment (subunit \( M_r \) 10,000), abolition of E3 binding, and dissociation of an active E1′ species (subunit \( M_r \) 100,000). Complete disassembly of OGDC following this proteolytic event indicates that its subunit binding potential resides in this segment of its E1 component. A putative peripheral subunit-binding domain has been identified at the site of tryptic attack (13). It is now apparent that limited proteolysis also promotes release of E1, implying that sequences critical for its tight interaction with the E2 core.

**FIG. 5.** Dissociation and separation of the constituent enzymes of bovine heart OGDC. Preparative dissociation of OGDC into active E2 and E1/E3 fractions was performed on an FPLC system (Amersham Pharmacia Biotech) using a Superose 6 column (100 ml) equilibrated with 50 mM MOPS/KOH, pH 7.6, 1 M MgCl\(_2\), 0.1% (v/v) Triton X-100 (see “Experimental Procedures”). Fractions were pooled on the basis of \( A_{280} \) peaks (A) and dialyzed extensively at 4 °C against multiple changes of the 20 mM MOPS/KOH, pH 7.0, 10 mM KCl, 0.1% (v/v) Triton X-100. Aliquots (1 ml) of the eluant fractions were subjected to trichloroacetic acid precipitation (B) and separated by 10% (w/v) SDS-PAGE prior to staining with Coomassie Blue. Lane L, OGDC column load (15 \( \mu \)g); lanes 14–34, trichloroacetic acid-precipitated samples from column fractions showing resolution of the constituent enzymes.

**FIG. 6.** Reconstitution of overall OGDC complex activity from purified E2 core and E1/E3 fractions: levels of reconstitution and component stoichiometry. Following the dissociation of OGDC as described in the legend to Fig. 5, the E2 and E1/E3 fractions were dialyzed into 50 mM MOPS/KOH, pH 7.4, 1 mM EDTA, 50% (v/v) glycerol and stored at 4 °C. E2 and E1/E3 fractions were pre-conditioned by a 1:1 dilution in 50 mM potassium phosphate buffer, pH 7.6, 3 mM NAD\(^+\), 2.7 mM cysteine-HCl, 2 mM MgCl\(_2\), 0.2 mM thiamine diphosphate, 0.02 mM CoASH and incubated for 15 min at 20 °C. To initiate reconstitution, increasing quantities of E1/E3 (0–40 \( \mu \)g) were added to cuvettes containing E2 (10 \( \mu \)g) under the appropriate assay solutions. The production of NADH was subsequently initiated by the addition of 14 \( \mu \)l of 100 mM 2-oxoglutarate. Activities are expressed as the percentage of reconstitution relative to the activity of the native OGDC complex before dissociation (A). The purity of the pooled fractions employed for this reconstitution study was determined by 10% (w/v) SDS-PAGE analysis and Coomassie Blue staining. Lane M, \( M_r \) marker proteins; lane 1, intact OGDC (10 \( \mu \)g); lane 2, E1/E3 fraction (15 \( \mu \)g); lane 3, E2 core (5 \( \mu \)g).
Dissociation of Mammalian OGDC—Although NaCl (1–2 M) could be employed successfully for releasing the E3 component from native OGDC, the E1/E2 subcomplex was stable to treatment with 4 M NaCl (results not shown). Attempts to use low (non-denaturing) levels of GdnHCl also proved fruitless. The successful disassembly of mammalian OGDC into active E2 and E1/E3 fractions employed high levels of MgCl₂ and slightly alkaline pH to achieve optimal dissociation with minimal denaturation of the individual enzymes. Under these dissociative conditions, it is clear that the E1 and E3 components are not interacting (Fig. 5).

An important criterion, upon which the success of the OGDC dissociation was judged, was the production of components which remained functionally active. Reconstitution analysis provided a good indication of whether this had been achieved. The extent of reconstitution (30%) was appreciably lower than observed with the E2/E3BP and E1/E3 fractions of PDC at 60–80% (24). However, it was also noted that dissociated E1 was unstable and lost 50–70% of its original activity within 12 h at 4 °C even when dialyzed rapidly into buffers at pH 6.5–7.0. Because this represents the minimum time required to prepare the enzyme, further work is required to improve its stability during the isolation procedure. It can be stored for 2–3 weeks in 50% (v/v) glycerol at −20 °C without further loss of activity.

Parallels with E3BP Function—It is interesting to note the physical and functional parallels between E1 of OGDC and E3BP of PDC (25). Although it was not possible to obtain as high levels of reconstitution of OGDC (30%) compared with PDC (60–80%), the two dissociation and reconstitution protocols are not strictly equivalent, with harsher treatment required to remove E1 from the E2 core of OGDC. Moreover, for PDC, no comparable method exists for removing E3BP from the E2 core and maintaining its activity. In view of the possible functional overlap between the two proteins i.e. participation in E3 binding, it is notable that both E1 of OGDC and E3BP of PDC are tightly but peripherally associated with their respective E2 cores. Recent structural studies of the E2/E3BP core of Saccharomyces cerevisiae PDC position the E3BP-binding sites at 12 large openings in the E2 core structure (26). Similar studies on mammalian OGDC will be necessary to shed light on whether its E1 homodimers are positioned similarly, although this complex is built on a 24-mer E2 lattice exhibiting octahedral symmetry. If this proves to be the case, it would be predicted that mammalian OGDC would be composed of six E1 homodimers attached to the surfaces of its cubic (24-meric) E2 core mediating the association of an equivalent number of E3 dimers. The absolute number of E1 and E3 homodimers associated with mammalian E2-OGDC core has not been determined unequivocally. However, studies of E. coli OGDC, which also has a 24-mer E2 core, in this case supporting the binding of both peripherally associated enzymes, report a chain ratio E1:E2:E3 of 0.5:1.0:0.5 (27). These values suggest the presence of six E1 and E3 dimers bound to the E2 core in this case.

Gel permeation of the isolated E1/E3 fraction from bovine heart OGDC, under associative conditions, detected the presence of a higher Mₙ assembly (303,000 Mₙ) corresponding to an E1:E3 subcomplex exhibiting 1:1 stoichiometry. Direct participation of the E1 component in E3 binding is borne out by analysis of cDNAs encoding the rat and human dihydrolipoamide succinyltransferase (E2) genes, which indicate that the domain typically involved in E3 binding is absent (14, 15). In the human gene for E2 of BCOADC, which displays a high degree of similarity to the corresponding OGDC gene, the domain responsible for E1/E3 binding is encoded within a single
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It is likely that loss of this exon has occurred during the evolution of mammalian E2-OGDC. Moreover, the evidence that E1 can physically interact with E3 in this complex is indicative that this E3 binding role now resides within the E1 component.

Subsequent immunological analysis confirmed that antigenic epitope(s) on E1 of OGDC also recognizes antigenic epitope(s) on E2 of PDC. Anti-E3BP serum was also able to detect E1-OGDC specifically, emphasizing the close structural similarity undoubtedly reflects the presence of sequences usually found in lipoyl domains and other common sequence elements. Because the E3 enzyme is common to all mammalian α-ketoacid dehydrogenase complexes, it is not surprising that similar sequence motifs/domain structures are present on the enzymes involved in its binding and catalytic function.

In summary, in mammalian OGDC the binding of E3 to the complex is not mediated by the E2 component, as in other α-ketoacid dehydrogenase complexes, because this protein lacks the domain involved in E3 interactions. In contrast to mammalian PDC, the E3 binding role in OGDC has not been transferred to a separate gene product. Instead, it would appear that the E1 of mammalian OGDC has acquired an additional structural role that is normally performed by the E2 or E3BP components in other prokaryotic and eukaryotic complexes. Recent identification of the odhA gene from Corynebacterium glutamicum encoding the E1 component of OGDC has revealed an N-terminal extension with sequence homology to E2s from PDC and OGDC, albeit to their C termini (30). Usuda et al. indicate that this may represent a degree of bifunctionality in this E1 protein. In our case, the catalytic role of mammalian E1-OGDC is supplemented by a second structural one, namely mediating the integration of the E3 component in optimal orientation to promote the required interaction with conformationally mobile lipoyl domains located on the oligomERIC E2 core. Current research involves overexpressing the native E1 enzyme of OGDC and various subfragments in E. coli with a view to mapping its domain organization more precisely, in particular those regions involved in maintaining critical subunit contacts.

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