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Sequences directing dihydrolipoamide dehydrogenase (E3) binding are located on the 2-oxoglutarate dehydrogenase (E1) component of the mammalian 2-oxoglutarate dehydrogenase multienzyme complex

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Sequences located in the N-terminal region of the high M_r 2-oxoglutarate dehydrogenase (E1) enzyme of the mammalian 2-oxoglutarate dehydrogenase multienzyme complex (OGDC) exhibit significant similarity with corresponding sequences from the lipoyl domains of the dihydrolipoamide acetyltransferase (E2) and protein X components of eukaryotic pyruvate dehydrogenase complexes (PDCs). Two additional features of this region of E1 resemble lipoyl domains: (i) it is readily released by trypsin, generating a small N-terminal peptide with an apparent M_r value of 10 000 and a large stable 100 000 M_r fragment (E1') and (ii) it is highly immunogenic, inducing the bulk of the antibody response to intact E1. This 'lipoyl-like' domain lacks a functional lipoamide group. Selective but extensive degradation of E1 with proteinase Arg C or specific conversion of E1 to E1' with trypsin both cause loss of overall OGDC function although the E1' fragment retains full catalytic activity. Removal of this small N-terminal peptide promotes the dissociation of dihydrolipoamide dehydrogenase (E3) from the E2 core assembly and also affects the stability of E1 interaction. Thus, structural roles which are mediated by a specific gene product, protein X in PDC and possibly also the E2 subunit, are performed by similar structural elements located on the E1 enzyme of the OGDC.

Key words: E1 component/E3 binding/lipoyl-like domain/ 2-oxoglutarate dehydrogenase complex

Introduction

Three analogous mitochondrial multienzyme assemblies, the pyruvate dehydrogenase complex (PDC), the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched-chain 2-oxoacid dehydrogenase complex (BCDC) catalyse key regulatory steps in carbohydrate and amino acid metabolism respectively (Patel and Roche, 1990; Perham, 1991). The conversion of pyruvate to acetyl CoA and NADH is controlled by the PDC in a reaction which results in the irreversible loss of carbohydrate precursors to the cell in mammals. The OGDC is responsible for the formation of succinyl CoA and NADH from 2-oxoglutarate, the rate-limiting step in the flux of intermediates in the latter stages of the citric acid cycle while BCDC catalyses the oxidative decarboxylation of branched-chain 2-oxoacids generated during the turnover of the branched-chain amino acids, leucine, isoleucine and valine (also threonine and methionine). Defects in this multienzyme complex give rise to the symptoms of Maple syrup urine disease.

Each complex is composed of multiple copies of three separate enzymes: a substrate-specific thiamine diphosphate-requiring dehydrogenase (E1) which catalyses decarboxylation of the appropriate 2-oxoacid and subsequent reductive acylation of covalently-attached lipoamide groups of distinctive oligomeric dihydrolipoamide acyltransferase (E2) core assemblies; E2 promotes the transfer of acyl groups of coenzyme A via an interacting network of lipoyl moieties while E3, dihydrolipoamide dehydrogenase, an FAD-linked enzyme, common to all three complexes, is responsible for the re-oxidation of the reduced lipoamide dithiols on E2 with the production of NADH.

PDC from eukaryotic sources is composed of a 60 subunit E2 core arranged in the form of a pentagonal dodecahedron (icosahedral symmetry) to which are attached 20-30 copies of an $\alpha_2\beta_2$ tetrameric E1 enzyme and six copies of E3, a homodimer (Barrera *et al.*, 1972). In contrast, in OGDC and BCDC, each E2 component forms a 24 subunit core structure exhibiting octahedral symmetry while their dehydrogenases (E1) are homodimers and tetramers of two non-identical subunits (α and β) respectively.

The general organization of the distinctive E2 subunits is similar to one or two (PDC) mobile lipoyl domains (~80 amino acids in length) located at their N-termini (Dardel *et al.*, 1991) joined to a small (20-30 amino acid) highly-conserved E3 binding domain by a flexible linker region which is rich in alanine, proline and acidic amino acids (Packman and Perham, 1986; Radford *et al.*, 1987). The C-terminal halves of the E2 polypeptides contain the sites of E1 interaction, the acyltransferase active sites and are responsible for maintenance of the characteristic core structures.

While the domain organization of the E2 core of PDC in prokaryotes is similar to that of eukaryotes, there can be variations in the organization of the E2 core and the E1 components. In Gram positive bacteria the quaternary structures of these two enzymes resemble that of the mammalian PDC complex while Gram negative organisms such as *Escherichia coli* possess an octahedral (24meric) core and homodimeric E1 components (for review see Perham, 1991). Two further distinctive features of the E2 polypeptide chains of *E.coli* are the presence of three lipoyl domains in tandem repeat at the N-terminus and the ability to cleave the E3 binding domain without inducing concomitant dissociation of E1 which is the case for all the icosahedral complexes. Similar structural variations have not been detected in the OGDCs studied to date but recent cloning and sequence analysis of the dihydrolipoamide succinyl-transferase (E2) from rat heart has revealed a highly unusual

feature, namely that this sequence lacks the putative E3 and/or E1 binding domain sequences present in all E2 core assemblies determined to date (Nakano *et al.*, 1991).

In 1985, the surprising observation was made that mammalian PDC contains an additional subunit, termed protein or component X (De Marcucci and Lindsay, 1985; Jilka *et al.*, 1986). Subsequently, protein X was shown to be a distinct lipoyl-bearing polypeptide which exhibited significant sequence similarity to E2, particularly in the N-terminal region and was catalytically active in that its single lipoyl domain participated in the acetylation reactions of the complex (De Marcucci *et al.*, 1986; Hodgson *et al.*, 1986; Neagle *et al.*, 1989; Rahmatullah *et al.*, 1989). Recent biochemical studies on bovine PDC (Gopalakrishnan *et al.*, 1989; Lawson *et al.*, 1991) and genetic studies on PDC from *Saccharomyces cerevisiae* where the gene encoding protein X has been cloned and sequenced (Behal *et al.*, 1989) have revealed that this protein appears to play a structural role in promoting the tight and specific interaction of the E3 component with the core assembly. Moreover, it is clear that the N-terminal lipoyl domain of protein X is not required for full catalytic activity of the complex since a truncated 35 000 M_r fragment of protein X from which the lipoyl domain (but not the E3 binding domain) has been removed either genetically or biochemically, by selective proteolysis, is sufficient to sustain the activity of the complex. This modified form of the complex exhibits altered properties including a lowered affinity for E3 and increased salt sensitivity, suggesting that although the E3 binding domain on protein X is still functional, stable interaction of E3 with the complex also requires the presence of the protein X lipoyl domain (Lawson *et al.*, 1991; Neagle and Lindsay, 1991).

Two puzzling aspects of these findings relate (i) to the requirement for a specific protein to mediate E3 binding in view of the presence of numerous putative E3 binding domains located on the E2 component of the PDC and (ii) to the absence of an equivalent protein in the mammalian OGDC and BCDC which utilize the identical E3 enzyme in catalysing a parallel series of reactions. In this report, it is demonstrated that this second function in OGDC is performed by protein X/E2-like sequences located on 2-oxoglutarate dehydrogenase (E1), one of the constituent enzymes of the complex rather than by a separate gene product.

Results

As illustrated in Figure 1 the three related multienzyme complexes PDC, OGDC and BCDC all exhibit distinct polypeptide profiles when their subunit compositions are analysed by SDS–PAGE. For OGDC, the three prominent Coomassie blue-stained bands represent the subunits of 2-oxoglutarate dehydrogenase E1, M_r 110 000, dihydrolipoamide dehydrogenase, E3, M_r 55 000 and dihydrolipoamide succinyltransferase, E2, M_r 48 000 respectively (Hunter and Lindsay, 1986). Similarly, for BCDC, three major protein bands are observed in this case representing the E2, M_r 50 000, E1α, M_r 46 000, and E1β, M_r 36 000 components of the complex (Clarkson and Lindsay, 1991). Purified BCDC is often characterized by the absence of E3 which tends to dissociate from the complex during its isolation (see Yeaman, 1989). Thus, addition of purified E3 is usually required for assay of overall complex activity.

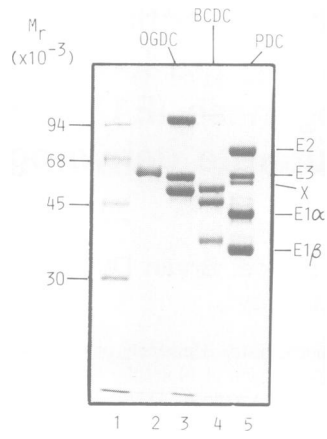

Fig. 1. Subunit profiles of the mammalian 2-oxoacid dehydrogenase complexes as examined by SDS–PAGE. Samples of the three purified complexes were subjected to electrophoresis on a 10% (w/v) SDS–polyacrylamide gel and stained with Coomassie blue. Samples applied to each lane were as follows: Lane 1, low M_r standards (10 μg); lane 2, purified pig heart E3 (5 μg); lane 3, bovine heart OGDC (15 μg); lane 4, bovine kidney BCDC (15 μg) and lane 5, bovine heart PDC (15 μg).

A more complex pattern of polypeptides is evident for PDC which contains the 51 000 M_r protein X subunit in addition to the normal complement of polypeptides, namely E2, M_r 74 000, E3, M_r 55 000, E1α, M_r 42 000 and E1β, M_r 37 000. It is clear from the differing patterns for the three complexes that they each contain distinctive E1 and E2 enzymes although they do, in fact, utilize the same E3 component in all cases.

Since protein X is unique to PDC, recent evidence indicating its involvement in promoting the high affinity binding of dihydrolipoamide dehydrogenase (E3) to the E2/X core assembly (Neagle and Lindsay, 1991) raises the question as to how a similar function is achieved in the OGDC and BCDC which both contain identical E3 enzymes. Figure 2 provides an important clue in respect of OGDC where N-terminal analysis of its high M_r E1 component reveals that the N-terminal sequence of 2-oxoglutarate dehydrogenase (E1) exhibits striking homology with the corresponding regions of the family of eukaryotic E2 and protein X sequences. These similarities include highly-conserved proline and glycine residues at positions 3 or 4 and 20 or 21 respectively and a variation of the characteristic PALSP/GTM motif, namely PFLSGTS. Interestingly, only protein X and E1 sequences lacked a conserved histidine at positions 3 or 4 and an N-terminal serine, both features of all eukaryotic dihydrolipoamide acetyltransferase enzymes (E2) analysed to date.

The presence of sequences showing considerable similarity to the N-terminal region of the lipoyl domain of protein X of PDC on the E1 component of the OGDC suggests the possibility that the functions of protein X are performed by equivalent structural elements located on one of the integral enzymes of the complex in the OGDC. Further evidence indicating that this represents a 'lipoyl-like' domain located at the N-terminus of 2-oxoglutarate dehydrogenase is presented in Figures 3 and 4. Selective proteolysis of OGDC with either trypsin (Figure 3A) or proteinase Arg C (Figure 3B) shows that the E1 component is the most proteolytically-sensitive subunit. In the case of proteinase

Source of enzyme	N-terminal amino acid sequence
* Bovine heart E2-PDC	S L P P H Q K V P L P S L S P T M Q A G T I A
Rat liver E2-PDC	S Y P V H M Q I V L P A L S P T M T M G T V Q R
Human liver E2-PDC	S L P P H Q K V P L P S L S P T M Q A G T I A R
S.cerevisiae E2-PDC	A S Y P E H T I I G M P A L S P T M T Q G M L A A
* Bovine heart X-PDC	A D P I - - K I L M P S L S P/G T M E E G N I (V) (K)
* Bovine heart E1-OGDC	T A P V - - - A A E P F L S G T S - - G N Y V E

Fig. 2. Comparative analysis of N-terminal amino acid sequences of eukaryotic acetyltransferase (E2) with protein X and the 2-oxoglutarate dehydrogenase (E1) enzyme of bovine heart OGDC. N-terminal amino acid sequencing of proteins was performed as described in Materials and methods. Regions of sequence identity are boxed as shown and hyphens represent spacing to allow alignment of the sequences. Amino acid residues in parentheses have only been identified tentatively. Asterisks beside a sequence indicate that these sequences were determined in Glasgow whereas the others were deduced from published nucleotide sequences.

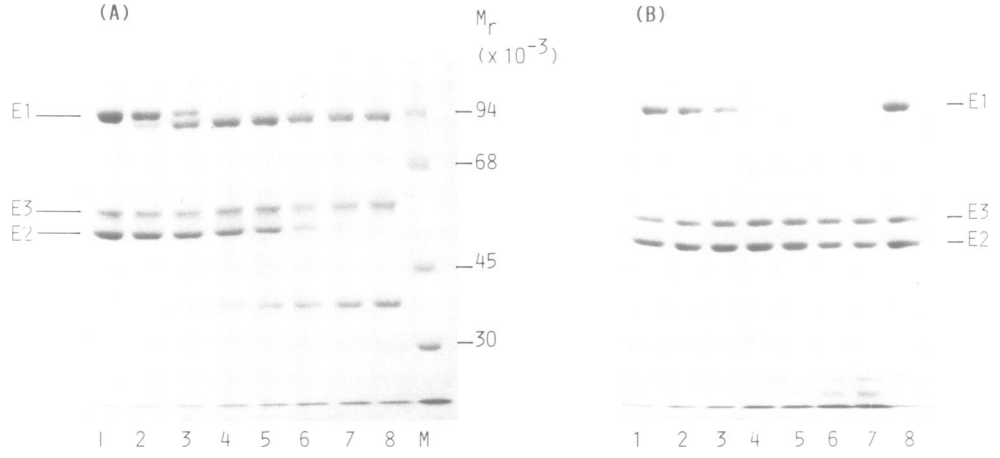

Fig. 3. Selective proteolysis of the 2-oxoglutarate dehydrogenase (E1) enzyme of OGDC with trypsin or proteinase Arg C. Bovine heart OGDC was diluted to a final concentration of 1 mg/ml with 50 mM potassium phosphate buffer, pH 7.6 containing 0.2 mM TPP, 1 mM $MgCl_2$ and 3 mM NAD^+. Digestion of OGDC was initiated at 30°C with either 0.1% (w/w) trypsin (**panel A**) or 9% (w/w) proteinase Arg C (**panel B**) which was added in 3% (w/w) aliquots at 0, 20 and 40 min respectively. Aliquots (15 μg) were withdrawn at the indicated times (see below), mixed with an equal volume of Laemmli sample buffer and resolved on 10% (w/v) SDS–polyacrylamide gels. Panel A: lanes 1–8, OGDC digested with trypsin for 0, 1, 3, 5, 7, 10, 20 and 40 min; lane M, low M_r standards (10 μg). Panel B: lanes 1–7, OGDC digested with proteinase Arg C for 0, 10, 20, 30, 40, 50 and 60 min; lane 8, control OGDC incubated for 60 min in the absence of proteases.

Arg C, the 2-oxoglutarate dehydrogenase enzyme is extensively degraded, producing a variety of low M_r peptides which can be detected visually and by immunoblotting with anti-E1 specific serum. No detectable cleavage of either E2 or E3 polypeptides is apparent even when analysed by immune replica analysis with the appropriate subunit-specific antibodies (not shown).

In contrast, low levels of trypsin promote a rapid, highly-selective cleavage of E1 producing a large stable, 100 000 M_r fragment, designated E1′, and a small peptide with an estimated M_r value of 10 000 which is not detected in this gel system. Its apparent M_r value was determined independently by Schägger and Von Jagow (1987) using SDS–PAGE which gives excellent resolution of peptides in the M_r range 2500–17 000. The above fragmentation pattern is reminiscent of the cleavage of dihydrolipoamide acyltransferases (E2) in which stable, lipoyl-bearing peptides and C-terminal intersubunit-binding domains are generated via specific cleavages within the flexible linker regions. It should be noted that at longer time intervals E2 of OGDC is degraded gradually with the appearance of a 35 000 M_r core fragment and a 15 000 M_r lipoyl domain fragment. The origins of these two peptides were confirmed by immunoblotting and N-terminal sequence analysis (not shown).

Figure 4 (A) reveals an additional feature of the small tryptic fragment derived from E1 which is also a characteristic of lipoyl domains, namely that it is extremely immunogenic, eliciting a high proportion of the total immune response to the original antigen i.e. the intact E1 polypeptide. Indeed the immune response to the 10 000 M_r peptide is considerably stronger than that against the large E1′ fragment which contains >90% of the original E1 sequence.

In Figure 4 (B) the N-terminal protein sequence of E1′ is also shown, indicating that it is distinct from the corresponding E1 sequence, thus confirming that the small tryptic fragment is derived from the N terminus of intact E1, the expected location for a lipoyl domain. This N-

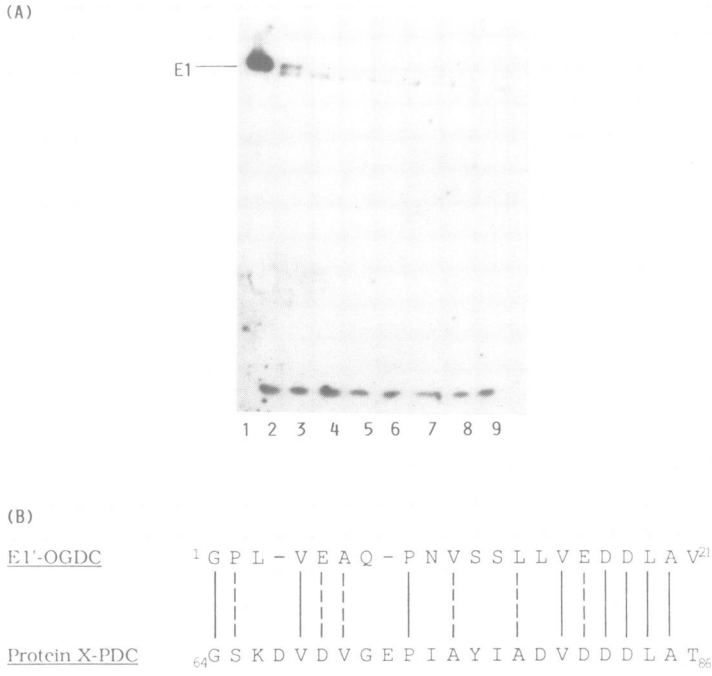

Fig. 4. Immune blotting analysis of the extent of 2-oxoglutarate dehydrogenase (E1) degradation during tryptic digestion of OGDC. OGDC was treated with 0.1% (w/w) trypsin as before (**panel A**) and samples (15 μg) were resolved on a 12.5% (w/v) SDS–polyacrylamide gel and stained with Coomassie blue. Proteins were then transferred electrophoretically onto nitrocellulose paper for incubation with anti-E1 serum (1:100 dilution). Bound antibodies were detected by autoradiography after incubation with ^{125}I-labelled protein A as described in Materials and methods. Lanes 1–9, OGDC incubated with trypsin for 0, 3, 5, 10, 20, 30, 40, 50 and 60 min respectively. **Panel B** compares the N-terminal amino acid sequence of the low M_r E1′ species which is formed by tryptic digestion of E1, with protein X sequence (residues 64–86 inclusive) from *S.cerevisiae*. Solid lines between residues represent sequence identity whereas dashed lines indicate conservative amino acid changes.

terminal sequence of E1′ also exhibits striking sequence similarity with residues 64–86 of the predicted protein X sequence from *S.cerevisiae* again confirming the presence of protein X-like sequences on the E1 component of OGDC. It is also clear from this result that such sequence homology is not confined solely to the released N-terminal peptide but continues into the E1′ fragment.

Sequences determined on E1 and E1′ are homologous to stretches of amino acid sequence located ~20 residues on the N-terminal and C-terminal sides respectively of the highly-conserved segment of the lipoyl domain which includes the active, lipoylated lysine residue. Therefore, the possibility that this region of the E1 component of OGDC contains a functional lipoamide group was assessed by incubating the complex with either [2-^{14}C]pyruvate (which can be loaded onto the E2 core assembly of the OGDC) or its natural substrate, [2-^{14}C]oxoglutarate in the absence of coenzyme A and analysing for the presence of radiolabelled subunits by SDS–PAGE and fluorography. As expected, in both cases, the E2 component of OGDC was radiolabelled owing to the formation of the covalently-bound [^{14}C]acetyl or [^{14}C]succinyl dihydrolipoamide intermediate; however, no similar incorporation of radioactivity into the E1 enzyme could be detected (not shown).

Figure 5 demonstrates the effects of trypsin treatment on E1 and overall OGDC activity under conditions (0.1% w/w) which lead (i) to the rapid conversion of E1 to E1′ within 10 min (as in Figure 3) and significant degradation of E2 at longer time intervals and (ii) under conditions (0.01% w/w) which lead to the slow production of E1′ over 60 min and no detectable proteolysis of the E2 component. It is apparent from Figure 5 that the E1′ fragment retains full enzymatic activity while there is progressive loss of intact

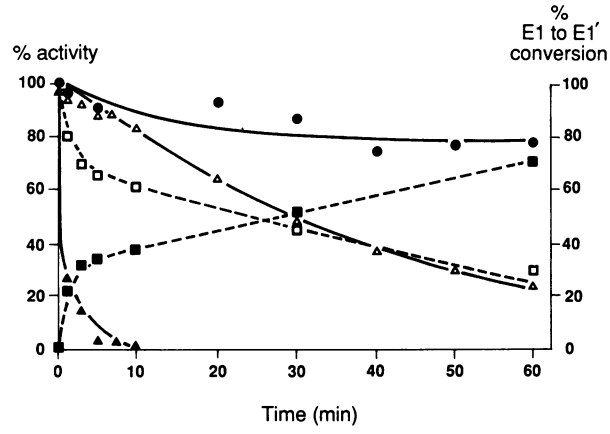

Fig. 5. Time course of tryptic digestion of OGDC; effects on E1 and overall complex activity. OGDC was incubated with 0.01% (w/w) trypsin (△) or 0.1% (w/w) trypsin (▲) as described previously and at times indicated samples (10 μg) were withdrawn and assayed immediately for overall complex activity. The effect of 0.1% (w/w) trypsin on 2-oxoglutarate dehydrogenase (E1) activity was also monitored at the indicated time points (●). All values represent the mean of triplicate assays which differed by <10% in all cases. Parallel OGDC samples (20 μg) were resolved on 10% (w/v) SDS–polyacrylamide gels, stained with Coomassie blue and the areas of the bands corresponding to E1 and E1′ were determined by densitometric scanning as described in Materials and methods. Alterations in the levels of E1 (□-□) and E1′ (■-■) are expressed as changes in their percentage contribution to the combined areas of the E1 plus E1′ bands at each time point. The combined areas themselves varied by <10% over the time course of tryptic digestion, indicating that the E1′ fragment was stable under these conditions.

complex activity which proceeds almost in parallel with the E1 and E1′ conversion as monitored by densitometric scanning. Interestingly, in the early stages of E1 digestion,

E1 to E1′ conversion appears to proceed at a faster rate than the loss of overall complex activity.

Since protein X has a structural role in binding E3 to the core assembly in PDC, it is important to establish if an equivalent function is performed by the protein X-like domains located on the E1 enzyme of OGDC. As shown in Figure 6 intact OGDC, trypsin-modified OGDC (E1 converted to E1′) and proteinase Arg C-modified complex (E1 degraded extensively) were subjected to gel filtration on a Superose 12 FPLC column to permit resolution of the high M_r E2 core assembly from dissociated E1 or E3 enzymes. It is apparent that in the native multienzyme complex, the vast majority of E3 co-elutes with the high M_r core assembly at the void volume while in the trypsin or proteinase Arg C-modified complex, E3 is dissociated from the core structure and elutes with an M_r value of 110 000, corresponding to free E3. Under these conditions, the elution profile of the E2 core and the integrity of individual E2 chains was monitored by immunoblotting, revealing that there was no proteolytic cleavage of E2 or dissociation of its oligomeric structure; however, the active E1′ fragment did also show a marked tendency to dissociate from the complex (not shown), indicating that the 'lipoyl-like' peptide on this component may also be involved in E1 binding.

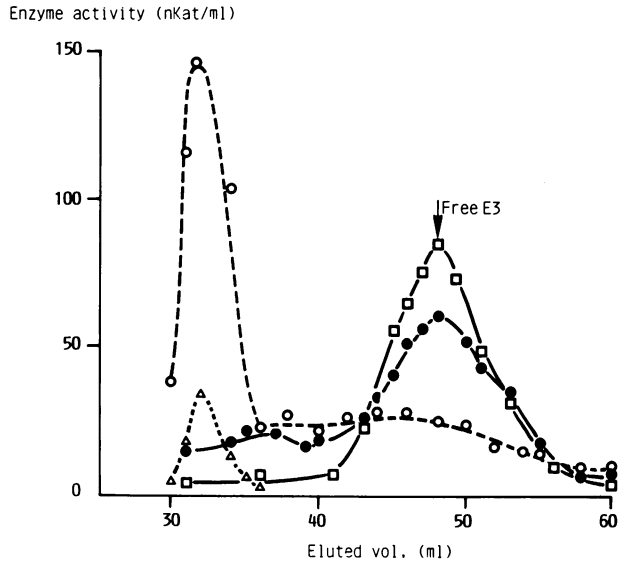

Fig. 6. Purified OGDC was diluted in column buffer (50 mM potassium phosphate, pH 7.6 and 10 mM NaCl) to give a final concentration of 1 mg/ml. Typically, 2 mg of protein was loaded onto the column for each experimental run as described in Materials and methods. Fractions (1 ml) were assayed for either overall complex activity or E3 activity as indicated below. Native OGDC was loaded onto the column and fractions were assayed for both overall complex activity (△-△) and associated E3 activity (○-○). OGDC was then treated with either 9% (w/w) proteinase Arg C for 60 min as described in the Figure 3 legend (□-□) or 0.01% (w/w) trypsin for 10 min (●-●), loaded onto the column and in both cases E3 activity was assayed in the collected fractions. A small proportion of the digested samples was analysed by SDS−PAGE to ensure that digestion had occurred as expected (not shown).

Discussion

In this paper, it is demonstrated that there are significant similarities between the N-terminal region of the 2-oxoglutarate dehydrogenase component (E1) of the OGDC from bovine heart and the equivalent lipoyl domain regions of eukaryotic protein X and dihydrolipoamide acetyltransferase (E2) sequences of the PDC. This segment of E1 has two further distinctive features which are normally characteristic of lipoyl domains which are that it is highly immunogenic and it is removed selectively by trypsin to yield a small stable peptide, M_r 10 000 and a large, stable E1′ fragment, subunit M_r 100 000 which retains full enzymatic activity. However, this 'lipoyl-like' fragment does not contain a functional lipoamide group and is much smaller than a normal lipoyl domain which has an apparent M_r value of 15 000. Consistent with this finding is the observation that the N-terminal sequence of E1′ aligns well with residues 64−86 of the yeast protein X sequence and does not exhibit the characteristics of a flexible linker, rich in alanine, proline and acidic amino acids. This indicates that the tryptic cleavage site on E1′ is located within the 'lipoyl-like' region itself. Further sequence analysis of this region of the E1 gene is in progress to determine to what extent it has retained the normal features of a functional lipoyl domain such as the highly-conserved sequence which contains the lipoylated lysine residue. Selective cleavage of E1 to E1′ with trypsin or extensive but specific degradation of E1 with proteinase Arg C both promote the dissociation of dihydrolipoamide dehydrogenase (E3) from the complex. The large E1′ fragment produced by trypsin also has a low affinity for the E2 core.

It is not clear why the more specific protease, proteinase Arg C catalyses a more extensive degradation of 2-oxoglutarate dehydrogenase than trypsin although it is necessary to employ 50- to 90-fold higher levels of the former enzyme to achieve complete disappearance of the intact E1 polypeptide. This raises the possibility that proteolysis of E1 is promoted by a minor contaminating activity present in the commercial preparations of Arg C; however, this seems unlikely as all batches of Arg C tested cleave the protein X subunit of PDC at only two specific sites, producing a stable 15 000 M_r lipoyl peptide and a 35 000 M_r inner domain fragment which is further degraded to a stable 30 000 M_r species (S.J.Sanderson, J.C.Neagle and J.G.Lindsay, unpublished observations). Arg C is also reported to cleave at Lys−Lys (Chartier *et al.*, 1989) and Lys−Arg bonds (Bousfield and Ward, 1988). It would seem, therefore, that Arg C is capable of cleaving the E1 enzyme at a number of sites at similar rates while low levels of trypsin promote a rapid and specific cleavage at a single highly susceptible site; however, the stable E1′ fragment is also rapidly converted to lower M_r products in the presence of 5−9% (w/w) trypsin.

Previous research has established conclusively that the protein X polypeptide of eukaryotic PDCs plays an important structural role in promoting optimal interaction of E3 with the E2 core structure (Powers-Greenwood *et al.*, 1989). There is also evidence that the lipoyl domain of protein X, which is normally acetylated and deacetylated during the catalytic process, is not required for overall complex activity which can be sustained by the presence of a truncated C-terminal fragment, containing approximately two-thirds of the mature polypeptide sequence (Lawson *et al.*, 1991; Neagle and Lindsay, 1991). In this modified form of PDC, however, E3 dissociates readily and the complex is markedly more sensitive to inhibition by NaCl.

As shown here for OGDC, extensive degradation of the E1 component with proteinase Arg C or specific removal

of a small N-terminal fragment with trypsin both cause the release of E3 from the core assembly indicating that 2-oxoglutarate dehydrogenase, in particular its N-terminal region is involved in promoting the stable interaction of E3 with the assembled complex. Since loss of overall OGDC activity is associated with the cleavage of E1 to E1′ and the dissociation of E3 (and E1′), it appears that this 'lipoyl-like' domain is essential for the maintenance of OGDC function in contrast to the lipoyl domain of protein X. Interestingly, proteolytic cleavage of E1 proceeds more rapidly in the initial stages than loss of overall OGDC activity. As the proteolysis of trypsin-sensitive sites on E1 homodimers is likely to be a random process, resulting primarily in the formation of E1−E1′ heterodimers initially, this observation may indicate that it is necessary to produce E1′ homodimers before the ability of individual E1 molecules to maintain overall complex activity is lost. A more precise mathematical analysis of this effect is underway to determine if this is the case. A further difference is that while the truncated form of protein X remains tightly bound to the E2 core assembly in PDC, in the OGDC, E1′ is much more loosely associated with the complex than native E1 (J.E.Rice and J.G.Lindsay, unpublished observations). However, in both PDC and OGDC, there is no apparent requirement to retain the catalytically-active lipoamide moiety on protein X or equivalent E1 sequences.

As the E2 core assembly of PDC contains highly-conserved putative E3 binding domains, it is still unclear why a specific subunit protein X has evolved to promote the high-affinity physical and functional interaction of E3 with this complex. In the case of OGDC, a variation on this theme has been demonstrated where sequences related to protein X are situated in the N-terminal region of 2-oxoglutarate dehydrogenase (E1), one of the constituent enzymes of the complex in a clear case of a 'domain shuffling'. These sequences are not essential for enzymatic function since the E1′ fragment retains full catalytic activity. In support of these observations, recent cloning and sequence analysis of the human dihydrolipoamide succinyltransferase E2 gene of the OGDC (Nakano *et al.*, 1991) has revealed that this polypeptide uniquely lacks the characteristic highly-conserved sequence elements implicated in E3 binding, a feature of all other prokaryotic and eukaryotic E2 core enzymes examined to date; moreover, the conserved region thought to be involved in E1 interaction with the core assembly is also absent. In this regard, the E1 component of OGDC exhibits a high affinity for the E2 core, only dissociating in the presence of 4−5 M urea (Reed and Willms, 1969), whereas in PDC and BCDC, their respective E1 enzymes are released by 1−2 M NaCl (Pettit *et al.*, 1973). Similar stringent incubation conditions are required to promote the dissociation of protein X in PDC, highlighting an additional common property of protein X of PDC and the E1 enzyme of the OGDC. The precise region of protein X involved in interaction with the core assembly has still to be determined although it is known to be located within the C-terminal two-thirds of the polypeptide. In contrast, E1′ of the OGDC is much more loosely associated with the E2 core than native E1, raising the possibility that release of E3 may be induced by conformational changes in or concomitant release of E1′. Such effects are reminiscent of the simultaneous release of E3 and E1 from icosahedral forms of PDC on selective cleavage of the E3 binding domain on the E2 core (Packman *et al.*, 1988). As removal of the lipoyl-like domain from E1 of OGDC also affects E1 binding, it may be directly involved in E3 and E1 interaction with the complex or alternatively this may be mediated by a putative E3 binding domain located within the protein X-like sequences on the E1′ fragment. Experiments are in progress to establish if there is any evidence for the existence of an E1′−E3 subcomplex.

It remains to be established if E3 interaction with the PDC is maintained solely via the protein X component or whether these polypeptides are involved mainly in stabilizing a lower affinity association of this enzyme with the respective acyltransferase core structures. In the case of PDC, it has been reported that E3 exhibits some affinity for both E2 and X subunits as measured by ELISA plate assays (Gopalakrishnan *et al.*, 1989); however, since there are an estimated 6−12 mol protein X/mol PDC which contains six E3 homodimers and 12 mol of both E1 and E3 enzymes per 24meric E2 core of OGDC, there is clearly the potential for a 1:1 stoichiometric interaction between these polypeptides in both complexes.

In the BCDC, dihydrolipoamide dehydrogenase (E3) appears to bind more weakly to the intact assembly, often resulting in its removal from the complex during purification (Clarkson and Lindsay, 1991). Whether a similar mechanism has evolved to promote its high-affinity association with the complex *in vivo* has still to be established. The evolution of a specific structural component to bind E3 may be related to the requirement for the polypeptides of eukaryotic PDCs to be translocated into the mitochondrial compartment in precursor form prior to assembly, a process which also involves interactions with at least two separate mitochondrial heat-shock proteins, hsp60 and hsp70 as obligatory steps in the maturation pathway. In this context, the mitochondrial cytochrome *c* oxidase complex in yeast requires the mitochondrially-coded subunit III for functional assembly *in vivo* while this polypeptide can be removed from the purified complex without affecting its enzymatic properties *in vitro* (Poyton *et al.*, 1988). Thus while heat-shock proteins associate transiently with oligomeric enzymes during their assembly to effect their functional maturation, in more complex macromolecular assemblies, it seems that integral structural proteins may be required to provide the necessary framework to optimize the orientation of the individual enzymes for full catalytic activity.

In this regard, it should be noted that PDC, with an M_r of 8.5×10^6, is larger than a mammalian ribosome, M_r value 4.5×10^6, where it is clear that functional reconstitution occurs in an ordered sequence of events. Thus core proteins (primary binding proteins) recognize specific binding sites on the large or small subunit RNA templates and in the process create new binding sites for the interaction of more peripherally-located components which bind at a later stage in the assembly pathway. As expected, omission of individual core proteins leads to dramatic effects on ribosomal assembly. These observations on the PDC and OGDC have wider implications for other complex assemblies containing multiple subunits, many of which have no assigned function as yet, such as in the respiratory chain complexes of the mitochondrial inner membrane.

Materials and methods

Materials

Dihydrolipoamide dehydrogenase (pig heart) and proteinase Arg C were from Boehringer Mannheim, Germany; trypsin (*N*-tosyl-L-phenylalanine

chloromethyl ketone treated) and DL-lipoamide were purchased from Sigma Chemical Co., Poole, Dorset, UK; Immobilon membrane was obtained from Millipore, Harrow, Middlesex, UK.

DL-Dihydrolipoamide was prepared by the reduction of DL-lipoamide with $NaBH_4$ as described previously (Kochi and Kikuchi, 1976). All other chemicals used were of analytical grade or the finest grade commercially available.

Methods

The 2-oxoglutarate dehydrogenase complex (OGDC) was purified from bovine heart by a previously reported modification (De Marcucci *et al.*, 1985) of an earlier procedure (Stanley and Perham, 1980). The overall activity of OGDC was determined by monitoring NADH formation spectrophotometrically at 340 nm according to the method of Brown and Perham (1976). The 2-oxoglutarate dehydrogenase (E1) activity of OGDC was measured by following the reduction of DCPIP (2,6-dichlorophenol-indophenol) at 600 nm (Khailova *et al.*, 1976). Dihydrolipoamide dehydrogenase (E3) activity of the complex was assayed as described by Jackman *et al.* (1990), in which enzymatic activity was followed by measuring NADH production at 340 nm.

Detailed methods for standard SDS–PAGE, antibody production and immunoblotting have been reported in a previous publication (De Marcucci and Lindsay, 1986). Analysis of low M_r fragments was performed on SDS–polyacrylamide gels employing the procedure described by Schägger and Von Jagow (1987). Coomassie blue-stained gels were scanned using a Shimadzu dual-wavelength flying spot scanner, model CS-9000, Kyoto, Japan. Parameters were set according to the manufacturer's instructions with computer analysis of peak areas. N-terminal sequencing of proteins was performed by resolving proteins by SDS–PAGE on a Biorad Mini Protean II gel apparatus and then blotting the gel onto Immobilon membrane according to the conditions outlined in Applied Biosystems user bulletin no. 25 (1986). After staining, protein bands were excised from the membrane with a scalpel and sequenced at the SERC funded protein sequencing facility at Aberdeen, using an Applied Biosystems model 477A gas phase sequencer with a 120A on-line PTH analyser and a modified version of the manufacturer's BLOTT-4 programme.

Gel filtration analyses were performed using a Superose 12 column (HR 16/50, ~100 ml bed volume) which was attached to a Pharmacia FPLC system. Collected fractions (1 ml) were assayed either for intact OGDC activity or released E3 activity or E1′ activity (not shown) as described above.

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