

Chain folding in the dihydrolipoyl acyltransferase components of the 2-oxo-acid dehydrogenase complexes from *Escherichia coli*

Identification of a segment involved in binding the E3 subunit

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The state of assembly of the pyruvate and 2-oxoglutarate dehydrogenase multienzyme complexes was examined after the dihydrolipoyl acyltransferase (E2) component of each enzyme system had been subjected to varying degrees of limited proteolysis. Dissociation of the dihydrolipoyl dehydrogenase (E3) component accompanied specifically the excision of a homologous segment of each E2 chain that connects the N-terminal lipoyl domain(s) with a C-terminal catalytic domain. The latter remains aggregated as a 24-mer and retains its capacity to bind the 2-oxo-acid decarboxylase (E1) component. The relevant segment of the E2o chain from the 2-oxoglutarate dehydrogenase complex was isolated and shown to be a folded protein which still binds to E3.

2-Oxo-acid dehydrogenase complex Binding domain Proteolysis

1. INTRODUCTION

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* comprises a structural core (24-mer) of dihydrolipoyl acetyltransferase (E2p, EC 2.3.1.12) subunits to which are bound multiple copies of pyruvate decarboxylase (E1p, EC 1.2.4.1) and a flavoprotein, dihydrolipoamide dehydrogenase (E3, EC 1.6.4.3). In the homologous 2-oxoglutarate dehydrogenase complex, the corresponding enzymes are dihydrolipoyl succinyltransferase (E2o, EC 2.3.1.61), 2-oxoglutarate decarboxylase (E1o, EC 1.2.4.2) and the same E3. Much is already known about the structure and mechanism of both complexes [1,2] but not, as yet, how E3 binds to two different proteins, E2p and E2o. This interaction is of particular interest in mapping the domains of the assembled en-

zyme complexes. Disassembly-reconstitution experiments suggest that the binding sites for E1 and E3 may overlap on the E2 core [3] while electron microscopy reveals E3 as binding to the faces of the octahedral E2 core with E1 bound along the edges [4]. The study is aided by knowledge of the amino acid sequence of each protein, inferred from the DNA sequence of the encoding genes, *aceF* (E2p) [5], *sucB* (E2o) ([6] with sequence amendment in [7]), and *lpd* (E3) [8]. Extensive sequence homology is evident between E2p and E2o [6], but this information provides no clues as to the likely site(s) of interaction of E3 with E2.

Experiments involving limited proteolysis have shown that the three tandemly arranged lipoyl domains of E2p, which form the N-terminal half of the polypeptide chain, or the single lipoyl domain of E2o, which is also N-terminal, can be removed from the enzyme complexes without significant dissociation of E1 or E3 [9–11]. We have now

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developed conditions of limited proteolysis which will cleave E2 in such a way as to release E3 also, leaving E1 still bound to the much truncated E2 core. The principal regions of E2p and E2o responsible for binding E3 have been identified and evidence is presented that this segment of polypeptide chain, after excision from E2o, retains a folded structure and its affinity for E3.

2. MATERIALS AND METHODS

2.1. Enzymes

Pyruvate dehydrogenase complex was purified from a constitutive mutant of *E. coli* K12 [12] and the 2-oxoglutarate dehydrogenase complex from *E. coli* K12(pGS156) [13]. Whole complex and E3 enzyme activities were determined by the NAD⁺-reduction assay [14]. The source of proteinases is given in [7].

2.2. Limited proteolysis

Pyruvate dehydrogenase complex (1–2 mg, 10 mg/ml in 20 mM sodium phosphate, pH 7, containing 2.7 mM EDTA, 0.02% NaN₃) was treated with 0.5% (w/w) *Staphylococcus aureus* V8 proteinase at 0°C. Samples (0.2 mg) were withdrawn at timed intervals and assayed for E3 activity. The assembly state of the enzyme complex in each sample was assessed by gel filtration on a TSK 3000SW column (7.5 mm × 60 cm) in 50 mM sodium phosphate, pH 7, at room temperature (21°C). Protein emerging at the void volume (V_0) was collected, as too was any dissociated E3 ($K_d \approx 0.37$). Each fraction was assayed for E3 activity and analysed by SDS-polyacrylamide gel electrophoresis. When the whole complex activity of the incubation had fallen to less than 5% of its initial value (about 2 h), the temperature was raised to 30°C for 60 min, after which time a final sample was removed.

2-Oxoglutarate dehydrogenase complex (0.5 mg, 10 mg/ml) was treated in a similar fashion with 0.2% (w/w) trypsin at 0°C. After about 20 min, the whole complex activity fell by about 98%. Digestion was arrested by adding soyabean trypsin inhibitor (5 µg) and the sample was gel filtered. The protein emerging at the V_0 of the column was collected and submitted to secondary digestion with 1% (w/w) *S. aureus* V8 proteinase. An equivalent concentration (0.4 mg/ml)

of intact complex was also digested with this proteinase to provide an assay for the course of the digestion of the inactive trypsin-treated sample. It was found necessary to carry out this digestion at room temperature to achieve proteolysis within 2 h at this lower substrate concentration.

The sample of doubly digested complex was again gel filtered and excluded protein and dissociated E3 were collected as before.

2.3. Analytical techniques

Details of methods used for SDS-polyacrylamide gel electrophoresis and amino acid sequence analysis are given in [7,15].

3. RESULTS

3.1. Identification of an E3-binding segment of E2

Treatment of the pyruvate dehydrogenase complex with *S. aureus* V8 proteinase at 0°C led to rapid degradation of E2p and inactivation of the complex, creating fragments of E2p of 37.2 and 34.5 kDa (fig.1). Neither cleavage was accompanied by significant loss of E3 from the assembled complex. However, prolonged digestion at the higher temperature (30°C) yielded larger amounts of a smaller E2 chain fragment of 29 kDa and this was accompanied by increasing dissociation of E3 but not E1 (fig.1). Direct sequence analysis has already identified the cleavage sites as Glu-292, Glu-318 and Glu-372, respectively [7]. A region of E2p between residues 319 and 372 therefore seems important in binding E3. Additional evidence was gained from a similar experiment in which the E2pE3 subcomplex was treated with trypsin. Cleavage of E2p at Lys-316 [15] caused little loss of E3 but prolonged digestion induced a cleavage at Lys-369 [7], which was accompanied by complete dissociation of the flavoprotein (Radford, S. and Packman, L., unpublished).

Treatment with trypsin similarly inactivated the 2-oxoglutarate complex by releasing the lipoyl domain; only a small amount (8%) of E3 was lost in the process. Digestion of the residual complex with *S. aureus* V8 proteinase released E3, as did similar treatment of native enzyme complex, leaving an E1-E2o (truncated) subcomplex (fig.2). Direct sequence analysis of the fragments has shown that trypsin cleaves E2o at Arg-100 and that *S. aureus* V8 proteinase cleaves at Glu-156 [7]. The segment

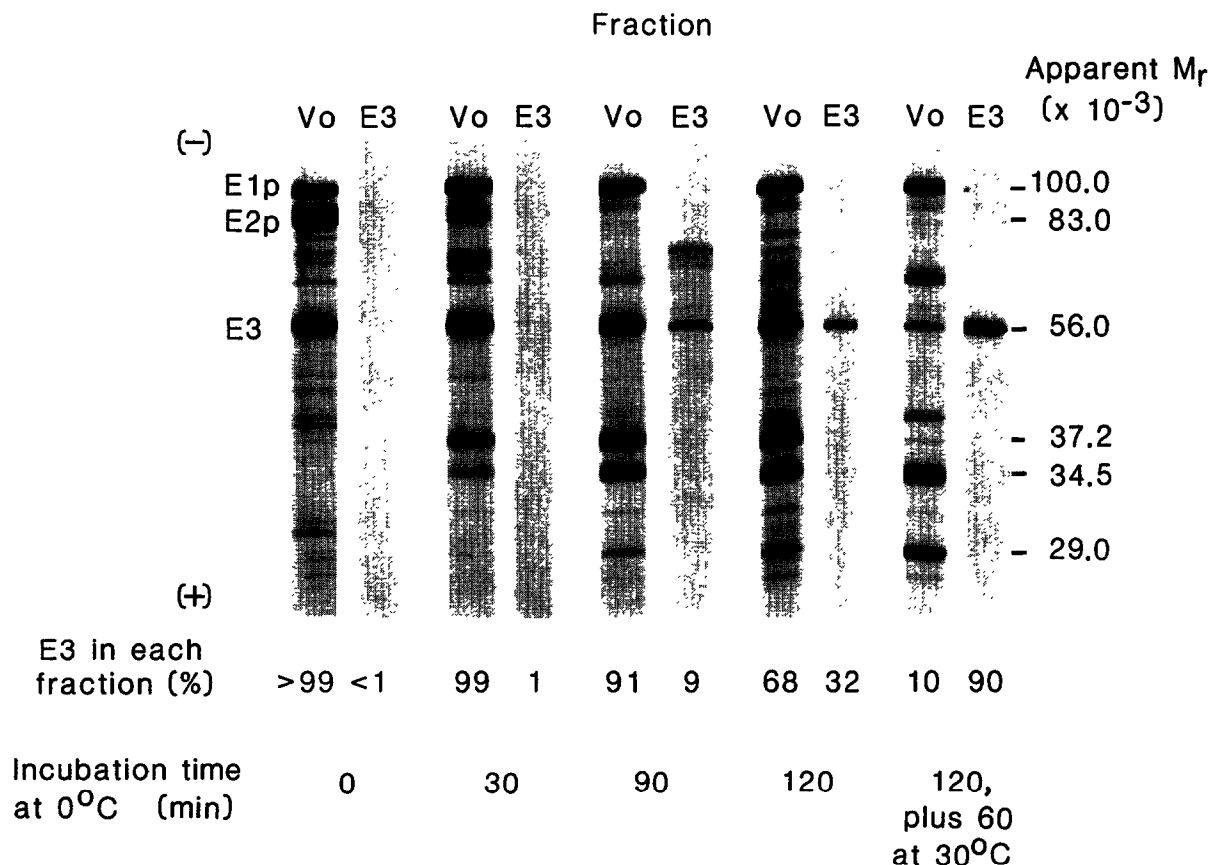


Fig.1. Digestion of pyruvate dehydrogenase complex with *S. aureus* V8 proteinase. Pyruvate dehydrogenase complex was digested with *S. aureus* V8 proteinase as described in section 2. At timed intervals, the assembly state of the complex was assessed by gel filtration. Samples of the protein at the void volume (V_0) of the column and any dissociated E3 were collected and analysed by SDS-polyacrylamide gel (20% T, 0.3% C) electrophoresis and by assays for E3 activity. Staining of the gels was with Coomassie blue followed by light staining with Ag to enhance the detection of the truncated E2 chains.

of E2o between residues 101 and 156 is therefore clearly important in binding E3 and it is significant that it is homologous with the similarly identified segment of E2p (fig.3).

3.2. Isolation of the E3-binding domain from E2o

To determine whether residues 101–156 of E2o exist as a discrete structural unit, a larger-scale experiment was performed. 2-Oxoglutarate dehydrogenase complex (10 mg, 20 mg/ml) was incubated with 0.1% (w/w) trypsin and gel filtered in 1% NH_4HCO_3 , pH 7.0 (on TSK 3000SW) or pH 7.8 (on Superose 12). The emergent complex was concentrated to 5 mg/ml if necessary, digested with *S. aureus* V8 proteinase, and gel filtered once

more, achieving separation of E3 from the E1o-E2 (truncated) subcomplex. Selected fractions of the column effluent, from E3 to the included volume (V_i), were pooled separately, freeze-dried and analysed by reverse-phase HPLC on ChromSpher C-18 support in 0.1% TFA with CH_3CN (1% per min) as organic modifier.

The major product was a single peptide fragment, eluting at 41% CH_3CN , whose N-terminal sequence (QQAS¹EEQN-) and amino acid composition were consistent with it comprising residues 101–156 of E2o. Significantly, the same fragment was recovered from two separate sections of the gel filtration profile; it was found as expected in those fractions approaching the V_i but

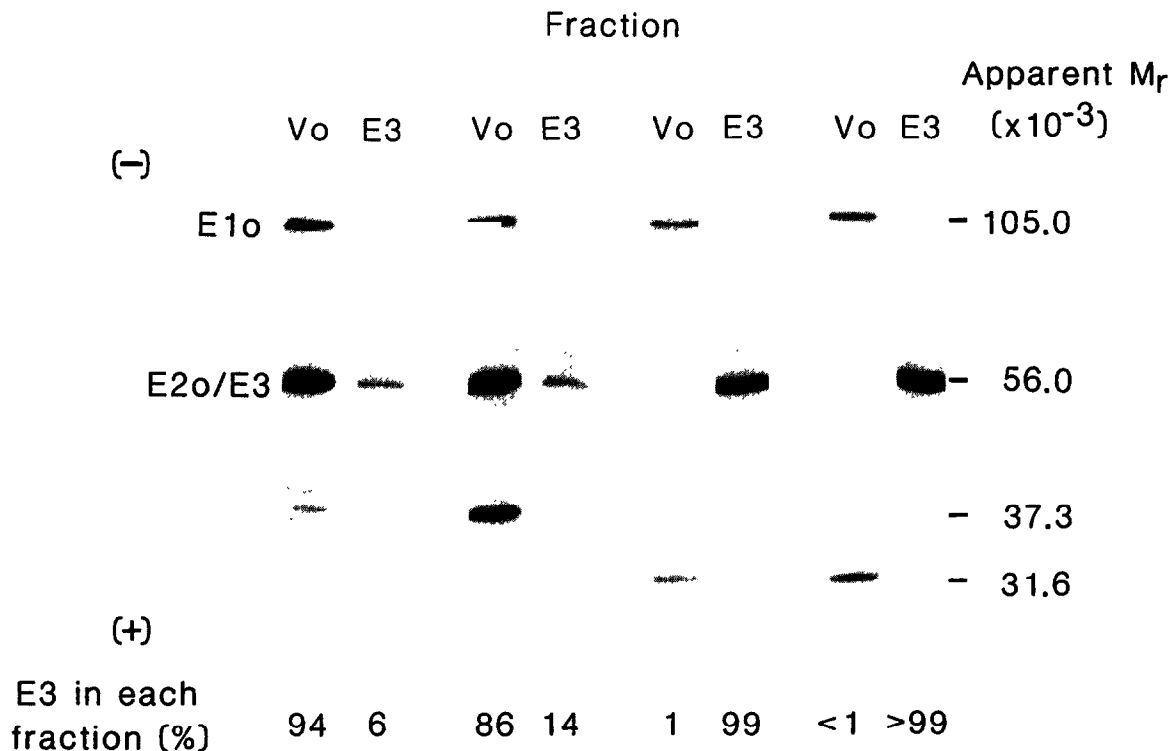


Fig.2. Limited proteolysis of 2-oxoglutarate dehydrogenase complex. Samples of 2-oxoglutarate dehydrogenase complex were treated with trypsin and/or *S. aureus* V8 proteinase as described in section 2. The products were analysed as described in the legend to fig.1. Gel filtration of 2-oxoglutarate dehydrogenase complex: A, untreated complex; B, complex treated with and inactivated by trypsin; C, the V_0 sample from B treated with *S. aureus* V8 proteinase; D, complex treated with *S. aureus* V8 proteinase. Note that E3 and E2o co-migrate in this gel system. Staining is with Coomassie blue only. The initial preparation of complex contains some E3 that dissociates on gel filtration (track A).

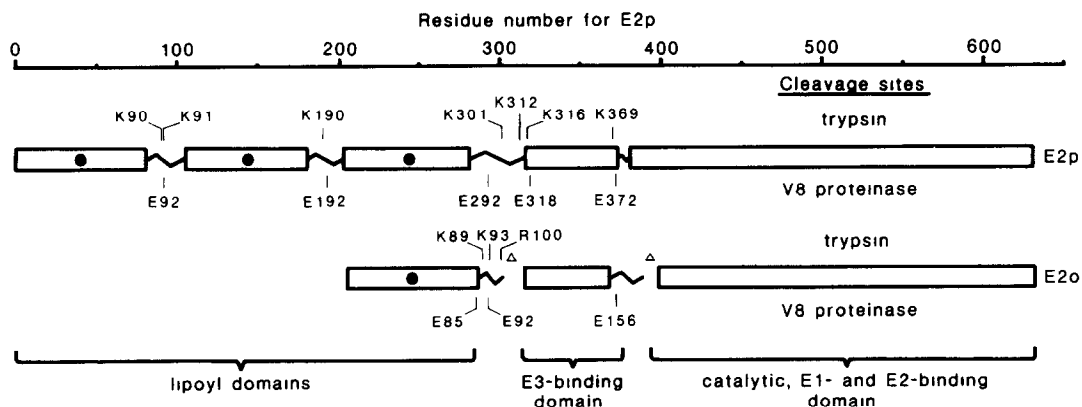


Fig.3. A model for the structure of E2p and E2o. The sequence of E2o is aligned with that of E2p to maximise the homology between the two proteins [6]. Δ, gaps of ≥ 5 residues in the sequence of E2o produced by this alignment. ●, lipoyc acid. ~, sequences rich in alanine, proline, lysine, glutamic acid and glutamine. The precise limits of the structural domains are unknown and those shown here are for illustrative purposes only.

also in the earlier E3-containing fractions. It was not detected in intervening fractions. Clearly, the segment of E2o comprising residues 101–156 is resistant to cleavage by trypsin and *S. aureus* V8 proteinase under the conditions used, despite containing several lysine, arginine and glutamic acid residues. This segment is therefore likely to be a folded structure and, since it elutes at least in part with E3 during gel filtration, it possesses residual affinity for the E3 protein. Thus it has the properties of an E3-binding domain.

On SDS-polyacrylamide gels, the E3-binding segment of E2o migrated with an apparent M_r value of 5500–6000, close to its computed M_r value of 6018 (residues 101–156). It stained poorly with Coomassie blue but exhibited the remarkable property of being resistant to subsequent staining with Ag. This explains why it was not detected earlier in *S. aureus* V8 proteinase digests of 2-oxoglutarate dehydrogenase complex. The amount loaded onto the gel from analytical digests was insufficient for Coomassie blue detection alone and subsequent Ag staining failed to indicate its presence.

4. DISCUSSION

It has hitherto not been possible to distinguish the binding of E1 and E3 to the E2 cores of the 2-oxo-acid dehydrogenase multienzyme complexes. We have now demonstrated that homologous segments of E2p and E2o are important in the binding of E3 and presumably in the assembly of the complexes. The isolated segment of E2o exhibits characteristics indicative of a folded and, at least in part, functional protein which we term the E3-binding domain. Although a comparable intact fragment has yet to be isolated from E2p, the conditions necessary for its generation (incubation for several hours at 37°C with trypsin or *S. aureus* V8 proteinase) may well be too harsh to allow high recovery of an undegraded product.

Inspection of the models for the structure of E2p and E2o (fig.3) shows each E3-binding domain to be located between segments of polypeptide rich in alanine, proline, lysine, glutamic acid and glutamine residues. Some of these segments have been implicated in polypeptide chain mobility by virtue of their sharp $^1\text{H-NMR}$ signals, fostering movement of the lipoyl domains [2,11,13,16].

Since proteolysis in these regions readily releases the E3-binding domain, it is conceivable that this domain and its associated E3 molecule experience some degree of motional freedom in the intact complex which may not be readily detectable by $^1\text{H-NMR}$ spectroscopy owing to the large size (102.5 kDa) of the bound E3 dimer. Any such movement might provide a basis for interpreting fluorescence data which suggest that E3 bound to the pyruvate dehydrogenase complex is quite mobile [17]. The identification of a discrete E3-binding domain should enable a more detailed analysis of the interaction of E3 with E2 to be made.

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