LIMITED PROTEOLYSIS OF 2-OXOGLUTARATE DEHYDROGENASE MULTIENZYME COMPLEX FROM BOVINE KIDNEY

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1. Introduction

The 2-oxoacid (pyruvate or 2-oxoglutarate) dehydrogenase complexes are composed of a lipoate acetyltransferase (E2) core to which multiple copies of a 2-oxoacid dehydrogenase (E1) and lipoamide dehydrogenase (E3) are bound (reviewed [1,2]). Recently, the molecular structure of the lipoate acetyltransferase component of bovine PDC has been elucidated by studies using limited proteolysis [3–5]. The acetyltransferase polypeptide chain is built from two separate folding domains, one containing the active center for transacylation as well as the E2 intersubunit binding sites (active domain) whereas the other comprises the enzyme-bound lipoyl residues (lipoyl domain). Little is known as yet on the structural properties of E2 from mammalian OGDC. This paper presents evidence indicating an analogous structural organization of mammalian OGDC-E2 as found for PDC-E2.

2. Experimental

2.1. *Enzymes*

OGDC was purified from bovine kidney mitochondria according to [6]. The isolation procedure was modified by the addition of a sucrose density gradient centrifugation step as in [7]. The complex had spec. act. 8–12 U/mg. PDC activity in these preparations was $\leq$1% of OGDC. Glutamate dehydrogenase, succinate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase activities were not detectable. The SDS gel electrophoresis pattern (fig.2) showed the 4 principal bands described in [8] together with varying amounts of contaminating material most of which probably resulted from degradation by endogenous proteases [8]. Estimated M_r of the principal bands was: band 1 (E1), 96 000; band 2 (unknown [8]), 90 000; band 3 (E3), 55 000; band 4 (E2), 52 000.

Papain, stem bromelain, and elastase were from Boehringer (Mannheim), trypsin (treated with L-tosylamido-2-phenylethyl chloromethyl ketone) from E. Merck (Darmstadt). PDC inactivase was a generous gift of Dr A. Lynen, Institut für Diabetesforschung, München. The sources of all other materials were as in [3,4,7].

2.2. *Enzyme assays*

OGDC overall activity (NAD reduction) and E3

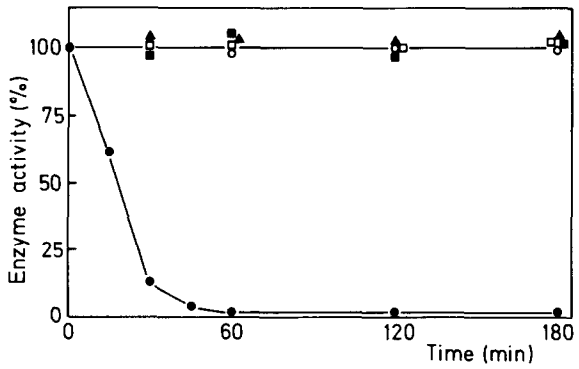

Fig.1. Effect of PDC-inactivase treatment on the overall and component activities of OGDC. OGDC (0.5 mg/ml) was incubated at 23°C with PDC inactivase (4.8 μg/ml) in 0.1 M potassium phosphate (pH 6.5) 2 mM cysteine hydrochloride, 1 mM EDTA. At the times indicated, samples were removed, mixed with leupeptin (0.14 mg/ml) and assayed. Overall activity (●); E1 (■); E2 (□); E3 (▲); control sample containing 0.1 mg leupeptin/ml, overall activity (○).

Abbreviations: PDC., pyruvate dehydrogenase complex; OGDC, 2-oxoglutarate dehydrogenase complex; SDS, sodium dodecylsulfate; M_r, relative molecular mass

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were assayed as in [7]. 2-Oxoglutarate dehydrogenase (E1) was assayed either spectrophotometrically as in [9] or by measuring the decarboxylation of [1-^{14}C]-oxoglutarate (NEN) under the conditions given in [10]. Lipoate succinyltransferase (E2) was assayed according to [8]. With all assays, 1 U corresponds to 1 μmol product formed or substrate consumed/min at 25°C.

2.3. *Treatment with proteases*

The experimental conditions for protease treatment of OGDC were identical as described for PDC in [3,7].

2.4. *SDS gel electrophoresis*

Electrophoresis was carried out either in cylindrical gels containing 7.5% acrylamide in the phosphate buffer system of [11] or in 12.5% acrylamide slab gels using the Tris–glycine system of [12].

3. Results

3.1. *Inactivation of OGDC by proteases*

In a way quite similar to bovine PDC [3,4,7] the overall activity of OGDC was destroyed by proteases such as rat liver lysosomal PDC inactivase [13] (fig.1), papain, elastase, or bromelain (not shown) without any loss of E1, E2, or E3 activities.

During inactivation, bands 1 (E1) and 4 (E2) of the SDS gel pattern disappeared (fig.2). The main product resulting from E1 cleavage was a 94 000 M_r fragment (band 1A) which, hence, was only ~2% smaller than the uncleaved E1 chain and evidently retained the total E1 activity (fig.1). The E2 polypeptide chain, in contrast, apparently was cleaved about in the middle to give fragments with app. M_r 29 500 (band 4A) and 27 000 (band 4B). Band 4A, in most cases, was stained only faintly with Coomassie blue and was not always detectable. With PDC inactivase, papain or elastase, cleavage of E2 was faster than E1 fragmentation. In contrast, during treatment with trypsin or bromelain, the E1 band was cleaved faster than E2.

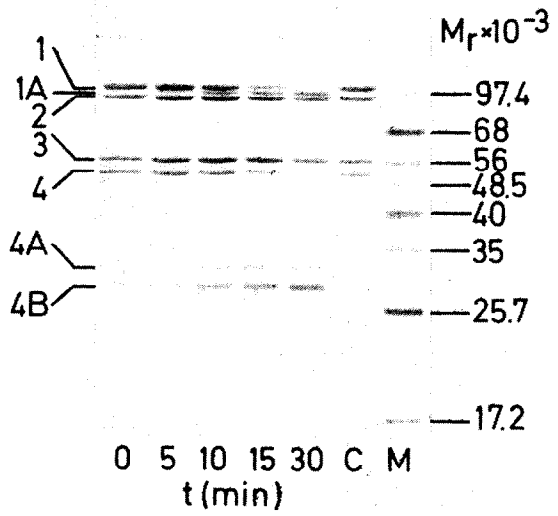
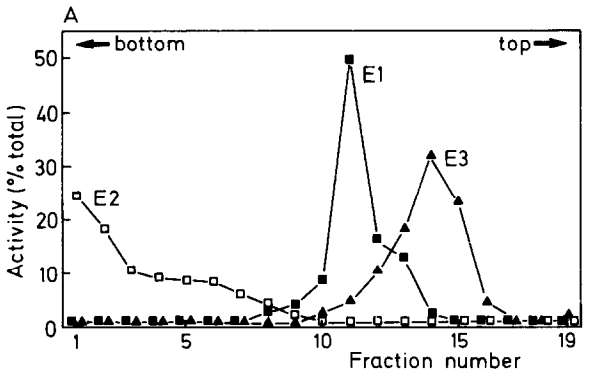


Fig.2. Fragmentation of OGDC by papain. OGDC (1.1 mg/ml) and papain (1.8 μg/ml) were incubated at 23°C under the same conditions as in fig.1. At the times indicated, samples were removed, mixed with leupeptin (0.1 mg/ml) and analyzed by SDS gel electrophoresis (Tris–glycine system). The residual overall activities were: 0 min, 100% (12.5 U/mg); 5 min, 63%; 10 min, 51%; 15 min, 31%; 30 min, 4.3%. The control sample (C, lane 6) contained additionally 80 μg/ml leupeptin (residual activity after 30 min, 94%). The extreme right lane (M) shows a mixture of marker proteins used for M_r estimation [4].

3.2. *Disassembly of OGDC after protease treatment*

Just as with bovine kidney PDC [3,4,7] OGDC was disassembled into its enzyme components during treatment with papain or elastase. This is demonstrated in fig.3 showing the results of sucrose density gradient centrifugation of papain-treated OGDC. Whereas E2 activity sedimented to the bottom of the gradient, E1 (M_r 216 000 [2]) and E3 (M_r 108 000 [2]) were found in the middle and top fractions. With OGDC treated with papain in the presence of the protease-inhibitor leupeptin, OGDC overall as well as E1, E2 and E3 activities were found together at the bottom of the gradient. The result in fig.3 furthermore proves that limited proteolysis does not affect the association of the E2 subunits to give the high M_r core structure. The SDS gel pattern of some gradient fractions is also shown in fig.3 again demonstrating the dissociation into the individual enzyme components. Fraction 1 contains nearly exclusively band 4B characterizing this 27 000 M_r fragment as the active domain of E2.

Since protease treatment induces the disassembly of the enzyme complex into its enzyme components these could be isolated, under very mild conditions, from papain- or elastase-treated OGDC, by fractional precipitation with ammonium sulfate similarly as described for PDC [3,4]. As shown in fig.4, the precipitate obtained at 36% $(NH_4)_2SO_4$ saturation contained both fragments formed from E2 as well as band 2 which represents a unknown component of kidney OGDC [8] and small amounts of E3. The 36–55% ammonium sulfate fraction mainly consisted of the



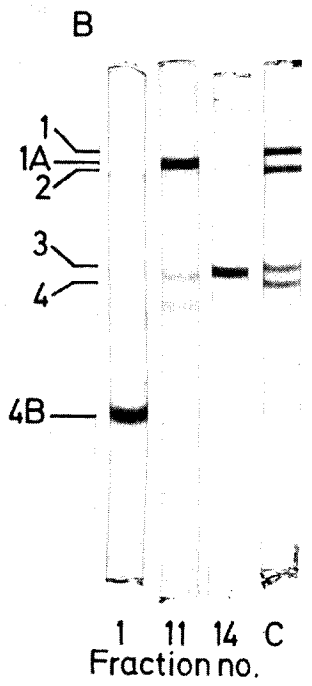


Fig.3. Sucrose density gradient centrifugation of papain-treated OGDC. The complex (3.7 mg/ml) was treated with 15 μg/ml papain for 15 min at 25°C under the conditions in fig.1 (resid. act. 0.03%). The sample was then mixed with 0.1 mg leupeptin/ml and layered on top of a linear 5–20% gradient of sucrose in 20 mM potassium phosphate (pH 7.0) 1 mM $MgCl_2$, 10 μM thiamin diphosphate, 0.1 mM EDTA, in the 38 ml tubes of a Beckman SW-27 rotor. Centrifugation was carried out at 131 000 × *g* for 16 h. Fractions of 2 ml were collected and analyzed for activity (A). To obtain a common scale, the values are plotted as a percentage of the total activities. (B) Aliquots of the fractions were mixed with an equal volume of double-concentrated sample buffer [11] and used for SDS gel electrophoresis (phosphate system). The extreme right gel (C) shows fraction no. 1 of a control experiment in which the complex was treated with papain in the presence of 0.1 mg leupeptin/ml.

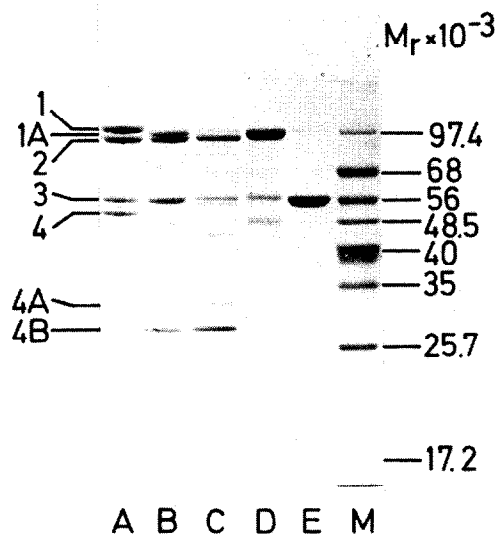


Fig.4. Fractionation of elastase-treated OGDC with ammonium sulfate. OGDC (5.4 mg/ml) was treated with 30 μg elastase/ml at 22°C in 0.1 M potassium phosphate (pH 7.0). After 15 min, the reaction was stopped by the addition of 34 μg elastatinal/ml (overall resid. act. 0.4%). The mixture was then diluted to 4 ml (3.7 mg OGDC/ml) and fractionated by the addition of appropriate volumes of saturated solution of ammonium sulfate in 50 mM potassium phosphate (pH 7.0), 1 mM $MgCl_2$, 10 μM thiamin diphosphate, 1 mM EDTA, or solid ammonium sulfate. Precipitates were collected by centrifugation (30 min at 35 000 × *g*) and used for SDS gel electrophoresis (Tris–glycine system). (A) Untreated OGDC; (B) elastase-treated OGDC before fractionation; (C) 0–36% ammonium sulfate fraction; (D) 36–55% ammonium sulfate fraction; (E) 55–90% ammonium sulfate fraction; (M) marker proteins [4].

E1 fragment (band 1A) with only little contamination by E3 and a 50 000 M_r fragment probably resulting from further cleavage of E1. The 59–90% fraction contained E3 in a high state of purity (400–570 U/mg).

4. Discussion

These results demonstrate that the effect of limited proteolysis on bovine kidney OGDC is quite analogous to bovine PDC. Various proteases preferentially attack the E2 component. The cleavage site, which may represent a hinge region between separate folding domains, apparently is located near the middle of the E2 chain (M_r 52 000 in SDS gel electrophoresis) thus giving fragments of app. M_r 29 500 and 27 000. The latter fragment can be isolated by sucrose density gra-

dient centrifugation and contains the active site for trans-succinylation as well as the E2 inter-subunit binding sites. This is very similar to bovine PDC where the E2 component can be cleaved, by a number of proteases, into a lipoyl fragment of M_r 28 600 [5] and an active fragment of $M_r \approx 26\,000$ [3–5]. However, the active OGDC-E2 fragment (band 4B) did not possess lipoate acetyltransferase activity (not shown).

PDC-E2 [14,15] and its lipoyl fragment [5] exhibit anomalous migration in SDS gel electrophoresis due to the acidic nature and swollen or extended structure of the fragment. Although earlier results are suggestive of similar anomaly for OGDC-E2 [16] this has not been clearly established. Thus, M_r of OGDC-E2 as well as its lipoyl fragment may have to be corrected.

Though the enzyme-bound [16] lipoyl residues, have not been localized in our experiments, it is most probable, by analogy with PDC, that the 29 500 M_r fragment (band 4A) represents the lipoyl domain of OGDC-E2. This domain can be imagined as an extension of the lipoyl-lysine flexible arm of the multienzyme complex [17]. Since lipoyl domains of similar size and similar structural properties now have been found in the E2 components of *Escherichia coli* PDC [17], bovine kidney [4,5] and heart [5] PDC, *E. coli* OGDC [5] and bovine kidney OGDC (this paper), this domain structure of the E2 component appears to be a general structural principle of the 2-oxo acid dehydrogenase complexes. Thus, the lipoyl domain performs the function of an 'acyl carrier protein' component within the multienzyme complex in a similar way as the acyl carrier protein components in fatty acid synthetases [18] or the carboxyl carrier proteins of biotin enzymes [19] which, in several cases, are also linked covalently to other components of the enzyme complexes [20,21].

Acknowledgements

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