Limited proteolysis of the pyruvate dehydrogenase multienzyme complex of *Bacillus subtilis*

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(Received 11 September 1984/Accepted 29 October 1984)

Limited digestion of the pyruvate dehydrogenase complex of *Bacillus subtilis* with either trypsin or chymotrypsin at 0°C inhibited its ability to decarboxylate pyruvate and 2-oxoisovalerate oxidatively, without causing disassembly of the complex. The proteinases selectively cleaved the E1 α subunits to form two fragments of M_r 31 500 and approx. 9500, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, both fragments remaining bound to the complex. Trypsin also caused a much slower cleavage of the E2 subunits, to form a fragment of apparent M_r 34 000. The inhibition of overall dehydrogenase-complex activity was accompanied by the apparent loss of the pyruvate-driven and 2-oxoisovalerate-driven E1 activities, which was found to be due to a large increase in the K_m for the 2-oxo acids: this change was correlated with the cleavage of the E1 α subunit.

The pyruvate dehydrogenase complex from *Bacillus subtilis* catalyses the oxidative decarboxylation of both pyruvate and 2-oxoisovalerate (Lowe *et al.*, 1983a), according to the following reaction:

It contains multiple copies of three enzymes that function successively in the mechanism: pyruvate decarboxylase (E1, EC 1.2.4.1), lipoate acetyltransferase (E2, EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3, EC 1.6.4.3), the E2 component forming a structural core to which the E1 and E3 components are bound tightly but non-covalently.

The pyruvate dehydrogenase complex from *B. subtilis* resembles that from *Bacillus stearothermophilus* and from mammalian sources, in that it contains multiple copies of four different types of polypeptide chain: E2, E3, E1 α and E1 β . The E2 component appears to comprise 60 copies of the E2 polypeptide chain arranged to form a structural core with icosahedral symmetry (Henderson *et al.*,

1979; Hodgson *et al.*, 1983). In these respects it differs from the complex from *Escherichia coli*, which only contains a single type of E1 subunit and is arranged around an E2 core of octahedral symmetry (Reed, 1974; Danson *et al.*, 1979).

Limited proteolysis has proved very useful in the study of the structure, assembly and enzymology of the 2-oxo acid dehydrogenase complexes. With the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes from *E. coli*, trypsin excises fragments of the E2 chains containing the lipoic acid moieties without causing disassembly (Hale & Perham, 1979; Bleile *et al.*, 1979; Perham & Roberts, 1981; Packman *et al.*, 1984). Similarly, the main effect of trypsin on the pyruvate dehydrogenase complex from *B. stearothermophilus* is cleavage of the E2 chains, but this is accompanied by loss of peripheral E1 and E3 subunits from the residual E2 core (Perham & Wilkie, 1980). However, treatment of the same complex with chymotrypsin cleaves lipoic acid-containing domains from the E2 chains, but disassembly does not ensue (Duckworth *et al.*, 1982). Trypsin also causes a slower cleavage of the E1 α chains (Perham & Wilkie, 1980). With mammalian pyruvate dehydrogenase complexes, the E1 α and E2 chains are again susceptible to limited proteolysis, but, in the complex from ox kidney, the E1 α chains are more susceptible than are the polypeptide chains of the E2 core (Kresze & Ronft, 1980; Kresze *et al.*, 1981).

Abbreviation used: SDS, sodium dodecyl sulphate.

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The pyruvate dehydrogenase and 2-oxoisovalerate dehydrogenase reactions appear to be catalysed by a single multienzyme complex in *B. subtilis* (Lowe *et al.*, 1983a). We have therefore subjected the *B. subtilis* complex to treatment with either trypsin or chymotrypsin to test further whether the pyruvate dehydrogenase and 2-oxoisovalerate dehydrogenase reactions can be separated and to compare the properties of this complex with those of the other 2-oxo acid dehydrogenase complexes previously studied.

Materials and methods

Enzymes and enzyme assays

Pyruvate dehydrogenase complex from *B. subtilis* was prepared and assayed as described elsewhere (Hodgson *et al.*, 1983; Lowe *et al.*, 1983a). E1 activity was measured by the reduction of 2,6-dichlorophenol-indophenol in the presence of 2 mM-pyruvate or 2 mM-2-oxoisovalerate (Lowe *et al.*, 1983b). E2 activity was measured by the conversion of acetyl phosphate and dihydrolipoic acid into acetyl-lipoic acid catalysed by phosphotransacetylase and the E2 component of the complex (Reed & Willms, 1966). E3 activity was measured by reduction of NAD⁺ in the presence of dihydrolipoamide (Hodgson *et al.*, 1983). 1-Chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated trypsin and α -chymotrypsin were from Worthington Biochemical Corp.

Limited proteolysis

Pyruvate dehydrogenase complex (1–5 mg/ml) was incubated with 1–5 μ g of trypsin or chymotrypsin

sin per mg of complex protein in 20 mM-sodium phosphate buffer, pH 7, containing 2 mM-EDTA at 0°C. At intervals, samples (2–4 μ l) were removed for assay of enzyme activity and other samples (15–20 μ l) were added to an equal volume of acetic acid. The latter were dried down *in vacuo* and then analysed by SDS/polyacrylamide-gel electrophoresis. For studies of the kinetics of trypsin-treated complex, soya-bean trypsin inhibitor was subsequently added at a ratio of 10 μ g/ μ g of trypsin.

Other techniques

Other techniques, such as ultracentrifugation and SDS/polyacrylamide-gel electrophoresis, were performed as described by Hodgson *et al.* (1983). Coomassie Blue-stained gels were scanned with a Transidyne 2955 densitometer connected to an Apple microcomputer for integration of peak areas.

Results

Limited proteolysis of *B. subtilis* pyruvate dehydrogenase complex

Incubation of the pyruvate dehydrogenase complex with either α -chymotrypsin (Fig. 1) or trypsin (Fig. 2) at a ratio of 1–5 μ g/mg of complex at 0°C led to inhibition of pyruvate dehydrogenase-complex activity and a selective cleavage of the E1 α chains. With chymotrypsin, during an incubation in which over 90% of the E1 α chains were proteolysed, no loss of E3, E2 or E1 β chains was observed (Fig. 1b). No changes in E3 or E2 activities were detected. Trypsin had effects similar to those of chymotrypsin except that some

Fig. 1. Digestion of pyruvate dehydrogenase complex (1 mg/ml) with chymotrypsin (5 μ g/ml) (a) Samples were assayed for pyruvate dehydrogenase-complex (●), 2-oxoisovalerate dehydrogenase-complex (▲), pyruvate-driven E1 (○) and 2-oxoisovalerate-driven E1 (△) activities. (b) Samples were added to acetic acid at the times indicated and analysed by SDS/polyacrylamide-gel electrophoresis (15% slab gel, Tris/glycine buffer). For comparison, a sample of trypsin-digested complex (cf. Fig. 2) was also run on the gel. The band of apparent M_r 9500 is not visible as it runs with the dye front on this system.

Fig. 2. Digestion of pyruvate dehydrogenase complex (1 mg/ml) with trypsin (1 µg/ml)

(a) Samples were assayed for pyruvate dehydrogenase-complex (●), 2-oxoisovalerate dehydrogenase-complex (▲), pyruvate-driven E1 (○) and 2-oxoisovalerate-driven E1 (△) activities. (b) Samples were added to acetic acid at the times indicated and analysed by SDS/polyacrylamide-gel electrophoresis (15% slab gel, Tris/glycine buffer). The band of apparent M_r 9500 is not visible as it runs with the dye front on this system. (c) The Coomassie Blue-stained gel was scanned at 550 nm to estimate the amounts of the polypeptide chains. The amounts of E1α (□), E1β (△), E2 (○) and the proteolytic fragment of M_r 31 500 (■) were normalized to a constant amount of E3 chain. For comparison, the pyruvate dehydrogenase-complex activity (●) is shown.

loss of E2 chains occurred, although this was still much slower than the cleavage of E1α chains (Figs. 2b and 2c).

With both proteinases, cleavage of the E1α chains (apparent M_r 42 500) was associated with the appearance of two new Coomassie Blue-staining bands on SDS/polyacrylamide-gel electrophoresis with apparent M_r of about 31 500 and 9500 respectively. These two bands were formed at about the same rate as the E1α band was lost (cf. Fig. 2b). The E2 chain was cleaved by trypsin to yield a faint band of M_r 34 000.

B. subtilis pyruvate dehydrogenase complex also catalyses the oxidative decarboxylation of branched-chain 2-oxo acids, such as 2-oxoisovaleric acid (Lowe *et al.*, 1983a). During proteolysis

of the complex, dehydrogenase-complex activities with pyruvate and 2-oxoisovalerate as substrates were lost at the same rate (Figs. 1 and 2). The effects of chymotrypsin (Fig. 1a) and of trypsin (Fig. 2a) on the pyruvate-driven and 2-oxoisovalerate-driven E1 activities were measured separately. The 2-oxoisovalerate-driven E1 activity fell in parallel with the loss of pyruvate-driven and 2-oxoisovalerate-driven dehydrogenase-complex activities. However, even after prolonged incubation with proteinase, inhibition of the pyruvate-driven E1 activity reached a maximum of about 30–50%, whereas the 2-oxoisovalerate-driven E1 activity was inhibited by more than 90%. This result appeared difficult to reconcile with the conclusions of Lowe *et al.* (1983a) that the two activities are

catalysed by the same enzyme, and so we examined it in more detail, as follows.

Apparently different effects of proteinases on pyruvate-driven and 2-oxoisovalerate-driven E1 reactions

Trypsin-treated enzyme complex (84% inhibited) displayed non-linear kinetics in that the double-reciprocal plot of E1 activity against pyruvate concentration was curved downwards at high substrate concentration. A satisfactory fit to the data could be obtained by assuming that 16% of the enzyme was non-proteolysed and was behaving with native $V_{\max}$ and K_m and that 84% of the enzyme was proteolysed and now displayed a near-normal $V_{\max}$, but a high K_m . On this assumption, in the E1 reaction, native enzyme has a $K_{m(\text{app})}$ for pyruvate of about 27 μM , whereas trypsin-cleaved enzyme has a $K_{m(\text{app})}$ for pyruvate of about 600 μM . Similar results were obtained with the chymotrypsin-cleaved enzyme.

In contrast, the double-reciprocal plots for the 2-oxoisovalerate-driven E1 activities of native and proteolysed enzyme complexes were linear (at least in the substrate range 0.7–28 mM), with an unchanged K_m (approx. 3.5 mM) for 2-oxoisovalerate. Proteolysis appears simply to lower the $V_{\max}$ for the 2-oxo acid.

These results suggest an explanation for the apparently different sensitivities of the two E1 reactions to proteinases. At the substrate concentration used in the standard assay (2 mM), the pyruvate-driven E1 reaction of native enzyme complex is saturated with substrate. In contrast, the proteolysed enzyme complex has an elevated K_m for pyruvate and is therefore assayed under non-saturating conditions. Since the $V_{\max}$ of proteolysed enzyme is little altered, a partial inhibition of pyruvate-driven E1 activity will be seen. For the 2-oxoisovalerate-driven E1 reaction, the standard assay is not carried out at saturating substrate concentration even with the native enzyme complex, and any increase in K_m will therefore cause a corresponding decrease in the measured enzyme activity. If the K_m for 2-oxoisovalerate were raised by the same factor as that for pyruvate (about 22-fold), the K_m for the proteolysed enzyme would be about 80 mM and hence not measurable within the useful range of substrate concentrations available. The branched-chain-oxo acid-driven E1 activity would thus appear to be inhibited to a greater extent than the pyruvate-driven E1 activity when measured at a single fixed substrate concentration, e.g. 2 mM. However, when measured at low substrate concentration, where enzyme activity is proportional to substrate concentration, both activities should be equally inhibited. This was found to be the case (Table 1). Furthermore, when the data in Figs. 1(a)

Table 1. E1 activity of trypsin-treated enzyme complex at various concentrations of 2-oxo acid

Concn. of 2-oxo acid in assay (mM)	E1 activity (% of activity of untreated enzyme)	
	Pyruvate- driven E1 activity	2-Oxoisovalerate- driven E1 activity
0.01	18	16
2	55	16
14	85	18

and 2(a) are replotted assuming that the maximum extent of inhibition of pyruvate-driven E1 activity is 40%, the plots of loss of activity of pyruvate-driven and 2-oxoisovalerate-driven E1 reactions can be superimposed on each other.

A similar explanation could account for the apparently different effects of trypsin and chymotrypsin on the pyruvate-driven E1 and overall complex activities, for which K_m^{Pyr} of E1 = 0.023 mM and K_m^{Pyr} of pyruvate dehydrogenase complex = 0.33 mM in the non-proteolysed enzymes (Lowe *et al.*, 1983a).

Quaternary structure of the proteolysed complex

After proteolysis with chymotrypsin, the pyruvate dehydrogenase complex (retaining 15% overall catalytic activity) was subjected to ultracentrifugation for 3.5 h at 40000 rev./min in a Beckman SW50.1 rotor, and 80–90% of the residual overall complex and E1 activities were recovered in the yellow pellet. Examination of the supernatant and pellet by means of SDS/polyacrylamide-gel electrophoresis revealed little protein in the supernatant and showed that the pellet contained both proteolytic fragments of the E1 α chain. This implies that the E2 core was still assembled in the form of a complex of high M_r , and that cleavage of the E1 α subunit did not affect the ability of the resulting fragments to bind to the complex. Identical conclusions were reached when the proteolysed complex was analysed by means of gel filtration on a column of Sephadex G-75.

Discussion

The pyruvate dehydrogenase complexes from *B. subtilis* and *B. stearothermophilus* differ somewhat in their response to limited proteolysis. The prime effect of trypsin on the *B. stearothermophilus* complex is cleavage of the E2 polypeptide chains, with accompanying loss of peripheral E1 and E3 subunits from the inner core and a slower cleavage of E1 α chains (Perham & Wilkie, 1980). With the enzyme complex from *B. subtilis*, however, E1 α chains are cleaved preferentially and there is no

disassembly. Similarly, the two complexes behave differently when exposed to chymotrypsin: with the *B. stearothermophilus* complex, cleavage of E2 chains predominates (Duckworth *et al.*, 1982), whereas with the *B. subtilis* complex cleavage only of E1 α chains occurs. The two complexes are alike in that the E3 and E1 β chains are extremely resistant to proteolysis.

In all these regards, the *B. subtilis* pyruvate dehydrogenase complex more closely resembles that from ox kidney (Kresze & Ronft, 1980) than that from *B. stearothermophilus*, suggesting that it may be a better model for the mammalian enzyme in some ways.

We have shown that the K_m for pyruvate in the pyruvate-driven E1 reaction of the *B. subtilis* complex is increased some 20-fold after the E1 α chains are cleaved by trypsin or chymotrypsin. It is likely that the same effect occurs with the 2-oxoisovalerate-driven E1 reaction, but this could not be verified since the K_m for 2-oxoisovalerate is already so high that the concentrations of substrate required to test this had adverse effects on the enzyme activity. However, proteolysis of the E1 α chains is associated with simultaneous inhibitory effects on the pyruvate-driven and 2-oxoisovalerate-driven E1 activities. This is good evidence that the E1 α chains are shared by both pyruvate dehydrogenase-complex and 2-oxoisovalerate dehydrogenase-complex activities. We have already demonstrated that the E2 and E3 subunits are shared by these two activities (Lowe *et al.*, 1983a). Thus the only polypeptide chain that could now differ between them is E1 β .

We are grateful to the Science Research Council for a research grant to R. N. P., and to the Medical Research Council for a research studentship to J. A. H.

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