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Wild-type and mutant forms of the pyruvate dehydrogenase multienzyme complex from *Bacillus subtilis*

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A simple procedure is described for the purification of the pyruvate dehydrogenase complex and dihydrolipoamide dehydrogenase from *Bacillus subtilis*. The method is rapid and applicable to small quantities of bacterial cells. The purified pyruvate dehydrogenase complex ($s^0_{20,w} = 73$ S) comprises multiple copies of four different types of polypeptide chain, with apparent M_r values of 59500, 55000, 42500 and 36000: these were identified as the polypeptide chains of the lipoate acetyltransferase (E2), dihydrolipoamide dehydrogenase (E3) and the two types of subunit of the pyruvate decarboxylase (E1) components respectively. Pyruvate dehydrogenase complexes were also purified from two *ace* (acetate-requiring) mutants of *B. subtilis*. That from mutant 61142 was found to be inactive, owing to an inactive E1 component, which was bound less tightly than wild-type E1 and was gradually lost from the E2E3 subcomplex during purification. Subunit-exchange experiments demonstrated that the E2E3 subcomplex retained full enzymic activity, suggesting that the lesion was limited to the E1 component. Mutant 61141R elaborated a functional pyruvate dehydrogenase complex, but this also contained a defective E1 component, the K_m for pyruvate being raised from 0.4 mM to 4.3 mM. The E1 component rapidly dissociated from the E2E3 subcomplex at low temperature (0–4°C), leaving an E2E3 subcomplex which by subunit-exchange experiments was judged to retain full enzymic activity. These *ace* mutants provide interesting opportunities to analyse defects in the self-assembly and catalytic activity of the pyruvate dehydrogenase complex.

Pyruvate dehydrogenase multienzyme complexes have been isolated from a wide range of organisms, and all have been found to contain multiple copies of three different enzymes: 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). Those obtained from Gram-negative bacteria, such as *Escherichia coli* (Reed, 1974), *Salmonella typhimurium* (Seckler *et al.*, 1982) and *Azotobacter vinelandii* (Bosma *et al.*, 1982), are composed of three different types of polypeptide chain corresponding to the three different enzymic activities and are assembled round an E2 core of octahedral symmetry (Reed, 1974; Danson *et al.*, 1979). On the other hand, those from Gram-positive bacteria, such as *Bacillus* (Henderson *et al.*, 1979; Visser *et al.*, 1980), and from mitochondria (Reed, 1974; Keha *et al.*, 1982) are composed of four different types of polypeptide chain, the E1 activity residing in E1α and E1β components, and are assembled round an E2 core of icosahedral symmetry.

Purification procedures have been described for the pyruvate dehydrogenase complex from *B. stearothermophilus* (Henderson *et al.*, 1979; Henderson & Perham, 1980) and from *B. subtilis* (Visser *et al.*, 1980). We found the former procedure to be inapplicable to the enzyme from *B. subtilis*, whereas the latter appears to give extremely low yields of enzyme per g of bacterial cell paste. In the present paper we describe a new purification procedure for the *B. subtilis* enzyme complex that is rapid and simple and gives a 30-fold higher yield than the earlier method. It also permits the purification of *B. subtilis* dihydrolipoamide dehydrogenase.

Freese & Fortnagel (1969) isolated two *ace* mutants of *B. subtilis* that were able to grow on a glucose minimal medium only in the presence of supplementary acetate. The lesions were found to be in the pyruvate dehydrogenase complex, but the sites of mutation were not further studied. Our new purification procedure for the *B. subtilis* enzyme has enabled us to characterize the pyruvate dehydrogenase complexes produced by these *ace* mutants

and to identify the lesions as occurring in the E1 component. This is an important step forward in our understanding of the structure and mechanism of the *Bacillus* pyruvate dehydrogenase complex and in the correlation of gene and protein structure that we are currently undertaking.

Materials and methods

Reagents

N-Ethyl[2,3-^{14}C]maleimide was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Lysozyme chloride (egg white, grade VI) and ribonuclease A (ox pancreas, Type 1-AS) were from Sigma. Deoxyribonuclease I (bovine pancreas, grade II) was obtained from Boehringer Mannheim, and soya-bean trypsin inhibitor from BDH.

Bacterial strains

B. subtilis strain 168 (auxotrophic marker *trp*C2) was kindly given by Dr. P. Oliver (Department of Genetics, University of Cambridge), and *B. subtilis* mutants 61141 and 61142 (auxotrophic markers *trp*C2, *met*C7, *ace*A) were generously given by Dr. E. Freese (National Institutes of Health, Bethesda, MD, U.S.A.). Growth of mutant 61142 on glucose minimal medium was fully dependent on acetate as originally described (Freese & Fortnagel, 1969). However, moderate growth of mutant 61141 was obtained on glucose minimal medium, although this growth was enhanced by addition of 10mM-acetate. The values of A_{650} at stationary phase for growth on liquid glucose minimal medium were 0.05, 0.8 and 2.5 for mutants 61142, 61141 and wild type respectively. We noted that subculture of mutant 61142 from anything except freshly grown plates produced many revertants fully and partly independent of acetate for growth on glucose minimal medium. It is likely that the mutant 61141 that we have used in these studies is a partial revertant, and we have designated it 61141R to distinguish it from the mutant 61141 originally described (Freese & Fortnagel, 1969).

Growth of cells

B. subtilis 168 *trp*$^-$ was routinely subcultured on Tryptose–blood agar base (Difco) and the *ace* mutants were cultured on the same medium supplemented with 10mM-acetate. Subculture of *ace* mutants from aged plates was avoided to minimize selection for revertants. Working stocks of nutritionally checked cultures of *ace* mutants were kept frozen at −70°C with 10% glycerol added. Auxotrophic requirements were determined on 0.5% (w/v) glucose minimal agar made with Spizizen salts (Anagnostopoulos & Spizizen, 1961). Liquid glucose minimal medium was made in the same way, but without agar.

To obtain cells as a source of pyruvate dehydrogenase complex, *B. subtilis* 168 was grown in L-broth [10g of Bacto-tryptone (Difco), 5g of yeast extract (Difco) and 5g of NaCl per litre of distilled water] and the *ace* mutants were grown in L-broth supplemented with 10mM-acetate. Pre-warmed medium (1 litre in 2-litre conical flasks) was inoculated to A_{650} of 0.05 with an exponentially growing culture of the required bacteria and incubated at 37°C with vigorous aeration to late exponential phase (A_{650} of 1.0–1.5). Samples were checked for purity and auxotrophic requirements. The grown cultures were placed in ice and harvested within 3h by centrifugation at 4°C at 8000*g* (Sorvall GS3 rotor, 7000rev./min) for 15min. The cell paste was stored at −20°C. Wild-type *B. subtilis* and the *ace* mutants gave approx. 3g and 2g of wet cell paste per litre of medium respectively.

On one occasion when a culture of wild-type cells was allowed to grow for 2h into stationary phase, a low amount only of pyruvate dehydrogenase complex activity was released by sonication. This activity was also unstable.

Enzyme assays

Pyruvate dehydrogenase complex activity, as the reduction of NAD^+ in the presence of pyruvate, and dihydrolipoamide dehydrogenase activity, as the reduction of NAD^+ in the presence of dihydrolipoamide, were assayed as described by Danson *et al.* (1978). The activity of E1 was assayed as the reduction of 2,6-dichlorophenol-indophenol in the presence of pyruvate, as described by Lowe *et al.* (1983). One unit of pyruvate dehydrogenase complex or dihydrolipoamide dehydrogenase activity was taken as the amount of enzyme required to reduce 1 μmol of NAD^+/min.

Protein determination

In the cell-free extracts, protein was determined by the biuret method (Gornall *et al.*, 1949). At other stages, protein was determined by precipitation with trichloroacetic acid followed by a modified Lowry procedure (Peterson, 1977).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Proteins were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in phosphate-buffered tube and slab gels (Shapiro *et al.*, 1967) or Tris/glycine-buffered slab gels (Anderson *et al.*, 1973). Proteins used as markers, with apparent M_r values, were: bovine serum albumin (68000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36000), *E. coli* pyruvate decarboxylase component (E1) (100000), *E. coli* dihydrolipoamide dehydrogenase (E3) (56000).

Inhibition of pyruvate dehydrogenase complex with N-ethyl[2,3-^{14}C]maleimide

To identify the lipoate acetyltransferase (E2) component, the pyruvate dehydrogenase complex from *B. subtilis* was treated with *N*-ethyl[2,3-^{14}C]maleimide (10 Ci/mol) in the presence of pyruvate, as described for the *E. coli* complex (Brown & Perham, 1976) and the *B. stearothermophilus* complex (Henderson *et al.*, 1979). The treated enzyme complex was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 5% (w/v)-acrylamide phosphate-buffered tube gels. After electrophoresis, the tube gels were sliced into 1 mm segments and each segment was counted for ^{14}C radioactivity in an LKB RackBeta scintillation counter, as described previously (Brown & Perham, 1976).

Subunit-exchange experiments

B. subtilis pyruvate dehydrogenase complex (0.9 mg/ml) was incubated with 0.67 mM-sodium pyruvate in a buffer containing 50 mM-potassium phosphate, 0.2 mM-thiamin pyrophosphate, 1 mM-$MgCl_2$ and 0.2 mM-*N*-ethylmaleimide, pH 7.0, at 0°C under N_2. After 60 min the pyruvate dehydrogenase complex activity was <2% of its starting value, the E3 activity was unchanged, and the E1 activity had only fallen by <10%. 2-Mercaptoethanol (final concn. 20 mM) was then added and the mixture was immediately gel filtered by a centrifuge gel-filtration procedure (Penefsky, 1977), in which the Sephadex G-25 (fine grade) was equilibrated with 50 mM-potassium phosphate/1 mM-$MgCl_2$/0.2 mM-thiamin pyrophosphate, pH 7.0. The eluted enzyme was diluted with the same buffer for subunit-exchange experiments.

Other techniques

Electron microscopy and analytical ultracentrifugation were performed as described by Henderson *et al.* (1979).

Results

Purification of pyruvate dehydrogenase complex from B. subtilis

All operations were performed at 0–4°C, unless otherwise stated.

(1) *Preparation of cell-free extract. B. subtilis* cell paste was suspended (1 ml of buffer added to 0.5 g of cell paste) in 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-EDTA, 0.15 mM-phenylmethanesulphonyl fluoride, lysozyme (6 mg/ml) and deoxyribonuclease I (5 μg/ml) and stirred for 2 h. Centrifugation for 15 min at 15000 *g* (Sorvall SS34, 11000 rev./min) yielded a large pellet and a yellow supernatant that contained no pyruvate dehydrogenase complex activity. The pellet was washed by suspending it in 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-EDTA and 0.15 mM-phenylmethanesulphonyl fluoride and then centrifuging again. Both supernatants were discarded.

The pellet was resuspended in the starting volume of 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-EDTA, 0.15 mM-phenylmethanesulphonyl fluoride and 5 μg of deoxyribonuclease I/ml. The suspension was sonicated in an ice/water bath for periods of 30 s with a Dawe Soniprobe type 7532A at an output of 80–100 W. Intervals of 5 min were allowed between periods of sonication to maintain the temperature of the suspension between 0 and 10°C. After each sonication, a sample (0.1 ml) of the suspensions was centrifuged in an Eppendorf micro-centrifuge at 10000 *g* for 2 min. The supernatant was assayed for pyruvate dehydrogenase activity. When this reached its maximum value (after two to five sonication periods), the whole extract was centrifuged for 1 h at 50000 *g* (Beckman SW27 rotor, 20000 rev./min). The yellow cell-free extract containing pyruvate dehydrogenase complex activity was retained; the small pellet was discarded.

(2) *Ammonium sulphate fractionation.* The protein concentration in the extract was adjusted to 10 mg/ml by adding 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-EDTA. The extract was stirred while solid $(NH_4)_2SO_4$ was gradually added to 45% saturation (277 g/l) and the pH was maintained at 7.0 by adding 0.75 M-NaOH. After 30 min stirring, the white precipitate was removed by centrifugation for 15 min at 12000 *g* (Sorvall SS34 rotor, 10000 rev./min). The supernatant retained almost all the pyruvate dehydrogenase complex activity.

The saturation of $(NH_4)_2SO_4$ in the supernatant was further increased by three increments of 5% each (33 g/l), as above. After each increase the suspension was stirred for 30 min and the precipitate collected by centrifugation. When the supernatants were assayed for pyruvate dehydrogenase complex activity, it was found that 85–90% of the enzyme activity had precipitated in two of the 5% cuts. The two corresponding precipitates were resuspended in 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-EDTA, and pooled in a total volume 0.15 that of the starting cell-free extract; resuspension was immediate, yielding a clear yellow solution with >80% recovery of enzymic activity. The solution was dialysed overnight against the same buffer, and a slight activation of the enzyme was usually observed at this stage.

For small-scale preparations, when less than 10 ml of cell-free extract was available, $(NH_4)_2SO_4$ was added as a saturated solution in 50 mM-sodium phosphate buffer/5 mM-EDTA, adjusted to pH 7.0.

Ammonium sulphate fractionation could be postponed to be the final stage of purification without

Table 1. *Summary of the purification of pyruvate dehydrogenase complex*
The 6mg of pyruvate dehydrogenase complex was isolated from 6.4g of *B. subtilis* 168 *trp*C2 cell paste, as described in the text, with a recovery of 53%.

Step	Volume (ml)	Total protein (mg)	Pyruvate dehydrogenase complex: Total activity (units)	Pyruvate dehydrogenase complex: Specific activity (units/mg)	Total dihydrolipoamide dehydrogenase activity (units)
1. Cell-free extract	18	360	91*	0.3	248*
2. Resuspended $(NH_4)_2SO_4$ precipitate	2.7	80	110	1.4	240
3. Ultracentrifugation pellet after dialysis	3.0	13	73	5.8	175
4. Concentrated Sepharose-2B peak	0.3	6	48	8.0	115

* The rates of NAD^+ reduction in the cell-free extract were non-linear owing to the presence of NADH oxidases. The measured rates may therefore be an underestimate of the true amount of enzyme present at this stage.

change in the specific activity or yield of enzyme. If this step is postponed, it is advisable to treat the cell-free extract with ribonuclease (step 5 below) before ultracentrifugation.

(3) *Ultracentrifugation.* The dialysed enzyme solution was diluted to 5–10mg of protein/ml and layered on top of a solution of sucrose (12.5%, w/v) that rested in turn on a cushion of sucrose (70%, w/w) that was about 10mm deep at the bottom of a centrifuge tube. All solutions were made up to 50mM-sodium phosphate buffer/5mM-EDTA, pH7.0. Centrifugation was performed for 5h at 90000*g* (Beckman SW27 rotor, 26000rev./min) or for 3–4h at 150000*g* (Beckman 42.1 rotor, 40000rev./min). A brown protein band at the interface of the 12.5%- and 70%-sucrose layers was drawn off and found to contain >90% of the pyruvate dehydrogenase complex activity originally loaded. No pyruvate dehydrogenase complex activity was found in either the 12.5%-sucrose or the buffer layers, but dihydrolipoamide dehydrogenase activity (20–50% of that loaded) was found there. This low-molecular-weight dihydrolipoamide dehydrogenase activity is presumably uncomplexed enzyme [cf. Lusty & Singer (1964)]. Sucrose was removed from the enzyme samples by dialysis for 2h against 50mM-sodium phosphate buffer/5mM-EDTA, pH7.0.

(4) *Gel filtration.* The enzyme complex was next loaded on to a column of Sepharose CL-2B, bed volume approx. 200 × the sample volume, and eluted with 20mM-sodium phosphate buffer/2mM-EDTA, pH7.0. An opalescent contaminant was excluded from the matrix and the pyruvate dehydrogenase complex emerged later approximately halfway between V_0 and V_t. Fractions containing pyruvate dehydrogenase complex activity were pooled and concentrated either by $(NH_4)_2SO_4$ precipitation [70% saturation (472g/l) with solid $(NH_4)_2SO_4$ at pH7.0 as above] or by ultracentrifugation for 3h at 150000*g* (Beckman 42.1 rotor, 40000rev./min).

The clear yellow solution of enzyme, in the minimum volume of 50mM-sodium phosphate buffer/5mM-EDTA, pH7.0 could be frozen rapidly at −70°C, and then stored at −20°C for several months without loss of activity.

(5) *Ribonuclease treatment.* The enzyme complex was usually pure after the gel-filtration step, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1), but occasionally it was contaminated with a species that had a high A_{260}/A_{280} ratio and gave multiple bands on gel electrophoresis. This was probably 70S ribosomes and could be removed by treatment with ribonuclease before concentrating the enzyme-containing fractions from the Sepharose CL-2B column, as follows.

Ribonuclease (1mg/ml in water) was boiled for 7min and then cooled at 4°C for 12h. It was added to the pooled column fractions at a final concentration of 32μg/ml. The mixture was kept at 10–15°C for 70min, during which time a fine white precipitate formed. This was removed by centrifugation for 25min at 30000*g* (Beckman 42.1 rotor at 20000rev./min). The supernatant was decanted and the enzyme complex was concentrated as described above.

A typical summary of the purification is shown in Table 1. The overall recovery of enzymic activity is about 50–60%, and the final specific catalytic activity of the enzyme is about 8–12 units/mg in the NAD^+-reduction assay and about 14–20 units/mg in the E3 assay.

Purification of dihydrolipoamide dehydrogenase from B. subtilis

Dihydrolipoamide dehydrogenase from *B. subtilis* could be purified from the supernatants obtained from the ultracentrifugation through 12.5% sucrose (step 3) used to purify the pyruvate dehydrogenase complex. The dihydrolipoamide dehydrogenase was concentrated from this solution by adding

$(NH_4)_2SO_4$ to 75% saturation, as described above (step 2). The yellow precipitate was collected by centrifugation for 15 min at 14000 *g* and dissolved in 0.05 the initial volume of 20 mM-sodium phosphate buffer/5 mM-EDTA, pH 7.0. After dialysis against the same buffer, the enzyme solution was treated with trypsin (final concn. 0.1 mg/ml) for 3 h at 22°C. The resistance of dihydrolipoamide dehydrogenase to proteolysis has been used previously to facilitate its purification from *E. coli* (Brown & Perham, 1972) and *B. stearothermophilus* (Henderson *et al.*, 1979). The trypsin treatment led to no loss of E3 activity; the trypsin was then inhibited by adding soya-bean trypsin inhibitor (final conc. 70 μg/ml).

The trypsin-treated sample (1.2 ml) was fractionated on a column of Sephacryl S-200 (37 cm × 1 cm) equilibrated with the same buffer and the dihydrolipoamide dehydrogenase activity was eluted as a symmetrical peak ($V_0 = 5$ ml, $V_e = 13$ ml) at a position consistent with an M_r of about 110000. Fractions containing E3 activity were pooled and concentrated by $(NH_4)_2SO_4$ precipitation as above, except that the enzyme was redissolved in and dialysed against 10 mM-potassium phosphate buffer, pH 7.0. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed the presence of one major band, apparent M_r about 55000, and several minor bands. The latter could be removed by passing the enzyme preparation through a column of spheroidal hydroxyapatite (2 cm × 1.5 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. The E3 activity was not retained by the column and emerged free of other proteins, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The specific catalytic activity of pure E3 was 125 units/mg of protein.

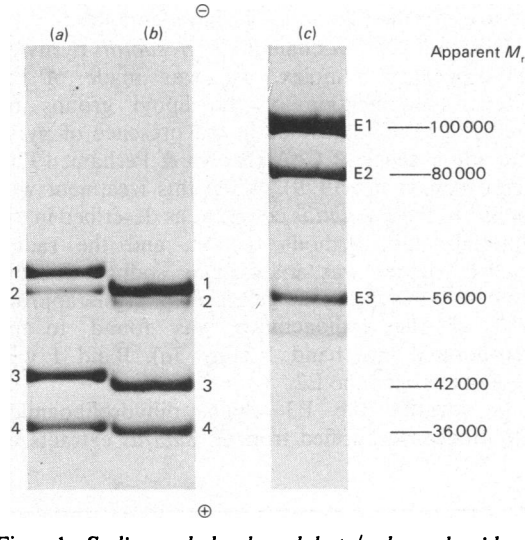

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of pyruvate dehydrogenase complexes from (a) B. subtilis, (b) B. stearothermophilus and (c) E. coli*

Samples of purified complexes were analysed by electrophoresis in phosphate-buffered sodium dodecyl sulphate/7.5% (w/v)-polyacrylamide gel. For full details see the text.

Molecular properties of the pyruvate dehydrogenase complex from B. subtilis

As expected from previous work on the pyruvate dehydrogenase complex from *B. stearothermo-*

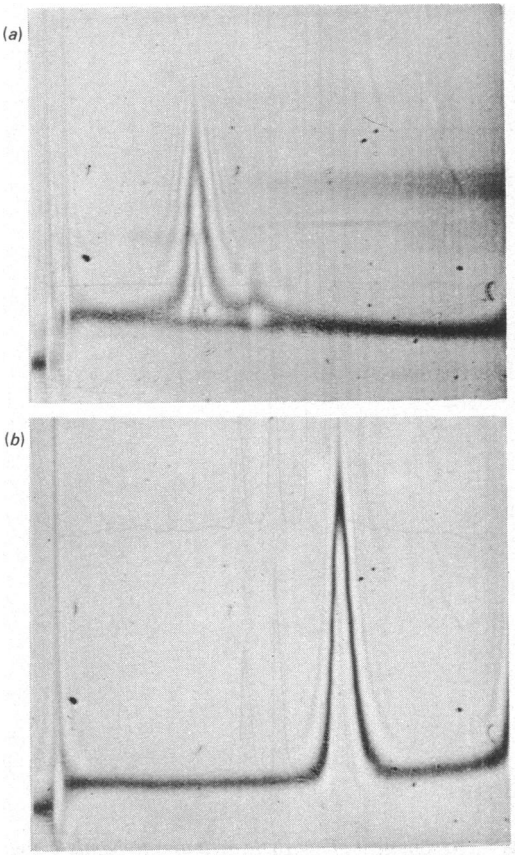

Fig. 2. *Ultracentrifugation of pyruvate dehydrogenase complex from wild-type B. subtilis and of E2E3 subcomplex from mutant 61141R*

Sedimentation was from left to right, at 24630 rev./min, in a Beckman model E ultracentrifuge. The buffer was sodium potassium phosphate, pH 7.0, containing 0.05 M-NaCl and 1 M-EDTA ($I = 0.1$ M). (*a*) Pyruvate dehydrogenase complex from wild-type *B. subtilis* (approx. 2 mg/ml; 18°C); (*b*) E2E3 subcomplex from mutant 61141R (approx. 2 mg/ml, 16°C).

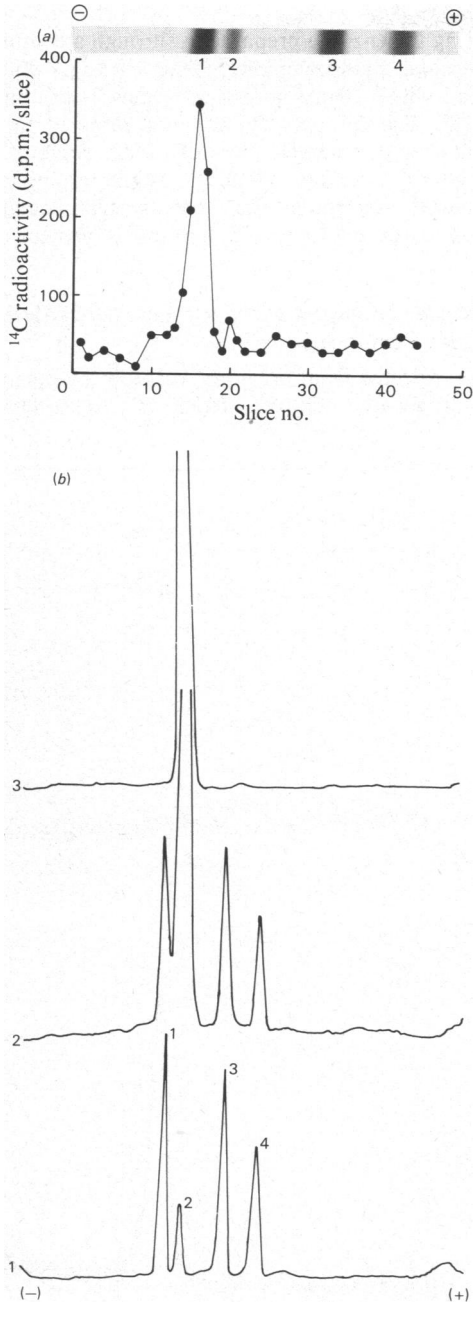

Fig. 3. *Assignment of catalytic activities to bands 1 and 2*

(*a*) Pyruvate dehydrogenase complex from *B. subtilis* (1.5 mg/ml) was incubated with *N*-ethyl[2,3-^{14}C]maleimide (0.5 mM, 10 Ci/mol) and sodium pyruvate (0.25 mM) at 30°C under N_2 in a buffer consisting of 40 mM-sodium phosphate, pH 7.0, 0.8 mM-NAD^+, 8 mM-$MgCl_2$ and 0.8 mM-thiamin pyrophosphate. The inhibited enzyme was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the gel was sliced and counted for ^{14}C radioactivity, as described in the Materials and methods section. (*b*) Pyruvate dehydrogenase complex and dihydrolipoamide dehydrogenase from *B. subtilis* were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the Coomassie Blue-stained gels were scanned in a Perkin–Elmer model 555 spectrophotometer at 550 nm. Track 1, enzyme complex; track 2, mixture of enzyme complex and dihydrolipoamide dehydrogenase; track 3, dihydrolipoamide dehydrogenase.

philus (Henderson *et al.*, 1979) and *B. subtilis* (Visser *et al.*, 1980), the *B. subtilis* enzyme purified by our procedure showed four protein bands when subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). The pattern was similar to, but not identical with, that observed for the *B. stearothermophilus* enzyme (Henderson & Perham, 1980) and was quite different from the three-band pattern of the *E. coli* complex (Fig. 1), the apparent M_r values for which are 100000, 80000 and 56000 [Danson *et al.* (1979) and references therein]. The apparent M_r values for the *B. subtilis* proteins estimated on phosphate-buffered 7.5% polyacrylamide gels were: band 1, 59500; band 2, 55000; band 3, 42500; band 4, 36000. On a Tris/glycine-buffered system (12.5% or 15% acrylamide) somewhat different M_r values were estimated: 66500, 63500, 42500 and 38000 for bands 1–4 respectively.

The value of $s^0_{20,w}$ for the *B. subtilis* complex was estimated to be 73S. At higher protein concentrations, we observed a second, smaller peak (Fig. 2*a*) that sedimented more rapidly ($s^0_{20,w} = 106$S). This value is consistent with the minor peak being a dimer of the major peak, as observed also for the pyruvate dehydrogenase complexes from *E. coli* (Danson *et al.*, 1979) and *B. stearothermophilus* (Henderson *et al.*, 1979).

In the electron microscope, negatively stained images of the *B. subtilis* complex resembled those of the *B. stearothermophilus* enzyme (Henderson *et al.*, 1979), suggestive of the icosahedral symmetry reported for the E2 core of the latter complex.

To identify the E2 chain in the *B. subtilis* pyruvate dehydrogenase complex, use was made of the selective modification of the lipoyl groups by *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate and absence of CoA (Brown & Perham, 1976; Henderson *et al.*, 1979). When this treatment was applied to the *B. subtilis* complex, as described in the Materials and methods section, and the radiolabelled enzyme was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, approx. 95% of the radioactivity was found to be incorporated into band 1 (Fig. 3*a*). Band 1 was therefore assigned to E2.

To identify the E3 chain, dihydrolipoamide dehydrogenase purified from *B. subtilis* extracts as

described above was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in parallel with a sample of *B. subtilis* pyruvate dehydrogenase complex (Fig. 3*b*). It was found to co-migrate with band 2 of the enzyme complex, implying that band 2 should be assigned to E3.

By inference, the remaining two bands for the *B. subtilis* enzyme, apparent M_r values of 42500 and 36000 (Fig. 1*a*) are tentatively assigned to E1α and E1β chains, in keeping with the structure of the mitochondrial pyruvate dehydrogenase complexes (Reed, 1974). The banding pattern of the *B. subtilis* complex therefore resembles that of the *B. stearothermophilus* enzyme (Henderson *et al.*, 1979) in structure and in function.

A steady-state kinetic analysis of the pyruvate dehydrogenase complex reaction gave a value of K_m for pyruvate of 0.4 ± 0.1 mM and $V_{max.}$ of about 12 units/mg, in the presence of NAD^+ (2.5 mM) and CoA (0.13 mM).

Purification of pyruvate dehydrogenase complex from B. subtilis ace mutant 61142

Freese & Fortnagel (1969) showed that the acetate-requiring *B. subtilis* mutant 61142 is highly deficient in overall pyruvate dehydrogenase complex activity. We assayed sonicated cells of mutant 61142 and found no overall pyruvate dehydrogenase complex activity and no E1 activity. On the other hand, E3 activity was readily detected at a value significantly above that of wild-type *B. subtilis* 168. As with the wild type, ultracentrifugation of the extracts from the mutant produced a yellow pellet that contained about half of the dihydrolipoamide dehydrogenase activity, implying the existence of an assembled E2 core to which this E3 was bound.

We therefore purified this putative complex containing E3, by using the dihydrolipoamide dehydrogenase activity as a marker, and applying the purification procedure described above for native *B. subtilis* complex with a modified order of steps. The purification was carried out as rapidly as possible and the fractionation with $(NH_4)_2SO_4$ was deferred until the end. This was to lessen the risk of the complex losing peripheral subunits before they could be identified by gel electrophoresis, since it was found that the mutation affected E1 binding to the core (see below). Pyruvate dehydrogenase complex from *B. subtilis* 168 *trp*C2 was also readily purified by this modified procedure.

After purification of the E3-containing complex by ultracentrifugation (step 3 of Table 1), sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that bands corresponding to all four subunits of the normal pyruvate dehydrogenase complex were present. The ratio of E1α and E1β subunits to E2 was similar to that of wild-type enzyme, but the ratio of E3 to E2 was higher than normal. The existence of an assembled but inactive pyruvate dehydrogenase complex, defective in E1 or E2 or both, was therefore thought likely. At the next step of purification, gel filtration on Sepharose CL-2B (step 4 of Table 1), the enzyme complex was eluted as a single symmetrical peak in the same position as wild-type enzyme, confirming its high M_r, but there appeared to have been a small loss of E1α and E1β subunits, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

When the eluted enzyme was subjected to $(NH_4)_2SO_4$ fractionation, E3 activity was precipitated both in the range 0–30% saturation and in the range 40–50% saturation. The 0–30% cut showed little change in the ratio of E1 : E2 chains, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, whereas the 40–50% cut showed a significant loss of E1 subunits. Re-precipitation with $(NH_4)_2SO_4$ led to almost total loss of E1 subunits, to form an E2E3 subcomplex (Fig. 4), and the latter was completely precipitable by $(NH_4)_2SO_4$ in the range 40–50% saturation. Treatment of wild-type pyruvate dehydrogenase complex with $(NH_4)_2SO_4$ caused no detectable loss of E1 chains.

To ascertain whether the dissociation of E1 subunits from the E2 core during the purification of pyruvate dehydrogenase complex from *B. subtilus* mutant 61142 was due to a mutation in the E1 subunits or in the E2 chains of the core, a subunit-exchange experiment was performed. Wild-type *B. subtilis* complex was inactivated by treatment with *N*-ethylmaleimide in the presence of pyruvate as described in the Materials and methods section. The resultant enzyme had 1.8% of the control complex activity, whereas the E1 and E3 activities were diminished by <10%. The inactivated complex was incubated with E2E3 subcomplex prepared from *B. subtilis* mutant 61142, which had no measurable E1 or overall complex activity. As shown in Fig. 5, pyruvate dehydrogenase complex activity gradually appeared in the mixture, rising to the value expected if active E1 subunits from the *N*-ethylmaleimide-treated complex were able to dissociate from this complex and associate with E2E3 subcomplex from *B. subtilis* mutant 61142, thereby generating an active complex. Thus the enzymic lesion in *B. subtilis* mutant 61142 must reside principally, and probably exclusively, in the E1 component of the pyruvate dehydrogenase complex.

Purification of pyruvate dehydrogenase complex from B. subtilis ace mutant 61141R

In the cell-free extract of *B. subtilis* 61141R cells, we detected pyruvate dehydrogenase complex activity and E1 activity equal to more than 45% of that of the wild type under comparable conditions. The activity of E1 (dichlorophenol-indophenol assay)

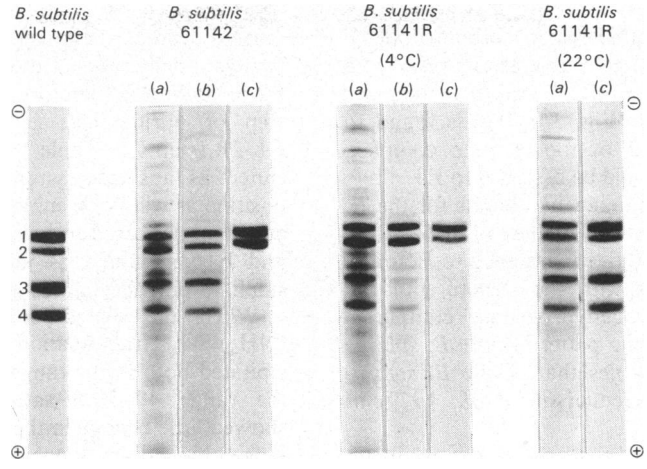

Fig. 4. *Purification of pyruvate dehydrogenase complexes from wild-type B. subtilis and mutants 61141R and 61142*
Samples at various stages of the purification were analysed by electrophoresis in phosphate-buffered sodium dodecyl sulphate/7.5% (w/v)-polyacrylamide gels. Track (*a*), samples taken from the ultracentrifugation pellet; track (*b*), samples taken after gel filtration on Sepharose CL-2B; track (*c*), samples taken after $(NH_4)_2SO_4$ fractionation. A gel of enzyme complex from wild-type *B. subtilis* is shown on the extreme left.

was about 10% of the pyruvate dehydrogenase complex activity, a ratio similar to that measured for the wild type, whereas the E3 activity was elevated 2–4-fold above that of the wild type.

The pyruvate dehydrogenase complex activity and the E1 activity declined rapidly when the extract was kept at 0–4°C, but the E3 activity remained constant. This cold-inactivation could be reversed by warming the extract to 22°C provided that the incubation at 0°C had not been prolonged.

To investigate these properties further we purified the enzyme complex at 0–4°C, as described for *B. subtilis* 61142, and at 22°C in the presence of 1mM-$MgCl_2$, 0.2mM-EDTA and 0.2mM-thiamin pyrophosphate, which greatly slowed the cold-inactivation.

(1) During the purification at 22°C, the enzyme complex from *B. subtilis* 61141R behaved similarly to that from the wild type, except that it was precipitated by 40–50% saturation with $(NH_4)_2SO_4$ rather than by the 50–60% needed for wild-type enzyme. The pure enzyme had a ratio of E3 to pyruvate dehydrogenase complex activities of about 7.8:1, in contrast with the value of about 1.4–2.4:1 for the preparation of wild-type enzyme. Analysis of the enzyme by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that it contained all four types of protein chain, but that, as expected, the E3 subunit was present in an unusually large proportion (Fig. 4). The K_m for pyruvate in the overall complex reaction was found to be 4.3 ± 1.0mM, with a $V_{max.}$ of about 6.4 units/mg, rather different values from those for the wild-type enzyme (K_m, 0.4mM; $V_{max.}$, 12 units/mg).

(2) During the purification at 0–4°C, the E1 and pyruvate dehydrogenase complex activities were both gradually lost at about the same rate. At the ultracentrifugation stage (step 3 of Table 1), all four types of subunit in the enzyme complex were observed in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but the ratios of E3:E2 and of E2:E1 subunits appeared abnormally high. During the gel-filtration step on Sepharose CL-2B a further loss of E1 subunits was observed, a process that was completed during the fractionation with $(NH_4)_2SO_4$. The final enzyme preparation, easily detected by means of its E3 activity (65 units/mg), was precipitated at 40–50% saturation with $(NH_4)_2SO_4$ and was found to consist of a pure E2E3 subcomplex (Fig. 4).

This subcomplex sedimented as a homogeneous peak in the ultracentrifuge, with an $s^0_{20,w}$ value of 52.5S. This value is considerably lower than that observed for the wild-type complex (73S), but demonstrated the high M_r expected for the subcomplex (Fig. 2*b*).

The location of the enzymic lesion in *B. subtilis* 61141R was checked by subjecting the E2E3 subcomplex to E1-subunit-exchange experiments, as described above for *B. subtilis* 61142 (Fig. 5). The recovery of enzymic activity by the E2E3 subcomplex demonstrated that, as with *B. subtilis* 61142, the lesion must lie principally, and probably exclusively, in the E1 polypeptide chain.

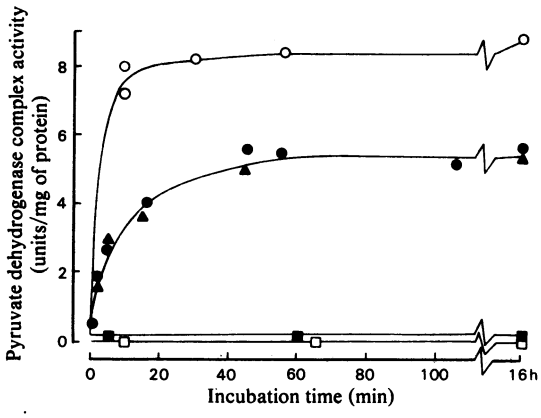

Fig. 5. *E1-subunit exchange between N-ethylmaleimide-inhibited pyruvate dehydrogenase complex and E2E3 subcomplexes*

Purified E2E3 subcomplex (0.042 or 0.21 mg/ml) from mutants 61141R or 61142 was incubated with *N*-ethylmaleimide-inhibited pyruvate dehydrogenase complex (0.17 mg/ml) from wild-type *B. subtilis* at 22°C in 50 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-$MgCl_2$ and 0.2 mM-thiamin pyrophosphate. Samples (3 μl) were assayed at intervals for pyruvate dehydrogenase complex, E3 and E1 activities. The E3 and E1 activities did not change during the incubation. The pyruvate dehydrogenase complex activities are expressed per mg of E2E3 subcomplex. ○, E2E3 subcomplex from mutant 61141R (0.042 mg/ml) plus wild-type complex; ●, E2E3 subcomplex from mutant 61141R (0.21 mg/ml) plus wild-type complex; ▲, E2E3 subcomplex from mutant 61142 (0.21 mg/ml) plus wild-type complex; □, E2E3 subcomplex from mutant 61141R (0.21 mg/ml); ■, wild-type complex (activity expressed per mg of protein).

Discussion

We have developed a simple, rapid, method for purifying the pyruvate dehydrogenase complex from *B. subtilis* which is applicable to small quantities of cells yet permits a yield some 30–40 times that of an earlier method (Visser *et al.*, 1980). Moreover, the enzyme that we describe has a higher specific catalytic activity. We suspect that much of the higher yield is due to our prior treatment of the cells with lysozyme, without which sonication was distinctly less effective in breaking the cell walls and releasing the enzyme complex in good condition. It is curious that the method that works so well for the purification of pyruvate dehydrogenase complex from the related *B. stearothermophilus* (Henderson & Perham, 1980) could not be applied to *B. subtilis*, because, for unknown reasons, the protamine sulphate used to fractionate the cell-free extract in that procedure caused irreversible inhibition of the *B. subtilis* enzyme.

The *B. subtilis* pyruvate dehydrogenase complex contains four types of polypeptide chain with apparent M_r values of 59 500 (band 1), 55 000 (band 2), 42 500 (band 3) and 36 000 (band 4), as judged by electrophoresis on phosphate-buffered sodium dodecyl sulphate/polyacrylamide gels (Fig. 1), in good agreement with those reported by Visser *et al.* (1980), who used a Tris/glycine-buffered system. However, it would appear from the published gel-electrophoretic patterns that enzyme prepared by our method contains more of the peripheral E1α, E1β and E3 subunits relative to the E2 core, This would be consistent with a value for $s^0_{20,w}$ of 73 S for our *B. subtilis* pyruvate dehydrogenase complex compared with the substantially smaller value of 61.7 S reported by Visser *et al.* (1980) and the value of 75 S for the *B. stearothermophilus* complex (Henderson *et al.*, 1979). It may be that the techniques of chromatography on ethanol–Sepharose and cellulose phosphate used by Visser *et al.* (1980) cause some loss of peripheral subunits, since it has been found that ion-exchange chromatography of *E. coli* pyruvate dehydrogenase complex can bring this about (Harrison, 1975). Thus we have no reason to doubt that the *B. subtilis* pyruvate dehydrogenase complex is based on an icosahedral E2 core.

By means of selective inactivation of the complex with *N*-ethylmaleimide in the presence of pyruvate (Fig. 3*a*) and of separate purification of dihydrolipoamide dehydrogenase (Fig. 3*b*), band 1 could be identified as E2 and band 2 as E3, leaving bands 3 and 4 to be assigned to E1α and E1β, respectively, as described for *B. stearothermophilus* (Henderson *et al.*, 1979). The identification of bands 3 and 4 was greatly strengthened by the observation that pyruvate dehydrogenase complex from *B. subtilis ace* mutant 61141R loses these subunits during purification (Fig. 4) and simultaneously loses its overall complex and E1 activities. The E2E3 subcomplex isolated from this mutant had no E1 activity. In almost all respects, therefore, the pyruvate dehydrogenase complex from *B. subtilis* closely resembles that from *B. stearothermophilus* and can be distinguished from its *E. coli* counterpart. However, the E3 component from *B. subtilis* is not strongly retained by hydroxyapatite, whereas E3 from *B. stearothermophilus* or *E. coli* is retained, signifying some difference.

The *B. subtilis ace* mutant 61142 (Freese & Fortnagel, 1969) was reported to require acetate for growth on glucose minimal medium and to have <1% of the pyruvate dehydrogenase complex activity of the parent strain. We have now shown that this mutant produces an assembled pyruvate dehydrogenase complex of high M_r that retains E3

activity but lacks E1 and overall complex activity. The E1 subunits, which are also inactive in the dichlorophenol-indophenol assay, are bound less tighly than in the native complex, since they were gradually lost during the fractionation with $(NH_4)_2SO_4$ (Fig. 4). Subunit-exchange experiments (Fig. 5) indicated that the resulting E2E3 subcomplex was potentially fully active, suggesting that the defect is limited to the E1 subunits.

The *B. subtilis ace* mutant 61141R used by us in the present studies behaved differently from mutant 61141 described by Freese & Fortnagel (1969) in that it was not completely dependent on acetate for growth on glucose minimal medium and it elaborated a functional pyruvate dehydrogenase complex. We found that, although the complex was originally in an assembled and active form, E1 activity (and overall complex activity) were lost during purification at 0–4°C. The loss of E1 activity was accompanied by dissociation of E1 subunits from the E2E3 subcomplex (Fig. 4). The subcomplex ($s^0_{20,w} = 52.5$ S) was judged potentially fully active by means of subunit-exchange experiments (Fig. 5), suggesting that with mutant 61141R, as with mutant 61142, the defect is limited to the E1 subunits. In addition to making the pyruvate dehydrogenase complex cold-labile, this defect in the E1 subunits affected the kinetics of the complex catalytic activity, since the K_m for pyruvate (4.3 mM) was raised 10-fold over the wild-type value (0.4 mM). The raised K_m, rather than the cold-lability of E1 subunit-binding, is probably the reason for the incomplete acetate-dependence of the 61141R mutant.

Exchange of E1 subunits (Fig. 5), is rapid compared with that found for the *E. coli* enzyme complex, where no exchange could be detected by this technique in 24h (Hale *et al.*, 1979). In this respect the *Bacillus* complex closely resembles that from ox kidney mitochondria, for which rapid E1 exchange has been reported (Cate & Roche, 1979).

The cell-free extracts of *B. subtilis* mutants 61141R and 61142 contained elevated amounts of both total and free dihydrolipoamide dehydrogenase activity compared with the wild type. Perhaps the structural defect in E1 is affecting the synthesis of the E3 component, as reported for *E. coli* (Henning *et al.*, 1969), or it could be that changed concentrations of metabolites are responsible. Furthermore the defective pyruvate dehydrogenase complexes elaborated by both mutants contained higher than usual ratios of E3 : E2 polypeptide chains.

The *B. subtilis* mutants 61141R and 61142 are valuable sources of active E2E3 subcomplex, devoid of E1 subunits, for studies of the assembly and catalytic activity of the pyruvate dehydrogenase complex *in vitro*. Further work will be necessary to characterize the nature of the defect in the E1 polypeptide chains in these mutants in terms of their protein chemistry and of the nucleotide sequences of their structural genes and controlling elements, but this offers a powerful approach to a richer understanding of the assembly and mechanism of the pyruvate dehydrogenase complex.

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