The Pyruvate Dehydrogenase Complex of *Pseudomonas aeruginosa* PAO. Purification, Properties and Characterization of Mutants

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(Received 12 May 1980)

The pyruvate dehydrogenase complex of *Pseudomonas aeruginosa* PAO was purified by affinity chromatography on ethanol–Sephacrose 2B followed by sucrose density gradient centrifugation. The overall purification was 130-fold based on enzyme activity. The purified complex contained three major and one minor polypeptide components when analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. These were identified by heat treatment, limited proteolysis and peptide mapping as pyruvate dehydrogenase (E1; M_r 92500), acetyltransferases (E2; major component, M_r 76000, and minor component, M_r 77800) and lipoamide dehydrogenase (E3; M_r 58000). The purified complex had a sedimentation coefficient of 48S and the specific activity for the overall reaction of the complex was 6.5 μmol substrate transformed (mg protein)⁻¹ min⁻¹ at the optimum pH (7.8) and 25 °C.

The lesions in four *ace* mutants lacking overall pyruvate dehydrogenase complex activity were identified after partial purification of the corresponding cell-free extracts. Three strains, designated *aceA* mutants, lacked pyruvate dehydrogenase activity (E1 component) and one strain, an *aceB* mutant, lacked the activity of the acetyltransferase (E2 component).

INTRODUCTION

The pyruvate dehydrogenase complex catalyses the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA, carbon dioxide and NADH. This multienzyme complex has been isolated from a number of sources (Reed, 1974; Bresters *et al.*, 1975; Guest, 1978; Henderson *et al.*, 1979). The bacterial complex consists of multiple copies of three enzymic components: pyruvate dehydrogenase (EC 1.2.4.1), dihydrolipoamide acetyltransferase (EC 2.3.1.12) and lipoamide dehydrogenase (EC 1.6.4.3).

Amongst the prokaryotes, three types of structural organization have been detected. In *Escherichia coli* the complex contains three different types of polypeptide chain: those specifying the pyruvate dehydrogenase (E1; M_r 96000 to 100000), the acetyltransferase (E2; M_r 65000 to 89000) and the lipoamide dehydrogenase (E3; M_r 56500) components (Reed, 1974; Vogel, 1977; Gebhardt *et al.*, 1978). The complex is assembled around a structural core comprising 24 E2 chains (Reed, 1974; Danson *et al.*, 1979). The polypeptide chain stoichiometry (E1:E2:E3) of the complex is not definitely known and ratios of approximately 2:2:1 (Reed, 1974; Fuller *et al.*, 1979; Angelides *et al.*, 1979) and 1 to 2:1:1 (Vogel *et al.*, 1972; Danson *et al.*, 1979) have been proposed. Recent estimates of the sedimentation coefficient and molecular weight are 60.2S and 6.1×10^6 (Danson *et al.*, 1979).

The pyruvate dehydrogenase complex of *Azotobacter vinelandii* is much smaller (19S; M_r 1×10^6) and contains four polypeptide chains (Bresters *et al.*, 1975). Two acetyltransferase isoenzymes (M_r 82000 and 59000) have been identified in addition to the pyruvate dehydrogenase (M_r 89000) and lipoamide dehydrogenase (M_r 56000) components. This complex can be transformed into an active three-component complex by removal of the lower molecular weight acetyltransferase (De Abreu *et al.*, 1977).

Substantial differences in the structures of the pyruvate dehydrogenase complexes of *Bacillus stearothermophilus* and other *Bacillus* species exist (Henderson *et al.*, 1979; J. Visser, unpublished observations; R. N. Perham, personal communication). The complexes of these Gram-positive bacteria resemble their mammalian counterparts with respect to their higher molecular weight (9.6×10^6), sizes of components and, particularly, the existence of two essential subunits for the pyruvate dehydrogenase component (Henderson *et al.*, 1979). The subunit molecular weights are: pyruvate dehydrogenase components (M_r 42000 and 36000), acetyltransferase (M_r 57000) and lipoamide dehydrogenase (M_r 54000).

Little is known about the pyruvate dehydrogenase complex of *Pseudomonas aeruginosa* PAO although four *ace* mutants lacking this activity have been isolated and their lesions located on the *P. aeruginosa* PAO linkage map (Jeyaseelan & Guest, 1980). This paper describes the purification and some properties of the pyruvate dehydrogenase complex of *P. aeruginosa* PAO. In addition, the lesions of the *ace* mutants were identified after partial purification of the corresponding complexes.

METHODS

Organisms. *Pseudomonas aeruginosa* strain PAO1 was obtained from Professor B. W. Holloway. The four *ace* mutants (PAO2851 to 4) lacking overall pyruvate dehydrogenase complex activity have been described previously (Jeyaseelan & Guest, 1980). Stock cultures of organisms grown for 16 h on slopes of nutrient agar were maintained at -20°C by rapid freezing in nutrient yeast broth (NYB) medium and revived in the same medium (Stanisich & Holloway, 1972). The wild-type strain of *E. coli* (H) and other strains used in the immunological studies have been described previously (Langley & Guest, 1977).

Growth of organisms and preparation of cell-free extracts. Batch cultures (6 l) of *P. aeruginosa* PAO1 were grown at 37°C in the minimal medium of Jeyaseelan & Guest (1980) with D-glucose (80 mM) as substrate in a Microferm fermenter (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Cultures of the *ace* mutants were grown in the same medium supplemented with potassium acetate (4 mM). The cultures were grown from a 15 ml inoculum of organisms grown overnight in NYB; they were stirred at 200 rev. min^{-1} and aerated at 0.2 l air min^{-1} . Cultures were harvested in late-exponential phase (after 16 h) at 15000 rev. min^{-1} and 4°C using the continuous action rotor of an HS18 centrifuge (M.S.E.). The bacterial paste (20 g wet wt) was washed in 500 ml potassium phosphate buffer (10 mM; pH 7.0) and resuspended in extraction buffer (120 ml). The extraction buffer contained potassium phosphate buffer (10 mM; pH 7.0), EDTA (2 mM), MgCl_2 (3 mM), phenylmethylsulphonyl fluoride (PMSF, 50 μM), 2-mercaptoethanol (5 mM), deoxyribonuclease I (5 $\mu\text{g ml}^{-1}$), ribonuclease (10 $\mu\text{g ml}^{-1}$) and thiamin pyrophosphate (TPP, 100 μM). The bacteria were disrupted with an ultrasonic disintegrator (150 W; M.S.E.) as 40 ml portions for two periods of 2 min with an interval for cooling. The extract was centrifuged at 25000 g for 15 min at 0°C and the supernatant fluid was dialysed at 4°C against potassium phosphate buffer (10 mM; pH 7.0) containing all components of the extraction buffer except the nucleases and TPP.

Enzymology. The pyruvate dehydrogenase complex activity was measured spectrophotometrically at 340 nm and 25°C in 1 ml cuvettes containing Tris/HCl buffer (100 mM; pH 7.8), pyruvate (5 mM), MgCl_2 (2 mM), dithiothreitol (2.5 mM), TPP (0.4 mM), CoA (130 μM) and NAD^+ (0.6 mM). The 2-oxoglutarate dehydrogenase complex activity was measured under similar conditions with 2-oxoglutarate as the substrate.

The pyruvate dehydrogenase (E1; EC 1.2.4.1) component was assayed spectrophotometrically at 420 nm and pH 6.3 with ferricyanide as the electron acceptor (Hager & Kornberg, 1961).

The dihydrolipoamide acetyltransferase (E2; EC 2.3.1.12) component was determined as described previously (Jeyaseelan & Guest, 1980).

The lipoamide dehydrogenase (E3; EC 1.6.4.3) component was measured at 25°C in 1 ml cuvettes containing potassium phosphate buffer (100 mM; pH 7.8), bovine serum albumin (0.01 %, w/v), EDTA (1 mM), reduced lipoamide (0.6 mM) and NAD^+ (0.36 mM).

For assays involving crude extracts, NAD^+ was replaced by 3-acetylpyridine-adenine dinucleotide (APAD⁺) (to prevent interference from NADH oxidase) and absorbances were recorded at 366 nm. However, this precaution was not necessary because the observed rates were the same with the two coenzymes. Enzyme specific activities are quoted in enzyme units (mg protein^{-1}), where one unit is the amount of enzyme transforming 1 μmol substrate min^{-1} . Protein concentrations were determined by the Lowry method with bovine serum albumin as the standard and all enzyme specific activities are based on this method even though absorbance at 280 nm was used to monitor the purification.

Affinity chromatography. The ethanol-Sepharose 2B matrix of Visser *et al.* (1978) was used as the first

step in the purification of the pyruvate dehydrogenase complex. The crude extract was loaded on a column (2.5 × 20 cm) equilibrated with potassium phosphate buffer (10 mM; pH 7.0) containing EDTA, PMSF, MgCl₂ and 2-mercaptoethanol at the concentrations described above (for extraction buffer). The column was eluted with the same buffer and fractions (10 ml) were collected at a flow rate of 30 ml h⁻¹. When all the unbound proteins had been removed the enzyme was eluted at the same flow rate with the same buffer but an increasing potassium phosphate concentration (10 to 80 mM, linear gradient). Fractions containing pyruvate dehydrogenase complex were pooled and the activity was concentrated by ultracentrifugation.

Ultracentrifugation. Pooled fractions from the ethanol-Sephadex column were centrifuged at 140 000 g for 4.5 h at 4 °C (40 000 rev. min⁻¹ in the 8 × 35 ml fixed angle rotor of the M.S.E. PrepSpin 50). The sediment was carefully dissolved in sodium phosphate buffer (30 mM; pH 7.0) containing EDTA (2 mM). Insoluble material was removed by low-speed centrifuging (2500 g for 10 min).

Sucrose gradients. Concentrated samples (1 ml) were applied to linear sucrose gradients (5 to 15 %, w/w) containing sodium phosphate buffer (30 mM; pH 7.0) and EDTA (2 mM). They were centrifuged at 140 000 g (39 000 rev. min⁻¹) for 3 h at 4 °C in a Beckman L2-65B preparative ultracentrifuge using a 6 × 14 ml (SW40) swing-out rotor. Fractions (1 ml) were collected and assayed for pyruvate dehydrogenase complex and lipoamide dehydrogenase activities. The sucrose concentration was determined with an Abbé refractometer. Samples were dialysed against sodium phosphate buffer (30 mM; pH 7.0) with EDTA (2 mM) to remove sucrose.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis in 10 % (w/v) polyacrylamide gels containing 0.1 % (w/v) sodium dodecyl sulphate was performed in the vertical slab gel apparatus described by Studier (1973) using a discontinuous sodium dodecyl sulphate buffer system (Laemmli, 1970). Gels were stained with Kenacid blue R. The molecular weights of the protein subunits were estimated by comparison with the following marker proteins: β -galactosidase (116 000), phosphorylase b (94 000), conalbumin, type I (78 000), bovine serum albumin (68 000), catalase (60 000), ovalbumin (43 000), carbonic anhydrase (29 000) and trypsin inhibitor protein (21 500). Samples of *E. coli* pyruvate dehydrogenase complex were also included as reference proteins and for comparison with the *P. aeruginosa* complex. The gels were scanned using a Unicam SP1809 scanning densitometer at 550 nm.

Sedimentation analysis. Sedimentation measurements were performed in a Beckman Spinco Model E ultracentrifuge at a rotor speed of 55 740 rev. min⁻¹ at 20 °C. Migration of the boundary was followed using Schlieren optics and the boundaries were measured at 4 min intervals. Sedimentation was in sodium phosphate buffer (30 mM; pH 7.0) containing EDTA (2 mM) and the protein concentration was 0.9 mg ml⁻¹.

Peptide mapping. Peptide mapping by limited proteolysis in sodium dodecyl sulphate and analysis by polyacrylamide gel electrophoresis was done according to Cleveland *et al.* (1977). Bands (1 × 5 mm) from stained gels were treated with 2 to 5 ng *Staphylococcus aureus* V8 protease for 15 min in the stacking gel above the second separating gel (15 % acrylamide).

Immunology. Antisera raised against the pyruvate dehydrogenase complex and the pyruvate dehydrogenase and lipoamide dehydrogenase components of *E. coli* were used in Ouchterlony double diffusion with crude extracts of *P. aeruginosa* as described previously (Guest & Creaghan, 1974).

Quantitative titrations of enzymic activities with antisera were carried out by incubating samples of crude extract, having complex activities of 2 μ mol h⁻¹, at room temperature for 30 min with different amounts of antisera (final volume 250 μ l). The mixtures were then centrifuged for 20 min at 10 000 g (4 °C) and the supernatants were tested for residual enzyme activity (Langley & Guest, 1974).

The antiserum raised against the *E. coli* pyruvate dehydrogenase component exhibited minor reactions with lipoamide dehydrogenase and two non-specific bacterial components. It was made monospecific by mixing the antiserum (3 ml) with protein (0.03 mg) derived from cell-free extracts of *E. coli* deletion mutants (KΔ18, E1-E2-E3⁻, and HΔ10, E1-E2-E3⁺; Langley & Guest, 1977) and removing the precipitates by sedimentation at 10 000 g.

Materials. Dihydrolipoamide was prepared by reducing 300 mg lipoamide (Sigma) in methanol plus water (6 ml plus 1.5 ml) with sodium borohydride (30 mg in 1.5 ml water) at 0 °C. The product was extracted with chloroform, dried and recrystallized from benzene/n-hexane (2.5:1, by vol.). Other substrates and coenzymes were purchased from Boehringer or Sigma. Pyruvate dehydrogenase complex of *E. coli* strain K1-1LR8-13 was prepared according to Visser *et al.* (1978). The sources of other chemicals, enzymes and proteins were: BDH - deoxyribonuclease I and ribonuclease; Boehringer - β -galactosidase, phosphorylase b and trypsin inhibitor (soybean); Koch-Light - catalase; Sigma - conalbumin, bovine serum albumin, ovalbumin, carbonic anhydrase and phenylmethylsulphonyl fluoride (PMSF).

Fig. 1. Affinity chromatography of the 2-oxoacid dehydrogenase complexes of *P. aeruginosa* PAO1 on ethanol-Sepharose 2B. Crude extract was prepared from 20 g wet wt bacteria and chromatographed according to the procedures described in Methods. Fractions of 10 ml were collected and assayed for pyruvate dehydrogenase complex activity (●), 2-oxoglutarate dehydrogenase complex activity (▲) and lipoamide dehydrogenase activity (□). The potassium phosphate gradient (-----) and absorbance at 280 nm (—) are indicated.

Fig. 2. Purification of pyruvate dehydrogenase complex of *P. aeruginosa* PAO1 by sucrose density gradient centrifugation. Samples (1 ml) concentrated after ethanol-Sepharose 2B chromatography were layered on linear sucrose gradients (5 to 15 %, w/w) and centrifuged at 140000 g for 3 h at 4 °C. Fourteen fractions of 1 ml were collected and assayed for pyruvate dehydrogenase complex activity (○) and lipoamide dehydrogenase activity (●). The sucrose gradient (-----) and protein concentration based on the Lowry method (△) are indicated.

RESULTS

The pyruvate dehydrogenase complex of P. aeruginosa PAO

Purification. The elution profile of the pyruvate and 2-oxoglutarate dehydrogenase complex activities and lipoamide dehydrogenase on ethanol-Sepharose 2B is shown in Fig. 1. The 2-oxoglutarate dehydrogenase complex was eluted first, at low ionic strength, followed by the pyruvate dehydrogenase complex at a higher ionic strength. About 80 % of the pyruvate dehydrogenase complex applied was recovered, free from contamination with 2-oxoglutarate dehydrogenase complex, with an overall purification of 12-fold. Lipoamide dehydrogenase activity was associated with both complexes. The pyruvate dehydrogenase complex was concentrated by sedimenting at 140000 g for 4.5 h and several non-sedimentable contaminants were also eliminated. Further purification was achieved by centrifuging on

Table 1. *Purification of the pyruvate dehydrogenase complex of P. aeruginosa* PAO1

The results of a typical purification starting with 20 g wet wt bacteria are shown. Enzymes were assayed as described in Methods using APAD⁺ with crude extracts, but NAD⁺ for other fractions. Abbreviations: pdhc, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase; E2, dihydro-lipoamide acetyltransferase; E3, lipoamide dehydrogenase.

Purification step	Total volume (ml)	Total protein (mg)	Activity assayed	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Yield (%)
Crude extract	120	1620	pdhc	81.6	0.05	100
			E1	32.4	0.02	100
			E2	40.8	0.025	100
			E3	56.4	0.035	100
Ethanol-Sephadex	105	101	pdhc	60.9	0.61	74.6
			E1	23.1	0.23	71.3
			E2	29.4	0.29	72.1
			E3	26.3	0.26	46.6
Sedimentation	6	27	pdhc	56.7	2.10	69.5
			E1	21.0	0.78	64.8
			E2	27.6	1.02	67.6
			E3	24.9	0.92	44.2
Sucrose gradient	18	7.2	pdhc	46.8	6.5	57.4
			E1	17.3	2.4	53.1
			E2	24.3	3.4	59.6
			E3	21.6	3.0	38.3

sucrose density gradients (see Methods) and an activity profile for the complex and lipoamide dehydrogenase is shown in Fig. 2. The specific activity of pooled fractions 4 to 6 was 6.5 units (mg protein)⁻¹, corresponding to an overall purification of 130-fold and a 57 % recovery of activity. Details of a typical purification are summarized in Table 1. There was no appreciable loss of individual component activities (Table 1) during the purification except for lipoamide dehydrogenase, which was distributed between the two complexes at the first step. Attempts to purify the ethanol-Sephadex fractions by calcium phosphate gel chromatography, gel filtration on Sephadex 6B (Henderson *et al.*, 1979) or affinity chromatography on propanol-Sephadex 2B and ethylene glycol-Sephadex 2B (J. Visser, unpublished observations), instead of by the final centrifugation procedure, were unsuccessful.

The purified complex was stable in sodium phosphate buffer (30 mM; pH 7.0) containing EDTA (2 mM) for up to 4 weeks at -20 °C. Under standard assay conditions the optimum pH for overall complex activity was 7.8 in Tris/HCl and potassium phosphate buffers. The activity fell steadily on either side of the maximum and was 50 % of the optimal rate at pH 6.8 and pH 8.8.

On analytical ultracentrifugation of the purified complex a single component having a sedimentation coefficient (*s*_{20, w}) of 48S was detected. After storage at -20 °C for 4 weeks the complex degraded into three components of 47, 13 and 12.5S, without loss of activity.

Molecular weights and identification of the subunits. Sodium dodecyl sulphate-polyacrylamide gel electrophoretic analysis indicated that the purified complex contained four polypeptides, although one was generally a minor component (Fig. 3). The molecular weights (*M*_r) of these components, as estimated from their rate of migration relative to reference proteins, were: 92 500, 77 800, 76 000 and 58 000. Under the same conditions the molecular weights of the components of the pyruvate dehydrogenase complex of *E. coli* were estimated to be 96 500 (E1), 83 000 (E2) and 56 000 (E3).

Attempts to identify the polypeptides by dissociating the complex were unsuccessful because it was unaffected by procedures such as treatment with alkali (pH 10) or potassium iodide (2 M), which normally dissociate the complexes of other bacteria (Bates *et al.*, 1977; Henderson *et al.*, 1979). However, evidence for the identities of the polypeptides was obtained

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of pyruvate dehydrogenase complex of *P. aeruginosa* PAO1. A sample of the purified complex (25 μ g protein) was analysed and the stained gel was scanned at 550 nm using a Unicam SP1809 scanning densitometer. The molecular weights of the polypeptide chains were estimated by comparison with standard marker proteins and the pyruvate dehydrogenase complex of *E. coli* (see Methods).

from their differential stabilities to heat and proteolysis with trypsin. Heating the partially purified complex (ethanol-Sephadex fraction) in buffer plus ammonium sulphate (5 %, w/v) for 5 min at 70 °C followed by centrifuging (25 000 *g* for 15 min at 4 °C) and dialysis of the supernatant fluid, totally inactivated the overall complex and its pyruvate dehydrogenase component (E1) and also eliminated the 92 500 polypeptide from the gel profile (Fig. 3). The other enzyme activities and polypeptide components were unaffected. A second similar heat treatment inactivated the acetyltransferase component (E2) and removed all of the 77 800 and most of the 76 000 polypeptides: most of the lipoamide dehydrogenase activity (E3 component) and the 58 000 polypeptide were retained. The identities of the E2 and E3 components were confirmed by limited proteolysis of the purified complex with trypsin [0.2 mg complex plus 5 μ g trypsin in 0.5 ml sodium phosphate buffer (30 mM; pH 7.0) for 1 to 20 min]. Within 5 min the acetyltransferase activity was lost and the 77 800 and 76 000 polypeptides were replaced by polypeptides of lower molecular weight. The 92 500 polypeptide disappeared less rapidly but the lipoamide dehydrogenase activity (E3 component) and the 58 000 polypeptide were completely unaffected after treating for up to 20 min. Thus, the largest (92 500) and smallest (58 000) polypeptides correspond to the pyruvate dehydrogenase (E1) and lipoamide dehydrogenase (E3) components, respectively, and one, or both, of the other polypeptides (77 800, E2a; 76 000, E2b) must represent the acetyltransferase (E2) component (Fig. 3).

Quantitative densitometry of the stained components of the purified complex (Fig. 3) suggested a molar ratio of E1:E2:E3 of 3:1:1 but this was not corrected for differences in the staining affinities of the individual components.

Peptide mapping. Stained components from sodium dodecyl sulphate-polyacrylamide electrophoresis gels of the purified *P. aeruginosa* and *E. coli* complexes were examined by limited proteolysis with *Staphylococcus aureus* V8 protease and one-dimensional electrophoresis. The E1 components of both species resembled each other in 10 out of 15 visible digestion products. The E2a and E2b components of *P. aeruginosa* gave identical fragments and these were very different from the fragmentation pattern obtained from the E2 component of *E. coli*. The E3 components of the two species also responded differently.

Immunology. The *E. coli* pyruvate dehydrogenase complex produced two precipitin lines

Fig. 4. Ouchterlony double diffusion in agarose. Centre wells (G, H and I) contained antiserum and lateral wells (A to F) contained crude extract equivalent to 100 μg protein: A, *P. aeruginosa* PAO1 (wild type); B, *E. coli* (wild type); C to F, *E. coli* deletion strains described by Langley & Guest (1977) [C, K Δ 27 ($\text{E1}^+\text{E2}^+\text{E3}^+$); D, C Δ 42 ($\text{E1}^-\text{E2}^+\text{E3}^+$); E, H Δ 10 ($\text{E1}^-\text{E2}^-\text{E3}^+$); F, K Δ 18 ($\text{E1}^-\text{E2}^-\text{E3}^-$)]; G, anti-*E. coli* pyruvate dehydrogenase complex (3 μl); H, anti-*E. coli* pyruvate dehydrogenase component, E1 (5 μl); I, anti-*E. coli* lipoamide dehydrogenase, E3 (3 μl).

Fig. 5. Effect of antibodies raised against the *E. coli* pyruvate dehydrogenase complex ($\circ$), its pyruvate dehydrogenase component, E1 ($\square$) and lipoamide dehydrogenase component, E3 ($\triangle$) on the overall pyruvate dehydrogenase complex activity of *E. coli* (----) and *P. aeruginosa* PAO1 (—). Titrations of enzymic activities with antisera were carried out as described in Methods.

in double diffusion with the homologous antiserum (Fig. 4a). These were identified as specific reactions involving pyruvate dehydrogenase (outer line, Fig. 4b) and lipoamide dehydrogenase (inner line, Fig. 4c) by using the corresponding antisera and appropriate strains. Because the E1 and E3 components surround the acetyltransferase core, antisera raised against the complex may not contain anti-E2 antibodies or these antibodies may be unable to penetrate the complex and react with the core. No immunological cross-reaction was observed by Ouchterlony double diffusion between *P. aeruginosa* and antisera raised against the *E. coli* pyruvate dehydrogenase complex or its E1 and E3 components. However, evidence for immunological cross-reactivity between the species was obtained in quantitative tests (Fig. 5). The overall pyruvate dehydrogenase complex activity of *P. aeruginosa* was precipitated by antibodies raised against the *E. coli* complex and its E1 component. The antiserum to the E3 component of *E. coli* was considerably less active, even after accounting for the proportion of E3 component that is associated with the 2-oxoglutarate dehydrogenase

Table 2. *Specific activities of the pyruvate dehydrogenase complex and components in P. aeruginosa PAO1 and ace mutants*

Enzymes in ethanol-Sepharose 2B fractions derived from 10 g wet wt bacteria were assayed as described in Methods. Abbreviations as in Table 1; ND, not detected.

Strain	Specific activity [units (mg protein) ⁻¹]			
	pdhc	E1	E2	E3
PAO1 (<i>ace</i> ⁺)	0.61	0.23	0.29	0.26
PAO2851 (<i>ace</i>)	ND	0.10	0.02	0.14
PAO2852 (<i>ace</i>)	ND	ND	0.23	0.19
PAO2853 (<i>ace</i>)	ND	ND	0.22	0.18
PAO2854 (<i>ace</i>)	ND	ND	0.22	0.18

complex in *P. aeruginosa*. Control serum had no effect on the enzyme activities. These results indicate that the pyruvate dehydrogenase components of *P. aeruginosa* and *E. coli* are related immunologically despite the negative precipitin reaction.

Characterization of the pyruvate dehydrogenase complex mutants

Previous attempts to define the lesions of *ace* mutants of *P. aeruginosa* by assaying for the individual components in crude cell-free extracts were inconclusive (Jeyaseelan & Guest, 1980). Further attempts at characterizing the mutants were made by partial purification of the corresponding pyruvate dehydrogenase complexes by affinity chromatography on ethanol-Sepharose 2B. The mutant complexes were isolated in the fractions eluted immediately after the 2-oxoglutarate dehydrogenase complex in separations analogous to that shown in Fig. 1. The elution profiles for the mutants were similar to the wild type strain except that the second peak of lipoamide dehydrogenase activity was not associated with any pyruvate dehydrogenase complex activity. This not only served to identify the fractions containing the mutant complexes, but also demonstrated that inactive complexes are assembled by the mutants, because uncomplexed lipoamide dehydrogenase is eluted at a different position (J. Visser, unpublished observation). Assays for specific components (Table 2) indicated that the *ace* mutants lacked either the pyruvate dehydrogenase component (PAO2852 to 4) or the dihydrolipoamide acetyltransferase component (PAO2851), and the corresponding genes were designated *aceA* (E1 component) and *aceB* (E2 component), respectively. Electrophoretic analyses of the partially purified preparations on sodium dodecyl sulphate-polyacrylamide gels showed that polypeptides corresponding to the defective components were present in each mutant, indicating that they are probably missense mutants.

DISCUSSION

The pyruvate dehydrogenase complex of *P. aeruginosa* was intermediate in size between those of *Escherichia coli* and *Azotobacter vinelandii* but possessed a single-chain pyruvate dehydrogenase component, typical of Gram-negative bacteria. It contained three major and one minor polypeptide chains, identified as pyruvate dehydrogenase (E1; M_r 92 500), acetyltransferase (E2a and E2b; M_r 77 800 minor and 76 000 major) and lipoamide dehydrogenase (E3; M_r 58 000). Compared with *E. coli*, the E1 and E2 components are smaller and the E3 component is larger. However, the relatively large difference in sedimentation coefficients (60 and 48S) is unlikely to stem from size differences of the components but rather from significant differences in the compositions of the complexes of the two species. Peptide mapping confirmed that the two polypeptides designated as acetyltransferase components probably have a common origin. Whether they are both invested with acetyltransferase activity was not investigated. In *E. coli* the sizes quoted for the acetyltransferase vary widely

(M_r 65000 to 89000) and this component is exceedingly prone to spontaneous and proteolytic degradation into enzymically active products as small as 35000 (Angelides *et al.*, 1979; Gebhardt *et al.*, 1978; Hale & Perham, 1979; Reed, 1974; Vogel, 1977; Vogel *et al.*, 1971). It is probable that the frequently reported 80000 to 83000 component is really a degradation product of a larger, 89000, native acetyltransferase (Gebhardt *et al.*, 1978). The *P. aeruginosa* complex was prepared in the presence of a protease inhibitor and EDTA to limit proteolysis. Nevertheless, the M_r 76000 component could be derived from the 77800 component, but no smaller components were observed. The existence of two acetyltransferase components (M_r 82000 and 59000) has been demonstrated for *Azotobacter vinelandii* (Bresters *et al.*, 1975) and preliminary studies indicate that two polypeptides (M_r 73000 and 65000) are associated with the acetyltransferase of *P. fluorescens* (J. Visser, unpublished observations) but it is not certain whether the two proteins have a common origin.

Immunological cross-reactivity between the *E. coli* and *P. aeruginosa* complexes was weak but could be detected by quantitative tests, particularly for the E1 components. It may be significant that the respective E1 components showed similar degradation patterns in peptide mapping. No similarities between the E2 and E3 components of the two species were detected other than the heat stability of lipoamide dehydrogenase.

Partial purification of the two complexes from mutants lacking pyruvate dehydrogenase complex activity permitted the identification of two classes of *ace* mutant. Three of the mutants, designated *aceA*, lacked pyruvate dehydrogenase activity (E1 component) and one mutant, designated *aceB*, was deficient in acetyltransferase activity (E2 component). Polypeptides corresponding to the defective components were detected and this is consistent with the mutants possessing missense mutations and assembling enzymically inactive complexes. Previous genetic studies with the *ace* mutants have shown that their mutations are closely linked (Jeyaseelan & Guest, 1980). Consequently, the results are consistent with the existence of an *ace* operon containing *aceA* (E1) and *aceB* (E2) genes similar to the *ace* operon of *E. coli* (Henning *et al.*, 1966; Guest, 1978).

We wish to thank Professor B. W. Holloway for his generous gift of *P. aeruginosa* PAO1 and K.J. acknowledges the receipt of a postgraduate award from The Commonwealth Commission in the United Kingdom.

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