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D- and L-Lactate Dehydrogenases of *Pseudomonas aeruginosa*

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During a study of the interaction between membrane-bound dehydrogenases and the electron-transport chain of *Pseudomonas aeruginosa* A.T.C.C. 15692 (Kemp, 1971), dehydrogenases for D- and L-lactate were mentioned. In the present communication these dehydrogenases are characterized and their role in the growth of *Ps. aeruginosa* on DL-lactate is examined.

The methods used were essentially those described by Kemp & Hegeman (1968). Cells were grown aerobically at 37°C on 20mM-DL-lactate, harvested at 0.4mg dry wt./ml, washed and resuspended in 50mM-sodium–potassium phosphate buffer, pH6.8, and disrupted by treatment with ultrasound. The disrupted cells were centrifuged for 10min at 10000*g* and the supernatant liquid was used as the crude extract. By centrifuging the crude extract for 90min at 140000*g* the high-speed supernatant and particulate fractions were obtained; the latter was washed by resuspending and centrifuging again.

There was no dehydrogenase activity (i.e. less than 0.002unit/mg of protein), assayed with NAD^+ or with $NADP^+$ (0.15mM), in either the crude extract or the supernatant fraction with D- or L-lactate (5mM) as substrate; the buffers used were 50mM-diethanolamine–HCl, pH9.0, 50mM-tris–HCl, pH8.0, and 50mM-phosphate, pH6.8. Pyruvate (5mM) did not stimulate the rate of oxidation of NADH or NADPH (0.15mM).

The crude extract did, however, catalyse the oxidation of D- and L-lactate with 2,6-dichlorophenol-indophenol, *N*-methylphenazonium methosulphate (phenazine methosulphate), ferricyanide and oxygen as acceptors. At least 95% of each of the activities was located in the particulate fraction, the results with which are shown in Table 1. Cytochrome *c* [horse heart; Boehringer Corp. (London) Ltd., London W.5, U.K.] was also used as an acceptor, but a concentration convenient for spectrophotometric assay (60μM) was very much less than the saturating concentration. Cyanide (1mM) inhibited the activity with oxygen as the acceptor by at least 90%. It had no effect on the initial rates with the other acceptors and preserved the linearity of the rate with dichlorophenol-indophenol, as it prevented the re-oxidation of reduced dichlorophenol-indophenol.

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A study of the distribution of D-lactate–dichlorophenol-indophenol dehydrogenase at different times during the treatment with ultrasound showed that the activity appeared initially in the fraction sedimented within 10min at 10000*g*, but with time was progressively recovered in fractions sedimented only after centrifuging for 20min at 38000*g* and for 90min at 140000*g*. After the cells had been broken with lysozyme–tris–EDTA (Campbell *et al.*, 1962), all of the activity was sedimented within 10min at 10000*g*, whereas all of the RNA detected by the orcinol method (Horecker *et al.*, 1953) remained in the supernatant liquid. It is concluded that the D-lactate–dichlorophenol-indophenol dehydrogenase and, by analogy, the other activities are located in the membrane of the cell rather than in discrete particles.

The reduction of cytochromes in the particulate fraction (2–5mg of protein/ml) was measured as the difference spectrum in a Cary 14 spectrophotometer equipped with a high-intensity light-source and a slide-wire for 0–0.1 absorbance. The reference sample was maintained fully oxidized with ferricyanide. A suspension reduced with dithionite gave an α-band peak at 552nm and a shoulder at 559nm, characteristic of cytochromes of the *b* and *c* type respectively (Peterson, 1970). Depending on the preparation, 30–60% of the cytochrome *c* was reduced immediately when D- or L-lactate (2mM) was added to a shaken suspension; when the oxygen in the suspension was exhausted, the cytochrome *c* became fully reduced and 25–40% of the cytochrome *b* was also reduced (see below). Similar results were obtained with NADH and with succinate in place of lactate. In a suspension rendered anoxic by bubbling nitrogen or treated with cyanide (1mM), but in the absence of any added substrate, the cytochrome *c* was again fully reduced; by contrast, no cytochrome *b* was reduced. On comparing a test suspension containing substrate or dithionite with a reference suspension containing cyanide a pure spectrum of cytochrome *b* was obtained, from which the degree of reduction could be measured much more easily than from the shoulder on the spectrum obtained with the fully oxidized suspension in the reference cuvette (see above). The reduction of cytochrome *c* in the anoxic state in the absence of added substrate was unexpected, but it is concluded from the reduction of cytochrome *c* in the steady state and from the

Table 1. *Activities of* D- *and* L-*lactate dehydrogenases with various acceptors*

The washed particulate fraction was assayed at 30°C in 20mM-sodium–potassium phosphate buffer, pH6.8; 1mM-cyanide was present except when oxygen was the acceptor. At zero time the extract was added and the blank rate measured; after 3min the substrate (2mM) was added and the net rate calculated. D-Lactate was the lithium salt from Calbiochem Ltd., London W.1, U.K., and L-lactate was the lithium salt made by neutralizing the free acid from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. A Cary 14 spectrophotometer was used for the spectrophotometric assays and an oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) for the assays by uptake of oxygen. From the electron equivalences shown, the rates are expressed as μmol of substrate disappearing/min.

Acceptor	Assay method	Electron equivalence	Specific activity (unit/mg) with D-Lactate	Specific activity (unit/mg) with L-Lactate
Dichlorophenol-indophenol (0.2mM)	600nm; $\Delta E = 20$ litre·mol^{-1}·cm^{-1}	$2e^-$	0.14	0.066
Phenazine methosulphate (1mM)	O_2 uptake; $[O_2] = 0.24$mM	$4e^-$	0.12	0.10
Ferricyanide (2mM)	420nm; $\Delta E = 2$ litre·mol^{-1}·cm^{-1}	$1e^-$	0.18	0.023
O_2 (0.24mM)	O_2 uptake; $[O_2] = 0.24$mM	$4e^-$	0.024	0.024

reduction of cytochrome *b* in the anoxic state that the D- and L-lactate dehydrogenases interact with the electron-transport chain.

However, it has been suggested that the oxidation of NADH by another fluorescent pseudomonad involves the formation of H_2O_2 by a flavoprotein oxidase (Lenhoff *et al.*, 1956); this possibility could also apply to the oxidation of lactate and was therefore examined. In Warburg manometer vessels in which the reaction with D-lactate (5mM) was stopped at 0 and 30min with $HClO_4$ (to 5%, w/v), the particulate fraction (5mg of protein) used 1.8μmol of O_2 (net) and 3.9μmol of D-lactate and formed 3.6μmol of pyruvate [assays described by Hullin & Noble (1953) and by Bergmeyer (1963)]. Because of the presence of catalase in the extract, this stoicheiometry would have resulted whether the oxygen had been reduced to H_2O_2 or directly to water. However, the stoicheiometry between oxygen consumption and pyruvate formation was not affected by the presence of azide (1mM), which decreased the catalase activity with 1mM-H_2O_2 to less than 20% of the rate of oxidation of D-lactate. H_2O_2 could also be removed by a D-lactate peroxidase; pyruvate was destroyed in the presence of H_2O_2, so the peroxidase could not be assayed by the formation of pyruvate; however, in an anaerobic incubation with the particulate fraction and azide (1mM), H_2O_2 (1mM), assayed with guaiacol (0.3mM) and horseradish peroxidase, disappeared more quickly in the presence of D-lactate (2mM) than in its absence, but the rate of disappearance was only 20% of the D-lactate oxidase rate in the presence of azide. Similar results were obtained with NADH. It is concluded that neither catalase nor a peroxidase account for the observed stoicheiometry of the oxidation of D-lactate, which is that expected for oxidation through the electron-transport chain.

The concentrations of the acceptors used in Table 1 were sufficient to give maximal rates. The oxidation of D-lactate was inhibited by concentrations of substrate above 2mM. By contrast, reciprocal Michaelis–Menten plots with L-lactate curved down at high lactate concentrations. With ferricyanide as the acceptor, 10mM-'L-lactate' gave an initial fast rate, which gave way to a slower but steady rate; as D-lactate reacted much faster than L-lactate with ferricyanide (Table 1), it seemed likely that the 'L-lactate' was contaminated with D-lactate and, by extrapolating the trace back to zero time, this contamination could be calculated as 0.1–2.0%, depending on the source of the chemical. Consistent with this explanation is the fact that heating the extract for 3min at 71°C, which destroyed 93% of the dichlorophenol-indophenol dehydrogenase activity for D-lactate but only 30% of that for 'L-lactate', left an activity that gave a much straighter reciprocal Michaelis–Menten plot.

Various observations show that there are two distinct dehydrogenases for the two lactate isomers, rather than one dehydrogenase and a racemase. (1) The rates with the two isomers were almost additive; insofar that they were not, contamination of the L-lactate (see above) was presumably responsible. (2) The different relative rates with the various acceptors (Table 1) suggest that there was only one dehydrogenase. With dichlorophenol-indophenol there was more D-lactate dehydrogenase than L-lactate dehydrogenase activity, so that the dehydrogenase would have to act directly on the D-isomer; however, the activity with the L-isomer was much

more resistant to heat. (3) Both activities were in the particulate fraction; other bacterial racemases are soluble. (4) After a 30min incubation of the particulate fraction with D-lactate (5mM), either aerobically or anaerobically, no L-lactate could be detected with muscle lactate dehydrogenase (less than 0.001 unit of racemase/mg of protein).

Both dehydrogenases were inducible, the activities in extracts from cells grown on succinate or choline being less than 2% of those from cells grown on lactate. Washed cells grown on the individual lactate isomers oxidized both isomers at similar rates to cells grown on DL-lactate, so it seems very likely that both dehydrogenases can be induced by either isomer. 3-Aminopropan-2-ol is converted into D-lactate in another pseudomonad (Higgins & Turner, 1969), but the strain used for the present study would not grow on that compound.

Lactate dehydrogenases of the type described in this paper have been found in other bacteria, but usually at least one enzyme is constitutive. They have only briefly been mentioned in pseudomonads (Higgins & Turner, 1969; Pichinoty *et al.*, 1968), except for *Pseudomonas natriegens* (Walker & Eagon, 1964), in which the induction pattern was quite different. However, the affinity of this strain with other pseudomonads is questionable (Baumann *et al.*, 1971).

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