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Short Communications

The Role of Imidazol-5-yl-lactate–Nicotinamide–Adenine Dinucleotide Phosphate Oxidoreductase and Histidine–2-Oxoglutarate Aminotransferase in the Degradation of Imidazol-5-yl-lactate by *Pseudomonas acidovorans*

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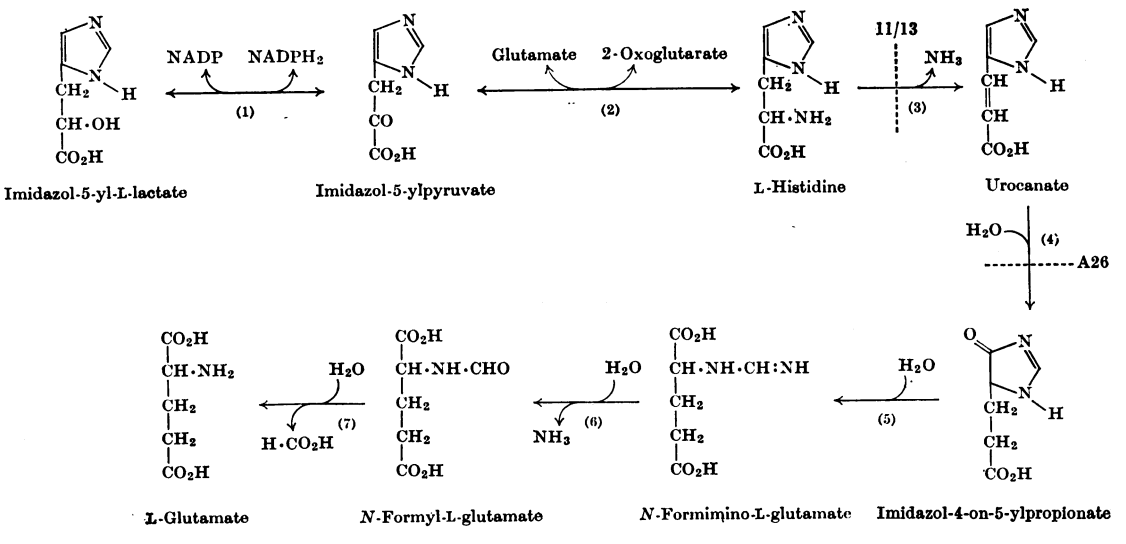
Imidazol-5-yl-L-lactate will serve as the sole source of carbon and energy for the growth of *Pseudomonas acidovorans* (Hassall, 1966). Washed cells of this organism [grown in a medium consisting of (g./l.): imidazol-5-yl-L-lactate, 2; $(NH_4)_2SO_4$, 1; KH_2PO_4, 2; $MgSO_4,7H_2O$, 0·2] were shown to oxidize imidazol-5-yl-L-lactate, L-histidine, urocanate, *N*-formimino-L-glutamate, *N*-formyl-L-glutamate and L-glutamate at similar rates; crude extracts of the cells were able to degrade all these compounds, with the exception of imidazolyl-lactate, to glutamate (Hassall, 1966). From these studies it was concluded that imidazolyl-lactate was metabolized to urocanate and that this was subsequently degraded to glutamate; the details of the reaction sequence from imidazolyl-lactate to urocanate remained obscure.

This communication presents evidence for the existence of two enzymes, imidazol-5-yl-lactate–NADP oxidoreductase (1) and histidine–2-oxoglutarate aminotransferase (2), involved in the degradation of imidazolyl-lactate as shown in Scheme 1.

The equilibrium position of the reaction catalysed by imidazolyl-lactate–NADP oxidoreductase is in favour of imidazolyl-lactate formation. The enzyme was therefore assayed spectrophotometrically at 340mμ in a reaction mixture containing $NADPH_2$ and imidazol-5-ylpyruvate. The system consisted of 5μmoles of imidazol-5-ylpyruvate, 0·5μmole of $NADPH_2$, 1ml. of 0·1M-KH_2PO_4 adjusted to pH7·0 with 5N-NaOH, 0·1ml. of enzyme preparation, and water to a total volume of 3ml. This, and other spectrophotometric assays, were carried out at 25° in a Gilford multiple-sample absorbance recorder fitted with a Unicam SP.500 monochromator. In crude extracts, the activity of the oxidoreductase with $NADH_2$ was 58% of that with



Scheme 1. Pathway of imidazolyl-lactate degradation by *Ps. acidovorans*. 'A26' and '11/13' refer to mutants described in the text.

$NADPH_2$ (namely 7 and 12 mμmoles/min./mg. of protein respectively).

Histidine–2-oxoglutarate aminotransferase was assayed initially by following the decrease in E_{282} of a reaction mixture containing imidazol-5-ylpyruvate (ε 2250) and L-glutamate. The system consisted of 1 μmole of imidazol-5-ylpyruvate, 10 μmoles of L-glutamate, 1 ml. of 0·1 M-KH_2PO_4 adjusted to pH 7·0 with 5 N-NaOH, 0·1 ml. of enzyme preparation, and water to a total volume of 3 ml. The activity of the enzyme in crude extracts, when assayed in this way, was 30 mμmoles/min./mg. of total protein. In the absence of glutamate no change in extinction occurred; no activity was observed if either L-alanine or L-aspartate replaced glutamate.

A more sensitive assay was used for the aminotransferase based on the method used by Spolter & Baldridge (1963) for the assay of histidine–pyruvate aminotransferase. The formation of the enol–borate complex of imidazolylpyruvate was followed spectrophotometrically at 293 mμ (ε 12000) on incubation of the enzyme with histidine and 2-oxoglutarate in borate–arsenate buffer; EDTA was added to the reaction mixture to inhibit histidase (histidine ammonia-lyase, EC 4.3.1.3). The complete system consisted of 2 m-moles of sodium borate, 20 μmoles of sodium arsenate, 80 μmoles of EDTA, 100 μmoles of L-histidine, 100 μmoles of 2-oxoglutarate and 0·1 ml. of enzyme preparation in a total volume of 3·5 ml. The pH was brought to 8·0 by the addition of 2 N-HCl to the borate–arsenate solution and the enzyme was preincubated with pyridoxal phosphate (150 μg./ml.) for 10 min. before the beginning of the assay. The activity in crude extracts was 150 mμmoles/min./mg. of protein. If the incubation with pyridoxal phosphate was omitted, the activity was lowered by 26%. No activity was observed if the 2-oxoglutarate was replaced by glyoxylate, pyruvate, 2-oxobutyrate or 2-oxoisocaproate.

The physiological role of these enzymes was studied with mutants of *Ps. acidovorans* prepared by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The mutants were isolated by plating samples of the treated culture on to agar plates containing 0·5 g. of histidine or urocanate/l., 0·02 g. of glutamate/l. and 0·5 g. of $(NH_4)_2SO_4$/l. Mutant cells unable to utilize the imidazole but able to grow on glutamate formed pin-point colonies.

Mutant A26 (urocanase$^-$) isolated in this way does not grow on imidazolyl-lactate or histidine. If it is grown on succinate in the presence of either of these compounds, urocanate accumulates in the medium. The activity towards any precursor of urocanate can be assayed spectrophotometrically, with whole cells, by following the increase in E_{277} due to urocanate (ε 19000). Cells grown on histidine (as nitrogen source) and succinate have activity towards imidazolyl-lactate only 40% lower than the activity of cells grown on succinate in the presence of imidazolyl-lactate. Washed cells of this mutant grown on histidine and succinate or imidazolyl-lactate and succinate accumulate urocanate from imidazolylpyruvate as well as from imidazolyl-lactate. Cell-free extracts of mutant A26 grown in the presence of imidazolyl-lactate show no change in E_{277} on incubation with imidazolylpyruvate unless glutamate is added to the system; a steady increase in extinction is then observed due to the formation of urocanate.

The accumulation of urocanate from imidazolyl-lactate by cell-free extracts of A26 (urocanase$^-$) was demonstrated chromatographically. The reaction mixture consisted of 10 μmoles of imidazolyl-lactate, 10 μmoles of glutamate, 0·5 μmole of NADP and 1 mg. of extract in a total volume of 0·6 ml. at pH 7·5. Samples of 0·1 ml. were removed at intervals between 0 and 60 min. after the start of the reaction. Each sample was heat-treated at 100° for 1 min. and 0·05 ml. of 2 N-HCl was then added to complete the precipitation of the protein. This was removed by centrifugation, and 0·05 ml. of the supernatant solution was chromatographed on Whatman no. 1 paper in the organic phase of 2-methylpropan-1-ol–formic acid–water (19:2:6, by vol.). The imidazole derivatives were detected by spraying the paper with diazotized *p*-chloroaniline and then holding it in ammonia vapour. Two different spots were visible, the first due to imidazolyl-lactate (R_F 0·09; orange) decreasing in intensity with time and the second due to urocanate (R_F 0·22; orange–brown) increasing in intensity.

When whole cells of the urocanase$^-$ mutant were incubated with imidazolyl-lactate in a similar system to that described above, but without added NADP or glutamate, chromatographic analysis showed an accumulation of imidazolylpyruvate (R_F 0·16; brown) as well as urocanate. This can be explained as being due to a lack, in the mutant, of sufficient glutamate, necessary for the complete transamination of imidazolylpyruvate to histidine.

Mutant 11/13 is a 'leaky' histidase$^-$ mutant with less than 10% of the inducible histidase activity of the parent organism. It grows readily on urocanate but only weak growth is discernible on histidine or imidazolyl-lactate after 4–5 days. Revertants of this mutant, selected as single colonies after heavy plating of the organism on histidine or imidazolyl-lactate plates and incubation for at least a week, grow readily on both histidine and imidazolyl-lactate. This suggests that a single mutation (histidase$^-$) is responsible for the lack of growth of 11/13 on histidine or imidazolyl-lactate.

Mutant 11/13 when grown on urocanate shows

fully induced activities of imidazol-5-yl-lactate–NADP oxidoreductase and histidine–2-oxoglutarate aminotransferase, and accumulates histidine when incubated with imidazolylpyruvate and glutamate. This is in agreement with the suggestion that urocanate is an inducer for the enzymes that metabolize imidazolyl-lactate (Hassall, 1966).

Imidazolyl-lactate–NADP oxidoreductase and histidine–2-oxoglutarate aminotransferase have also been reported in *Escherichia coli* B grown in the presence of L-histidine (Hedegaard, Brevet & Roche, 1966; Wickramasinghe, Hedegaard & Roche, 1967; Cortese, Brevet & Hedegaard, 1968). However, whereas these authors suggest that the enzymes function in *E. coli* B for the degradation of histidine via imidazolyl-lactate, it would appear that in *Ps. acidovorans* they function to degrade imidazolyl-lactate and imidazolylpyruvate via histidine. A pathway for the degradation of histidine or imidazolyl-lactate other than through the reaction catalysed by urocanase is most unlikely in this organism, since mutant A26, lacking this enzyme, will not grow on histidine.

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