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Properties and Function of Fumarate Reductase (NADH) in *Streptococcus lactis*

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Abstract

The fumarate reductase (NADH) present in cell-free extracts of *S. lactis* C10 was purified approximately 100-fold by chromatography on DEAE-cellulose in the presence of the non-ionic detergent Teric X-10, and some of the properties of this partially purified enzyme were characterized. Fumarate was able to act as a terminal electron acceptor and decreased the amount of lactate formed and oxygen used during the metabolism of pyruvate by resting cells of *S. lactis*. Anaerobic growth of *S. lactis* on glycerol was not observed and fumarate reduction was not coupled with glycerol-3-phosphate oxidation.

Introduction

The enzyme fumarate reductase (NADH) (EC 1.3.1.6) has been shown to catalyse the reduction of fumarate to succinate (fumarate+NADH+H^+→succinate+NAD^+) in *Streptococcus faecalis* (Jacobs and Vandemark 1960; Aue and Deibel 1967; Faust and Vandemark 1970), *Proteus rettgeri* (Kroger 1974), *Escherichia coli* (Miki and Lin 1973), *Mycobacterium phlei* (Bogin *et al.* 1969) and *Saccharomyces cerevisiae* (Hauber and Singer 1967). This enzyme is quite distinct from succinate dehydrogenase (EC 1.3.99.1), which catalyses the oxidation of succinate to fumarate in that (i) the succinate dehydrogenase catalysed oxidation of succinate is reversible, while the reduction of fumarate by fumarate reductase (NADH) is essentially irreversible; (ii) the flavin moiety (FAD) of succinate dehydrogenase is covalently bound to the enzyme, while the flavin moiety of fumarate reductase (NADH) is not (Singer *et al.* 1973); (iii) both enzymes have been detected in cells of *E. coli* (Hirsch *et al.* 1963) and mutants of *E. coli* have been isolated in which only one of the enzyme activities has been lost (Spencer and Guest 1973).

Fumarate reductase (NADH) is generally found in obligate or facultative anaerobes, while succinate dehydrogenase is generally found in aerobic organisms. The reduction of fumarate to succinate has been shown to be coupled with the oxidation of reduced pyridine nucleotides (Aue and Deibel 1967; Miki and Lin 1973) and under anaerobic conditions fumarate may replace O_2 as a terminal electron acceptor in some organisms (Jacobs and Vandemark 1960; Miki and Lin 1973). Fumarate reductase (NADH) has been coupled to oxidative phosphorylation in *S. faecalis* (Faust and Vandemark 1970), to the anaerobic oxidation of glycerol in *S. faecalis* (Jacobs and Vandemark 1960) and *E. coli* (Miki and Lin 1973), and to

the anaerobic transport of lactose in membrane vesicles of *E. coli* (Konings and Kaback 1973).

In the present investigation, fumarate reductase (NADH) was isolated and partially purified from *S. lactis* and its role in the metabolism of this organism studied.

Materials and Methods

Bacteria

S. lactis strains C10 and C2 (obtained from stock cultures at the CSIRO Dairy Research Laboratory, Highett, Vic.) were maintained by daily subculture in sterile skim milk (10% w/v) and grown in quantity in broth containing tryptone (Oxoid), 30 g/l; yeast extract (Difco), 10 g/l; lactose, 30 g/l; KH_2PO_4, 5 g/l and 'Lab-Lemco' powder (Oxoid), 2 g/l. The medium was filtered and autoclaved at 121°C for 10 min. The lactose was autoclaved separately before being added to the sterile medium.

The broth was inoculated from the skim milk cultures and incubated at 30°C until the cells reached the early stationary phase. The pH of the broth was maintained at 6·3 by the addition of 10 M NaOH controlled by a magnetic valve connected to a Radiometer Titrator Model TTT 11b (Radiometer, Copenhagen NV, Denmark). The cells were harvested from the growth medium by centrifugation at 24 000 *g* for 10 min at 4°C, washed twice with potassium phosphate buffer (0·1 M, pH 6·5) and resuspended in the same buffer at a concentration of 1 g (wet wt)/5 ml buffer.

Cell-free extracts were prepared by extruding the cells through a French pressure cell (French and Milner 1955) at a pressure of 154 MPa. Unbroken cells and cellular debris were removed by centrifugation at 35 000 *g* for 30 min at 4°C.

Assay of Enzyme Activities

Fumarate reductase (NADH) (hereafter called fumarate reductase) activity was measured anaerobically in a Thunberg-type cuvette by a modification of the method of Massey and Singer (1957). The reaction mixture (3·0 ml) contained (in μmoles): reduced flavin adenine dinucleotide ($FADH_2$), 0·7; sodium fumarate, 50; KH_2PO_4 (pH 6·5), 230; and cell-free extract. The reaction was followed by measuring the change in absorbance at 450 nm and the amount of flavin oxidized was determined using the molar extinction coefficient ε_{450} (FAD–$FADH_2$) = 11 330 M^{-1} cm^{-1} (Dairson *et al.* 1969).

Glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) activity coupled to fumarate reduction was assayed as described by Miki and Lin (1973).

Succinate dehydrogenase. Succinate oxidation was measured using the maximum concentration of the reagents described by Aue and Deibel (1967).

Partial Purification of Fumarate Reductase

Treatment with streptomycin sulfate

Nucleic acids were removed from the cell-free extracts of *S. lactis* C10 by treatment with streptomycin sulfate as described previously (Mou *et al.* 1972), and the resultant solution was dialysed overnight against phosphate buffer (0·1 M, pH 6·5).

Fractionation with ammonium sulfate

Sufficient solid ammonium sulfate was added to the dialysate to give a 25% (w/v) saturated solution. After standing at 4°C for 2 h the precipitate was removed by centrifugation (30 000 *g* for 20 min) and the supernatant brought to 60% saturation by the addition of solid ammonium sulfate. After standing at 4°C for 2 h, the precipitate obtained after centrifugation at 35 000 *g* for 20 min was dissolved in the minimum amount of phosphate buffer (0·1 M, pH 6·5) and dialysed overnight against the same buffer.

Treatment with Teric X-10

Preliminary experiments indicated that the enzyme, obtained at this stage of the purification, was totally excluded from Sephadex G200 and Sepharose 4B, which suggested a molecular weight

n excess of 20×10^6. It seemed likely therefore that the enzyme had either aggregated or was associated with particulate materials still present in the cell-free extracts. The enzyme was dissociated from its high molecular weight form by treating the ammonium sulfate fraction with 0·5% (w/v) Teric X-10 (I.C.I. Australia, Ltd.) which has been used to solubilize membrane proteins (Umbreit and Strominger 1973; Osborne *et al.* 1974).

Chromatography on DEAE-cellulose

Portions (*c.* 400 mg protein) of the 25–60% ammonium sulfate fraction (treated with Teric X-10) were applied to a 2·5 by 54 cm glass column packed with DEAE-cellulose (Whatman) which had been equilibrated with phosphate buffer (0·1 M, pH 6·5). The fumarate reductase did not bind to the DEAE-cellulose and was eluted with the void volume of the column, as was the Teric X-10. The enzyme was concentrated using a Diaflo ultrafiltration cell containing an XM-50 membrane, and applied to a second DEAE-cellulose column (2·5 by 54 cm) equilibrated with phosphate buffer (0·1 M, pH 6·5) containing 0·5% (w/v) Teric X-10. Approximately half of the protein which did not bind to the first DEAE-cellulose column (equilibrated in the absence of Teric X-10) bound to the second DEAE-cellulose column (equilibrated in the presence of Teric X-10). However, the fumarate reductase activity was still eluted with the void volume of the column. The fumarate reductase obtained after the second DEAE-cellulose column was used without further purification and is referred to as the partially purified fumarate reductase.

Estimation of protein

Protein in solutions which did not contain the non-ionic detergent Teric X-10 was measured by the method of Lowry *et al.* (1951). Protein in the presence of Teric X-10 was estimated by the method of Udenfried *et al.* (1972). Egg white lysozyme (Sigma, grade I) was used as the protein standard.

Absorbance spectra

These were obtained on a Cary Recording Spectrophotometer, model 14 (Applied Physics Corporation, Manvovia, California).

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide gels (5% w/v) were prepared by dissolving 2·5 g acrylamide, 0·07 g *N,N'*-methylene-bis-acrylamide, 100 mg ammonium persulfate and 0·1 ml β-dimethylaminopropionitrile in 50 ml of 0·1 M Tris buffer, adjusted to pH 9·6 with boric acid. Excess ammonium persulfate was removed from the gels (in 50 by 6 mm tubes) by pre-electrophoresis in the pH 9·6 Tris–boric acid buffer for 1 h at 5 mA per tube. Samples (50–100 μl) containing 0·02–1·0 mg protein, 15% (w/v) sucrose and bromophenol blue were layered onto the gels and a current of 5 mA per tube was applied until the bromophenol blue marker reached the bottom of the gel. Gels were stained for protein with 0·1% (w/v) amidoschwarz in 7% (v/v) acetic acid for 2 h before destaining with 7% acetic acid. Fumarate reductase activity was located by immersing the gels in a stoppered tube containing the reaction mixture described previously for the estimation of fumarate reductase. The enzyme was located where yellow bands indicated that the reduced flavin present in the reaction mixture had been oxidized.

Utilization of Fumarate and Excretion of Succinate by Growing Cells

S. lactis C10 grown in protein–digest broth at 30°C and pH 6·3 and continually sparged with a gas mixture containing 95% N_2 and 5% CO_2, was used to inoculate two growth vessels each containing 400 ml of protein–digest broth. After incubation for 1 h, fumarate (final concentration 10 mM) was added to one of the vessels and an equal volume of distilled water was added to the other. Samples were removed at regular intervals and the absorbance at 650 nm was determined as a measure of cell growth. The cells were removed by centrifugation and the amount of fumarate in the supernatant was estimated by the method of Williamson and Corkey (1969). Succinate was determined by gas chromatography as described by Hillier and Jago (1978*a*).

Effect of Fumarate on Pyruvate Metabolism

S. lactis C10 was grown to early stationary phase in protein–digest broth at 30°C and pH 6·3, harvested by centrifugation at 4000 *g* for 10 min, washed, and resuspended in 0·2 M NaH_2PO_4 (pH 5·5 containing 3·3 mM $MgCl_2$) at a concentration of 20 mg (dry wt)/ml. The cells were incubated at 30°C in Warburg flasks which contained (in μmoles): NaH_2PO_4 (pH 5·5), 298; $MgCl_2$, 5; sodium pyruvate, 60; fumaric acid (adjusted to pH 5·5 with 1 M NaOH), 15; and 10 mg (dry wt) of cells. The reaction was stopped by rapidly cooling the contents of each flask in ice-water. The cells were removed by centrifugation at 35 000 *g* for 15 min at 4°C and the supernatants were assayed for residual pyruvate and products of pyruvate metabolism.

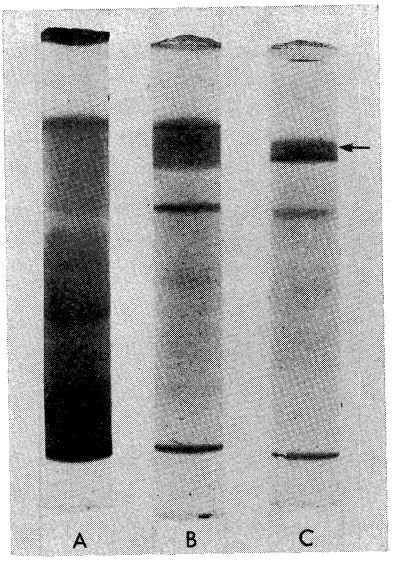


Fig. 1. Electrophoretic patterns on acrylamide gels of fumarate reductase extracted from *S. lactis* C10 at various stages of purification as set out in the following tabulation (enzyme activity expressed as nmoles $FMNH_2$ oxidized per minute per milligram protein):

Fraction	Volume (ml)	Total protein (mg)	Specific Activity	Purification (fold)
Cell-free extract	80	1856	25·7	1
Ammonium sulfate, 25%–60% (gel A)	25	952·5	41·6	1·6
DEAE-cellulose, no Teric X-10 (gel B)	12·0	23·8	1473	57·3
DEAE-cellulose, plus Teric X-10 (gel C)	9·5	8·7	2686	104·6

Arrow indicates bands containing fumarate reductase activity.

Estimation of Pyruvate and the Products of Pyruvate Metabolism

Pyruvate was estimated by the enzymic method of Von Korff (1969).

Acetate was estimated as a volatile acid as described by Anders and Jago (1970) except that the distillate was titrated to pH 7·0 with 5 mM NaOH delivered from a Radiometer autoburette (model ABU 1c).

Lactate was estimated by a modification of the enzymatic method of Scholz *et al.* (1959). The specific activity of the lactate dehydrogenase used was 815 units/mg and the change in optical density was measured after incubation at 25°C for 1 h.

Carbon dioxide evolution and *oxygen* uptake were determined manometrically as described by Umbreit *et al.* (1957). The values for CO_2 evolution were corrected for the retention of CO_2 in solution as bicarbonate.

Acetoin, butane-2,3-diol, diacetyl and *ethanol* were determined quantitatively by gas chromatography as described by Coventry *et al.* (1978).

Results

Properties of the Partially Purified Fumarate Reductase from S. lactis *C10*

The specific activity of the partially purified fumarate reductase used in this study was approximately 100 times greater than that initially found in the unpurified cell-free extracts but still exhibited a number of bands on polyacrylamide gels (Fig. 1).

pH optimum

The effect of pH on fumarate reductase activity in partially purified cell-free extracts is shown in Fig. 2. Two distinct peaks of activity (at pH 5·6 and 6·6) were observed in phosphate buffer, but only a single peak of activity was observed in phosphate–citrate buffer. The pH profiles obtained with unpurified cell-free extracts were essentially the same as those obtained with the partially purified cell-free extract. The reason for the single peak of activity in phosphate–citrate buffer is not known.

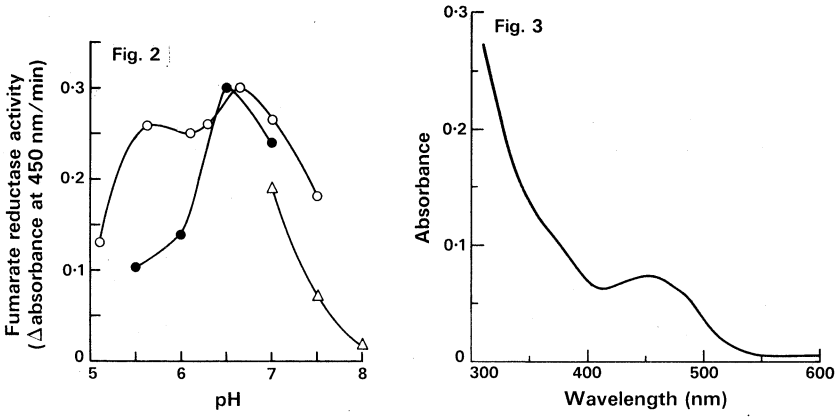


Fig. 2. Effect of pH on the activity of fumarate reductase extracted from *S. lactis* C10. The assay conditions are described under Methods. ○ 0·1 M phosphate. ● 0·1 M phosphate–citrate. △ 0·1 M Tris–HCl.

Fig. 3. Absorbance spectrum of fumarate reductase extracted from *S. lactis* C10. The absorbance spectrum of the fumarate reductase fraction (0·9 mg protein/ml) was recorded as described under Methods.

Specificity of substrates

Several fumarate analogues were tested for their ability to act as substrates or inhibitors of the partially purified fumarate reductase (Table 1). Maleic acid was 90% as effective a substrate as fumarate, but cinnamic and acrylic acids were poor substrates which suggested that the substrate must be a dicarboxylic acid. Citraconic and mesaconic acids, in which a methyl group replaces a hydrogen atom on fumarate, were even less effective as substrates. Mesaconic acid was the most inhibitory of

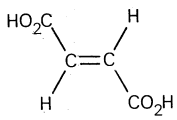
the analogues, while succinate, the product of the reaction, was not inhibitory unless preincubated with the enzyme. No oxidation of succinate to fumarate was detected using the phenazine methosulfate assay described under Methods.

Table 1. Effect of fumarate analogues on the activity of fumarate reductase

Analogues were present at the same concentration as fumarate and, where indicated, were preincubated with $FADH_2$ and enzyme for 15 min at 30°C. N.P., no preincubation of analogue with enzyme; P, analogue preincubated with enzyme and $FADH_2$

Analogue[A]	Activity as a substrate (%)	Inhibition (%) N.P.	P	Analogue[A]	Activity as a substrate (%)	Inhibition (%) N.P.	P
1. Fumaric acid	100	0	0	6. Mesaconic acid	4	61	61
2. Maleic acid	91	—	—	7. Tiglic acid	0	0	0
3. Cinnamic acid	10	0	27	8. Crotonic acid	0	27	30
4. Acrylic acid	11	0	8	9. Succinic acid	0	0	30
5. Citraconic acid	5	0	20	10. Malonic acid	0	7	8

[A] Structures as follows: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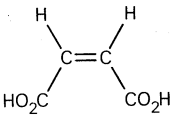
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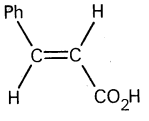
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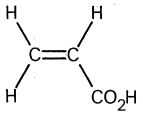
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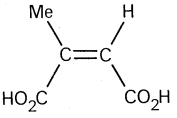
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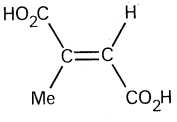
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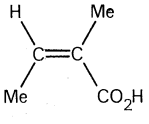
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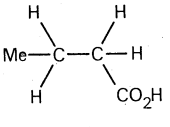
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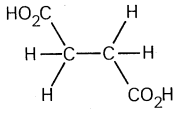
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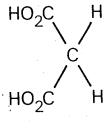
(10)

Absorbance spectrum

The absorbance spectrum (in the range 300–600 nm) of the partially purified enzyme (which still exhibited at least three protein bands on polyacrylamide gels) is shown in Fig. 3. The spectrum was similar to that obtained for the fumarate reductase from yeast (Tisdale *et al.* 1968), and the absorbance maximum at 450 nm suggested the presence of a flavin group. This was shown by the method of Burch (1957) to be an FAD molecule which was not covalently bound to the protein.

Effect of Fumarate on the Metabolism of S. lactis

Fumarate has been shown to act as an electron acceptor and enable organisms such as *E. coli* and *S. faecalis* to grow anaerobically on glycerol (Jacobs and

Vandemark 1960; Miki and Lin 1973). However, *S. lactis* does not ferment glycerol aerobically and no growth was observed anaerobically in broth containing 2% (w/v) glycerol and 2% (w/v) sodium fumarate. Moreover, cell-free extracts prepared from cells grown anaerobically on lactose or glucose contained no glycerol-3-phosphate dehydrogenase activity in the presence or absence of fumarate. It would appear, therefore, that fumarate reductase activity is not associated with anaerobic glycerol-3-phosphate dehydrogenase activity in *S. lactis*.

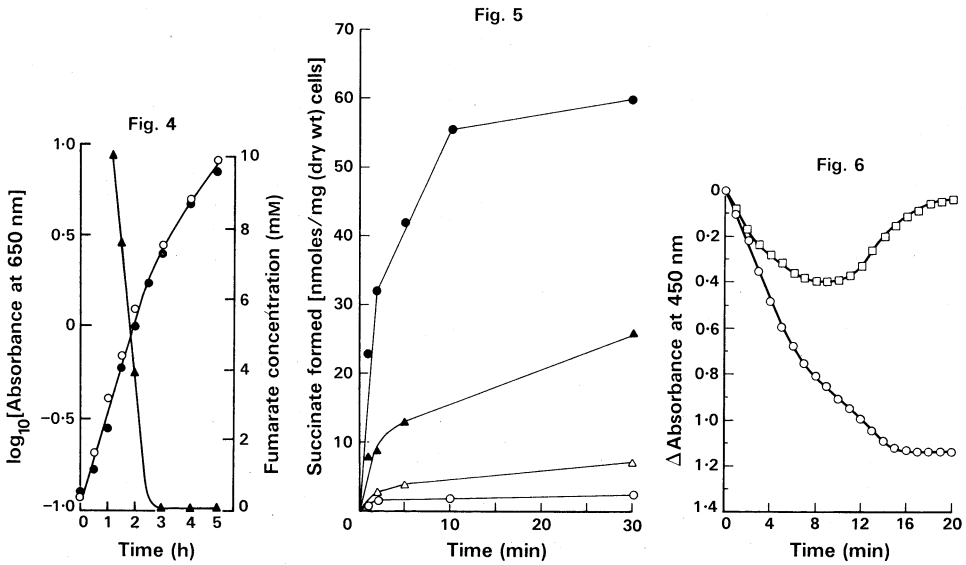

Fig. 4. Utilization of fumarate by growing cells of *S. lactis* C10. The organism was grown anaerobically at 30°C and pH 6·3 in protein–digest broth, as described under Methods. Fumarate (final concentration 10 mM) was added 1 h after incubation had commenced. At intervals, portions (2·0 ml) of the culture were taken and cell growth and residual fumarate were estimated as described under Methods. ● Growth in the absence of fumarate. ○ Growth in the presence of fumarate. ▲ Concentration of fumarate in growth medium.

Fig. 5. Effect of glucose and pyruvate on the conversion of fumarate to succinate by resting cells of *S. lactis* C10. The strain was incubated at 30°C with 20 μM [1,4-^{14}C]fumarate (2·5 μCi/μmole) in the presence or absence of 20 mM glucose or 20 mM pyruvate, as described under Methods. Samples were taken at the times indicated and the amount of succinate produced was determined, as described under Methods. ● With glucose. ○ Without glucose. ▲ With pyruvate. △ Without pyruvate.

Fig. 6. Effect of fumarate on the reduction of FAD by NADH in the presence of cell-free extracts of *S. lactis* C10. The reaction mixture (3·0 ml) contained (in μmoles): potassium phosphate (pH 6·5), 230; NADH, 0·68; FAD, 0·7; unpurified cell-free extract, approximately 3 mg protein, and where indicated, sodium fumarate, 50. The reaction was carried out under anaerobic conditions at 30°C. The reduction of FAD was followed by measuring the absorbance at 450 nm. ○ Absence of fumarate. □ Presence of fumarate.

Utilization of Fumarate

Hillier and Jago (1978*a*) have shown that *S. lactis* C10 can produce succinate from [^{14}C]bicarbonate and that the ratio of succinate formed to lactate formed can be as high as 1 in 100 (Hillier and Jago 1978*b*). While the mechanism by which

S. lactis synthesizes succinate is not known, it is known that fumarate is readily converted to succinate by growing cells of *S. lactis* (Hillier and Jago 1978*b*). The effect of fumarate on the growth of *S. lactis* C10 is shown in Fig. 4. While fumarate was rapidly used by the organism, there was no increase in the rate of growth in the presence of fumarate. Growth in the presence of fumarate also had no effect on the specific activity of fumarate reductase.

To determine the conditions under which resting cells were able to convert fumarate to succinate, *S. lactis* C10 was incubated with fumarate in the presence of glucose or pyruvate (Fig. 5). The metabolism of both glucose and pyruvate involves the formation of NADH which could then act as a source of reduced cofactors for the fumarate reductase reaction. Hillier and Jago (1978*b*) have shown that the fumarate reductase activity in *S. lactis* C10 was dependent on the presence of reduced FMN or FAD. No activity was observed in the presence of NADH or NADPH. However, since the NADH produced by the metabolism of glucose or pyruvate was able to reduce the fumarate, it is obvious that the cells must be able to convert NADH to $FADH_2$. This was probably due to the action of the NADH oxidase present in cell-free extracts of *S. lactis* C10 which has been shown to be flavin-linked (Anders 1967). When unpurified cell-free extracts were incubated anaerobically with NADH and FAD, the FAD was reduced (Fig. 6). When the experiment was repeated in the presence of fumarate, the FAD was partly reduced and then reoxidized, presumably via the fumarate reductase reaction (Fig. 6). It seemed likely therefore that fumarate could spare the NADH oxidizing reaction involved in the metabolism of glucose and pyruvate. The effect of fumarate on the products formed from pyruvate is shown in Table 2. Under aerobic conditions the utilization of oxygen

Table 2. Effect of fumarate on the metabolism of pyruvate by resting cells of *S. lactis* strains C10 and C2

Incubations were carried out as described under Methods and the results are expressed as μmoles of product produced or substrate used per 100 μmoles of pyruvate used

Substrate/product	Strain C10		Strain C2		Strain C2[A]	
	With fumarate	No fumarate	With fumarate	No fumarate	With fumarate	No fumarate
Pyruvate used	100	100	100	100	100	100
Fumarate used	28·6	0	54·7	0	84·7	0
O_2 used	14·3	31·9	—	—	—	—
CO_2 evolved	88·9	88·6	—	—	—	—
Acetate produced	76·2	83·9	68·7	68·6	93·2	33·3
Lactate produced	11·8	23·0	9·4	7·8	14·7	20·0
Acetoin produced	1·8	0	0	0	0	0
Butane-2,3-diol produced	6·8	0	0	0	0	32·6

[A] Incubated anaerobically.

and formation of lactate by strain C10 were markedly decreased in the presence of fumarate, while the level of acetate remained almost constant. Under anaerobic conditions, the production of acetate was markedly increased in the presence of fumarate, while the levels of butane-2,3-diol decreased in the presence of fumarate. The increased acetate production in the presence of fumarate was probably due to the coupling of fumarate reduction with the oxidation of the NADH produced by the pyruvate dehydrogenase system.

Discussion

The fumarate reductase from *S. lactis* C10 was purified approximately 100-fold by treatment with the non-ionic detergent Teric X-10, to dissociate the enzyme from some form of high molecular weight aggregate, and by ion-exchange chromatography on DEAE-cellulose. Since the fumarate reductase from some organisms have been shown to be associated with the particulate fraction of the cells (Faust and Vandemark 1970; Singh and Bragg 1975), it is possible that the Teric X-10 was required to solubilize the enzyme from a membrane.

The activity of *S. lactis* C10 fumarate reductase showed two distinct pH optima (at pH 5·6 and 6·6) when assayed in phosphate buffer, but only a single peak of activity (at pH 6·5) when assayed in phosphate–citrate buffer. A similar effect was observed with the fumarate reductase from *Propionibacterium pentosaceum* (Lara 1959) which had dual pH optima at pH 5·25 and 6·4 when assayed in acetate and phosphate buffers, but only a single pH optimum at pH 5·45 when assayed in citrate buffer. Dual pH optima have also been observed in the fumarate reductase purified from *Micrococcus lactilyticus* (Warringa and Guiditta 1958). The relative activity of the enzyme at various pH values remained constant throughout the purification procedure and after partial denaturation of the enzyme by heat, acid and alkali, suggesting that the dual pH optima were not due to two distinct enzyme species. It seems unlikely that the two peaks of activity are due to changes in the ionic form of fumarate since, even at pH 5·6, most of the fumarate (p*Ka* 3·03 and 4·54) would be in the fully ionized form.

The specificity of the fumarate reductase from *S. lactis* C10 was very similar to that of the enzyme from *S. faecalis* (Aue and Deibel 1967) and like the fumarate reductases from other organisms (Aue and Deibel 1967; Hauber and Singer 1967), was relatively insensitive to inhibition by succinate and did not oxidize succinate to fumarate. The fact that maleic acid was an effective substrate for fumarate reductase suggested that the enzyme was not specific for carboxyl groups in the *trans* position. However, the enzyme appeared to be specific for dicarboxylic acids, since acrylic acid was not an effective substrate.

The fumarate reductase isolated from *S. lactis* C10, unlike the enzyme isolated from other organisms including *S. faecalis* (Faust and Vandemark 1970), did not oxidize reduced NAD. However, crude cell-free extracts of *S. lactis* C10 were able to oxidize NADH anaerobically in the presence of FAD, with the production of $FADH_2$. As this cofactor was reoxidized in the presence of fumarate, the combined action of these enzymes thus couples the anaerobic oxidation of NADH with the reduction of fumarate to succinate. A similar sequence of reactions has been shown to occur in *S. faecalis* (Deibel and Kvetkas 1964).

The addition of fumarate to the growth medium has enabled some strains of *S. faecalis* and *E. coli* to grow anaerobically on glycerol (Gunsalus 1947; Jacobs and Vandemark 1960; Mika and Lin 1973). However, *S. lactis* C10 was unable to grow on glycerol under aerobic conditions or anaerobically in the presence of fumarate. Fumarate has also been shown to divert the normal fermentation of glucose by *S. faecalis* away from the production of lactate and ethanol to increased levels of CO_2, formate, acetate and acetoin (Deibel and Kvetkas 1974). Similarly, in this investigation, fumarate was shown to alter the pyruvate metabolism of resting cells of *S. lactis* under both aerobic and anaerobic conditions. Coupling of fumarate reduction

to the oxidation of reduced pyridine nucleotides appears therefore to substitute for oxygen (under aerobic conditions) or for the lactate dehydrogenase or D(−)-butanediol dehydrogenase reactions (under anaerobic conditions) as a terminal electron acceptor. The increased anaerobic production of acetate in the presence of fumarate suggests that the level of this compound in maturing cheese could be increased by adding small amounts of fumarate at the time of salting.

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