COMPARATIVE BIOCHEMISTRY OF THE BIOLOGICAL REDUCTION OF FUMARIC ACID*

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Fumaric acid can be utilized by a number of microorganisms as the terminal hydrogen acceptor for biological oxidation of various organic compounds and molecular hydrogen (*e.g.*, see KREBS[1]). The capacity to reduce fumarate to succinate in this manner provides such organisms with a mechanism for energy-yielding oxidations in the absence of molecular oxygen and this process is consequently of interest with regard to the energy metabolism of facultative and strict anaerobes.

In aerobic organisms, the oxidation of succinate to fumarate is effected by succinic dehydrogenase and it is generally assumed that the terminal catalyst required for fumarate reduction in anaerobic bacteria is either the same enzyme, acting in reverse, or a similar enzyme which may be operationally designated as a "fumaric reductase". The succinic dehydrogenases of yeast and mammalian tissues catalyze the reduction of fumarate to succinate, but at a slow rate as compared with the forward reaction[2]. FISCHER *et al.*[3] claimed the demonstration of a unidirectional fumaric reductase enzyme in yeast, but recent studies[2] have shown that this activity can probably be accounted for by a conventional succinic dehydrogenase acting in reverse.

Certain anaerobic bacteria, however, contain a reductase enzyme which reduces fumarate rapidly and oxidizes succinate at a comparatively low rate[4]. In organisms containing hydrogenase and reductase, molecular hydrogen can serve as the electron donor for reduction of fumarate[1,5,6]. The enzyme complex responsible for this reaction in *Escherichia coli* and similar bacteria is generally associated with the insoluble particulate fraction of such cells [4,7] and consequently has been difficult to analyze from an enzymic standpoint. On the other hand, the system is soluble, to a large extent at least, in extracts of the strict anaerobe *Micrococcus lactilyticus*. In the present report, the properties of the reductase in anaerobic bacteria are described and compared with those of the enzyme responsible for fumarate reduction in aerobic organisms. The purification and a study of the mechanism of action of the *M. lactilyticus* reductase will be reported elsewhere in a collaborative paper between this laboratory and the Enzyme Division of the Edsel B. Ford Institute for Medical Research[8].

EXPERIMENTAL

Cultivation of organisms and preparation of extracts

M. lactilyticus (strain 221) was grown in deep stationary culture at 37 C in a medium[9] containing: sodium lactate, 2%; peptone, 2%; yeast extract, 0.2%; NaCl, 0.5%; K_2HPO_4, 0.25%. Tap

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water was employed to make the medium to volume and the pH was adjusted to 7.3 before autoclaving. After 16 hours, the cells were harvested in a Sharples centrifuge and ground with Alumina A301 (2.5 g alumina per g wet weight of cells). After extracting the mixture with distilled water (1.5 ml per g wet weight of cells), alumina and cell debris were removed by centrifugation at 16,000 × *g* for 10 min. The supernatant fluid was further clarified by centrifugation at 20,000 × *g* for 1 hour and the extract was then stored under a hydrogen atmosphere at −20° C.

Clostridium pasteurianum (strain W5) was grown on the complex medium described by WILSON *et al.*[10] and cell-free extracts prepared as noted above. *C. butylicum* (Northern Regional Research Laboratory No. B-593) was cultivated in the same medium and the harvested cell paste ground with glass and extracted according to the procedure of PECK AND GEST[11]. The particulate preparation from *E. coli* was prepared according to GEST[7] and dried cells of *C. kluyveri* were kindly supplied by Dr. G. DAVID NOVELLI.

Assays

Hydrogen or oxygen utilization and carbon dioxide production were measured at 30° C, in most instances using Warburg vessels of approximately 10 ml capacity. Succinic dehydrogenase activity was determined manometrically employing methylene blue[12], phenazine methosulfate[13], or ferricyanide[14]. Oxygen was the final electron acceptor and hydrogen peroxide was assumed to be the final product with the two dyes. Succinic dehydrogenase activity was also determined by following the reduction of ferricyanide at 400 mμ (Beckman spectrophotometer (Model DU)) in the presence of cyanide[12].

Protein was determined by dry weight, the trichloroacetic acid method of BÜCHER[15] and the Folin-phenol method of LOWRY *et al.*[16]

RESULTS AND DISCUSSION

Activities in crude preparations; assay of fumaric reductase activity

Although intact cells of *M. lactilyticus* reduce fumarate with molecular hydrogen, the crude cell-free extract prepared by grinding with alumina ordinarily does not catalyze this reaction. Rapid utilization of hydrogen with concomitant reduction of fumarate is observed, however, if the extract is supplemented with catalytic amounts of "one-electron" viologen dyes (benzyl or methyl)[4]. This observation suggests that a factor required for coupling of hydrogenase with fumaric reductase is destroyed or diluted to a low level during preparation of the extract. The viologen dye apparently initiates the overall reaction by substituting for a natural electron carrier as follows:

$$H_2 + 2 \text{ viologen} \rightleftharpoons 2 \text{ reduced viologen} + 2H^+ \quad (1)$$

$$2 \text{ reduced viologen} + 2H^+ + \text{fumarate} \longrightarrow 2 \text{ viologen} + \text{succinate} \quad (2)$$

$$H_2 + \text{fumarate} \longrightarrow \text{succinate} \quad (3)$$

Reaction (1) is catalyzed by hydrogenase and is not readily reversible using the benzyl dye ($E_0' = -0.359$V). The methyl dye has a lower redox potential ($E_0' = -0.446$V) and with this dye, reaction (1) is readily reversible[11].

Reaction (2) is ascribed to fumaric reductase and is apparently irreversible since viologen dyes are not reduced by the extract in the presence of succinate.

The foregoing coupled reactions provide a method for the quantitative assay of fumaric reductase activity. In order to ensure that the reductase is the limiting component in the assay mixture, an excess of hydrogenase is added. Any hydrogenase preparation which rapidly reduces viologen dyes and does not exhibit fumaric reductase activity may be utilized for this purpose. Crude extracts of *Clostridium butylicum*[11] or *C. pasteurianum* meet these requirements. The latter is generally preferable because the hydrogenase is usually present in higher specific activity and is less subject to inactivation that that of *C. butylicum*. In the presence of excess hydrogenase, hydrogen utilization is proportional to fumaric reductase (in *M. lactilyticus* extract)

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concentration as illustrated in Fig. 1*. Similar proportionality between activity and enzyme concentration has been found with purified reductase preparations using either benzyl or methyl viologens[8]. Fumaric acid is the only acceptor which we have found to be reduced by this system; maleate, the *cis*-isomer of fumarate, is not reduced.

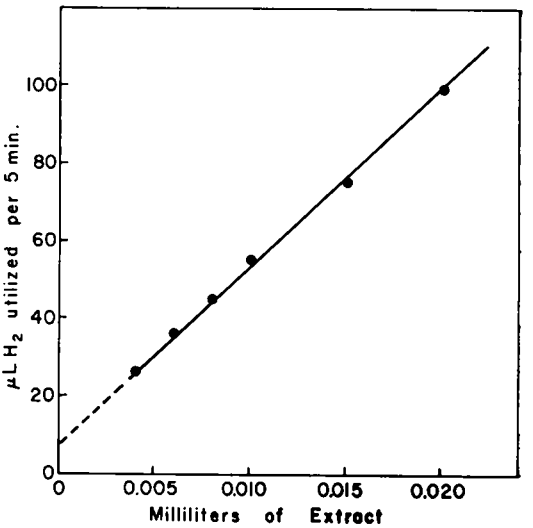

Fig. 1. Relationship between enzyme concentration and fumaric reductase acticity as measured by coupling with hydrogenase. Conditions: 0.0625 *M* phosphate pH 7.3, 20 μmoles fumarate, 1.2 μmoles benzyl viologen, 0.2 ml *C. butylicum* extract, *M. lactilyticus* extract as indicated, 0.1 ml 20% KOH in center well. Final fluid volume, 1.2 ml; gas phase, hydrogen; temp., 30 C.

The crude *M. lactilyticus* extract exhibits succinic dehydrogenase activity with methylene blue, phenazine methosulfate, ferricyanide or oxygen serving as electron acceptor (see Table I). The reaction with oxygen is inhibited by cyanide. Cytochrome *c* is not reduced by the preparation and in this connection it is of interest that cytochrome *b* appears to be absent from intact cells of *M. lactilyticus*[17].

TABLE I

SUCCINIC DEHYDROGENASE AND FUMARIC REDUCTASE ACTIVITIES IN BACTERIAL PREPARATIONS

Activity	*μmoles substrate utilized/10 min/mg protein*	
	M. lactilyticus extract	*E. coli particles*
Fumaric reductase[a]	8.6	8.1
Succinic dehydrogenase with:		
phenazine methosulfate[b]	0.089	0.57
methylene blue[c]	0.13	0.57
ferricyanide (optical)[d]	0.267	—
ferricyanide (manometric)[e]	0.039	0.185

[a] 75 μmoles phosphate buffer pH 7.6, 40 μmoles fumarate, 1.2 μmoles benzyl viologen, 0.1 ml *C. pasteurianum* extract, 0.1 ml 20% KOH in center well. Total volume, 1.2 ml; gas phase, hydrogen.

[b] 75 μmoles phosphate buffer pH 7.6, 40 μmoles succinate, 2 mg phenazine methosulfate, 1 μmole KCN, 0.1 ml 20% KOH in center well. Total volume, 1.2 ml; gas phase, air.

[c] 50 μmoles phosphate buffer pH 7.6, 40 μmoles succinate, 2.4 μmoles methylene blue, 8 μmoles KCN. Total volume, 1.2 ml; gas phase, air.

[d] 75 μmoles phosphate buffer pH 7.6, 40 μmoles succinate, 1.5 μmoles $K_3Fe(CN)_6$, 10 μmoles KCN, Total volume, 3.0 ml; gas phase, air; temperature, 25 C.

[e] 30 μmoles $NaHCO_3$, 40 μmoles succinate, 15 μmoles $K_3Fe(CN)_6$. Total volume, 3.2 ml; gas phase, 5% CO_2 plus 95% N_2.

* A detailed analysis of this method and description of its application for assay of reductase activity in a variety of preparations will be published elsewhere. The reductase activities indicated in Fig. 1 are not necessarily maximal, but refer to the specific conditions employed.

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Table I summarizes the relative fumaric reductase and succinic dehydrogenase activities typically observed in *M. lactilyticus* extract and in *E. coli* particles. It is evident that in these bacterial preparations the reductase activity greatly exceeds the succinic dehydrogenase activity. It should be noted that the phenazine methosulfate method is considered to be a primary assay for mammalian succinic dehydrogenase[18]. Depending on the method of assay of the latter enzyme, the activity ratio: fumaric reductase/succinic dehydrogenase varies from 30 to 220 in *M. lactilyticus* extract and from 14 to 40 in the *E. coli* preparation. In heart muscle and yeast preparations[2], on the other hand, succinic dehydrogenase activity greatly exceeds that of fumaric reductase, *e.g.* highly purified mammalian heart succinic dehydrogenase shows a ratio of about 0.1 (see ref. [19]). Even if it is assumed that the most valid ratios for the bacterial enzymes are the lower values (*i.e.*, 14 to 30), the striking difference between these and the ratio observed for mammalian and yeast succinic dehydrogenases suggests a basic difference in properties of the latter enzymes and the bacterial enzyme responsible for fumarate reduction.

In contrast with the crude *E. coli*, yeast, and mammalian preparations, the fumaric reductase and succinic dehydrogenase activities in the *M. lactilyticus* extract are soluble and remain in the supernatant fluid after centrifugation at 120,000 × *g* for 2 hours in the Spinco centrifuge.

Identification of the reaction product

An extract of *M. lactilyticus* supplemented with excess *Clostridium* hydrogenase and benzyl viologen, was incubated with 10 μmoles of fumarate under an atmosphere of hydrogen. As shown in Table II, approximately 9.1 μmoles of hydrogen were consumed. At this point the linear utilization of H_2 ceased abruptly. The reaction mixture was deproteinized by addition of acid and after neutralization and concentration the succinic acid content of an aliquot was determined using mammalian succinic dehydrogenase as described by UMBREIT *et al.*[20]. As indicated, 9.2 to 9.6 μmoles of succinic acid were found. Similar results have been obtained using the purified reductase[21]. It may be concluded that succinate is the only product of the reaction. This result and the linear course of the reaction indicates that the reductase possesses a high affinity for fumarate and, in contrast with mammalian succinic dehydrogenase, that the reaction product does not significantly inhibit activity of the enzyme.

TABLE II

IDENTIFICATION OF THE REACTION PRODUCT AS SUCCINATE

Expt.	*μmoles H_2 utilized*	*μmoles succinate found*
1.	9.10	9.60
2.	9.18	9.23

Conditions: 125 μmoles phosphate buffer pH 7.3, 10 μmoles fumarate, 1.2 μmoles benzyl viologen, 0.2 ml *C. butylicum* extract, 0.03 ml *M. lactilyticus* extract. Total volume, 2.4 ml; gas phase, hydrogen; temperature, 30 C. After cessation of H_2 uptake, the mixture was acidified with 0.2 ml of 5*N* H_2SO_4.

Effects of malonate and succinate

Both the oxidation of succinate and reduction of fumarate by mammalian preparations are appreciably inhibited by malonate, fumarate reduction being somewhat less

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sensitive to this competitive inhibitor[22]. In addition, both reactions are inhibited by the reaction product[22]. Thus, during the assay of soluble mammalian succinic dehydrogenase the forward reaction rate is linear only if the fumarate formed is continuously removed[18]. Yeast succinic dehydrogenase shows similar properties[2].

The succinic dehydrogenase activity in *M. lactilyticus* extract is inhibited by malonate to approximately the same degree as the mammalian enzyme, *i.e.*, about 70% with a malonate/succinate ratio of 1 : 4. In contrast with mammalian heart succinic dehydrogenase, however, the reductase activity in the *Micrococcus* preparation is relatively insensitive to this inhibitor. Thus with a malonate/fumarate ratio of 10 : 1, the reaction is inhibited only to the extent of 26%; with a 20 : 1 ratio, the inhibition is only 29%. Similar results have been observed with the purified reductase[8] and with the *E. coli* particulate preparation.

Although the succinic dehydrogenase activity in *M. lactilyticus* extract is inhibited by fumarate to the extent of 44% when the fumarate/succinate ratio is 1 :4, the reverse reaction (*i.e.*, fumarate reduction) is not inhibited by succinate. In fact, stimulations up to 50% have been observed in the presence of succinate with both crude and partially purified[21] reductase preparations. This effect is observed only with relatively large amounts of succinate and is not shown by ashed samples of the acid. The identity of the stimulatory factor is still uncertain.

The poor ability of malonate and succinate to inhibit the activity of the *Micrococcus* reductase can be explained by the fact that the enzyme has an unusually low affinity for these compounds and a high affinity for fumarate[8].

Coupling of hydrogenase with fumaric reductase

Although the *M. lactilyticus* extract prepared by alumina grinding does not reduce fumarate with hydrogen unless supplemented with viologen, intact cells and extracts made by sonic oscillation do catalyze the overall reaction[23]. These observations suggest that extracts obtained by the grinding procedure are deficient with respect to an intermediary carrier which is replaceable by viologen dyes. It was found that the alumina extract showed a slow, but definite, utilization of H_2 with fumarate (in absence of viologens) when supplemented with an extract from *Clostridium kluyveri* (extract made by grinding dried cells with alumina). Dialysis of the *C. kluyveri* extract, which had a high flavin content, resulted in loss of the stimulatory factor. The concentrated dialysate and supernatant fluid from boiled *C. kluyveri* extract, on the other hand, were both active in promoting the overall reaction. Addition of purified free flavins in low concentration to the crude *M. lactilyticus* extract did not initiate the reduction of fumarate with H_2, but both riboflavin and flavin mononucleotide did activate the system when added in relatively high concentration (0.1 to 2 mg per ml). The activities observed with these supplements were approximately proportional to flavin concentration, and analysis of the data by the Lineweaver-Burk method indicated a maximal velocity of approximately 400 μl H_2/15 min/0.1 ml enzyme extract with the mononucleotide.

The hydrogenase of *M. lactilyticus* appears to be a flavoprotein[24] and the enzyme reduces flavins under an atmosphere of hydrogen[25]. In connection with the foregoing results it is also of interest that free flavins, in relatively high concentrations, can mediate hydrogen transport between two flavoprotein oxidases from *Lactobacillus delbrückii* (*viz.*, lactic and pyruvic oxidases[26]).

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It may be noted that the hydrogenase of *M. lactilyticus* does not reduce pyridine nucleotides[23,24] and these carriers also do not initiate reduction of fumarate by H_2 in the crude alumina extract. In addition, reduced pyridine nucleotides are not oxidized by the extract upon addition of fumarate under anaerobic conditions.

SUMMARY

The biological reduction of fumaric acid is catalysed by succinic dehydrogenase, acting in reverse, or by a similar enzyme which can be provisionally designated as a fumaric reductase. Microorganisms which also contain hydrogenase can reduce fumaric acid with molecular hydrogen, and experiments with cell-free extracts from the anaerobic bacterium *Micrococcus lactilyticus* indicate that the coupling between hydrogenase and reductase can be mediated by free flavin. Reductase activity can be assayed in cell-free preparations by measuring utilization of molecular hydrogen in the presence of excess hydrogenase and a viologen dye serving as electron carrier.

Yeast and mammalian succinic dehydrogenases reduce fumarate at a low rate as compared with their ability to oxidize succinate. On the other hand, the reductase enzyme of *M. lactilyticus* reduces fumarate rapidly and oxidizes succinate slowly. Although malonate and succinate markedly inhibit the reductase activity shown by yeast and mammalian succinic dehydrogenases, the activity of the *M. lactilyticus* reductase is in comparison not appreciably inhibited by these compounds. The present results indicate that the reductase activity of *M. lactilyticus* cannot be ascribed to a conventional succinic dehydrogenase such as is found in the tissues of mammals and other aerobes and suggest the possibility that the bacterial reductase may be a similar enzyme modified to meet the physiological requirements of anaerobic growth.

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