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LINKAGE OF FORMATE HYDROGENLYASE WITH ANAEROBIC RESPIRATION IN *PROTEUS MIRABILIS*

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The linkage between the enzyme system catalysing formate hydrogenlyase and reductases involved in anaerobic respiration in intact cells of anaerobically grown *Proteus mirabilis* was studied. Reduction of nitrate and fumarate by molecular hydrogen or formate was possible under all growth conditions; reduction of tetrathionate and thiosulphate occurred only in cells harvested at late growth phase from a pH-regulated batch culture and not in cells harvested at early growth phase or in cells grown in pH-auxostat culture. Under all conditions, cells possessed the enzyme tetrathionate reductase. We conclude that linkage between tetrathionate reductase (catalysing also reduction of thiosulphate) and the formate hydrogenlyase chain is dependent on growth conditions. During reduction of high-potential oxidants such as fumarate, tetrathionate (when possible) or the artificial electron acceptor methylene blue by formate, there was no simultaneous H_2 evolution due to the formate hydrogenlyase reaction. H_2 production started only after complete reduction of methylene blue or fumarate, in the case of methylene blue after a lag phase without gas production. In preparations with a low fumarate reduction activity this was accompanied by an acceleration in CO_2 production. During reduction of thiosulphate (a low-potential oxidant) or of tetrathionate in the presence of benzyl viologen (a low-potential mediator) by formate, H_2 was evolved simultaneously. From this we conclude that formate hydrogenlyase is regulated by a factor that responds to the redox state of any electron acceptor couple present such that lyase activity is blocked when the acceptor couple is oxidised to too great an extent.

Introduction

The enterobacterium *Proteus mirabilis* possesses a versatile network of electron-transfer pathways located in its cytoplasmic membrane. This network enables the organism to oxidise either NADH, hydrogen or formate, and to reduce oxygen or, anaerobically, a variety of alternative electron acceptors like nitrate, fumarate, chlorate, thiosulphate, trithionate and tetrathionate [1–3]. In addition to this, like other enterobacteria (e.g., *Escherichia coli*) *P. mirabilis* possesses the ability to split formate via the formate hydrogenlyase reaction into CO_2 and H_2 [1].

For descriptive purposes, the enzymes and cofactors involved in these electron-transfer reactions may be divided into an aerobic respiratory chain analogous to that in *E. coli*, a set of reductases (comprising nitrate reductase, chlorate reductase, fumarate reductase and tetrathionate reductase) and a formate hydrogenlyase chain containing formate dehydrogenase, hydrogenase and one or more intermediate factors [1], much like that in *E. coli* [4–5]. These systems are interconnected in such a way that 'cross-reactions' like reduction of fumarate by formate, or reduction of nitrate by hydrogen are possible.

The exact pathways through this network of redox catalysts are as yet unknown; some components (e.g., the reductases [7,8] and hydrogenase [9]) have been purified, but largely this system is still terra incognita.

One important feature is that the biosynthesis of some components of the system is regulated by the presence of some of its substrates: both oxygen and nitrate repress the synthesis of hydrogenase and of the reductases, with the exception of nitrate reductase that is induced by nitrate, and repressed by oxygen [1,3,10,11].

Recently, redox systems that are able to form hydrogen (by reduction of protons) have gained interest, as they may afford ways to produce large amounts of hydrogen for use as fuel [12–14]. Coupling of a hydrogenase via redox mediators to chloroplasts photolysing water has resulted in the splitting of water into oxygen and hydrogen [15–17]. These attempts have not as yet been very successful in a quantitative way, due to instability of the biological components involved, and to complications caused by the oxygen which is evolved together with the hydrogen [13,14].

The possible use of formate as one of a set of mediators [18] in a biological system for the photoproduction of hydrogen prompted us to take a closer look at the formate hydrogenlyase of *P. mirabilis*. We have studied the question under what conditions oxidation of formate by the redox system of anaerobically grown *P. mirabilis* leads to the production of hydrogen, and not the reduction of alternative, thermodynamically favoured electron acceptors. We show that reduction of H^+ to H_2 is regulated by a 'switch' that possibly is under redox control, and that reduction of tetrathionate and thiosulphate involves an as yet unknown factor, the formation of which is a function of the growth conditions.

Materials and Methods

P. mirabilis strain S 503 was grown anaerobically at 37°C in a medium containing 0.5% glucose, 0.5% NaCl, 0.5% yeast extract (Difco) and 0.8% nutrient broth (Oxoid 2) at pH 7. Cells were grown in a Microferm fermentor (New Brunswick Scientific Co.) either as 10-l pH-regulated batch cultures or in large-scale pH auxostat culture as described previously [19]. After harvesting, cells were washed in 25 mM Tris-HCl buffer, pH 7.25, and either used immediately or stored at −80°C.

Enzyme activities were measured manometrically (at 5-min intervals) in Warburg flasks at 37°C in 300 mM sodium/potassium phosphate, pH 6.5. In cases where in addition to uptake or release of hydrogen also CO_2 or H_2S was measured, the direct method of Umbreit et al. [20] was used to calculate the amounts of the different gases. Solubilities of $CO_2 + HCO_3^-$ and of $H_2S + HS^-$ at 37°C and pH 6.5 were taken to be 1.419 and 3.158, respectively. 15% KOH in the center well of the Warburg flasks served to absorb both CO_2 and H_2S; 50% $CdSO_4$ was used to absorb only H_2S. Due to inhibition of $H_2 + CO_2$ production in the formate hydrogenlyase reaction by H_2S, the calculated amounts of H_2S have been progressively underestimated, and have only qualitative significance.

Protein was determined using the method of Lowry et al. [21].

Total thiosulphate and tetrathionate reductase activities were determined according to the method of Pichinoty et al. [22,23] using a crude hydrogenase preparation from *Desulphovibrio vulgaris* (strain Vechten).

Succinate was determined as described by Van Verseveld and Stouthamer [24], but with malonate as internal standard.

Results

Reduction of methylene blue by formate

When cells of *P. mirabilis* are anaerobically supplied with formate together with the artificial electron acceptor methylene blue, two reactions occur. As Fig. 1a shows, there is an initial phase in which there is only CO_2 evolution that stops after the blue colour of oxidised methylene blue has disappeared and an amount of CO_2 is produced equal to 85% of the amount of methylene blue added (the remainder of the methylene blue is reduced by endogenous reduction equivalents). Hence, during this phase methylene blue (MB) has been reduced according to:

$$MB + HCOOH \rightarrow MBH_2 + CO_2\uparrow \quad (1)$$

After a short lag phase in which there is no gas evolution at all, in a third phase H_2 and CO_2 are produced in a 1 : 1 ratio, indicating that this gas evolution is due to the formate hydrogenlyase reaction:

$$HCOOH \rightarrow H_2\uparrow + CO_2\uparrow \quad (2)$$

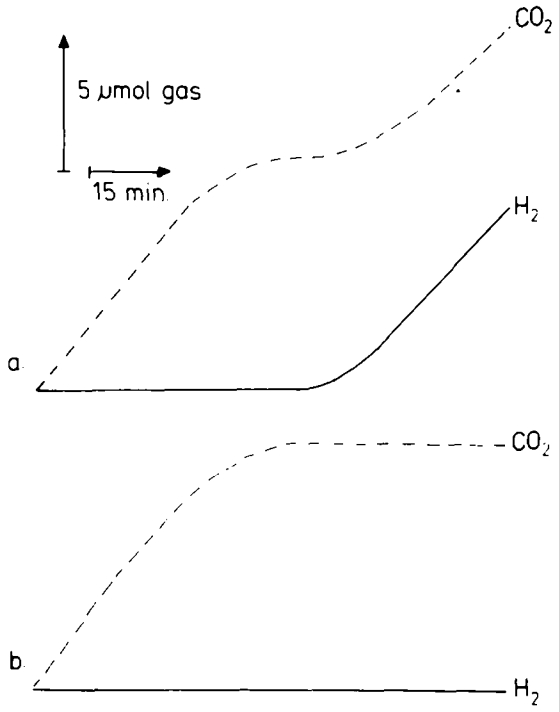

Fig. 1. Reduction of methylene blue by formate. The reaction was started by addition of 50 µmol formate to the main compartments of the Warburg flasks containing 10 µmol methylene blue and 1.1 mg protein of pH-auxostat-grown cells. The gas phase in the flasks was N_2, and the total volume 2.8 ml. (———) H_2, (- - - - -) CO_2. (a) No further additions, (b) 28 µmol formaldehyde present.

This is confirmed by Expt. b in Fig. 1 which has been done in the presence of 10 mM formaldehyde, a potent inhibitor of the formate hydrogenlyase reaction in *P. mirabilis* (50% inhibition at 1 mM, cf. Ref. 25 : 16% inhibition at 1 mM in *Sarcina ventriculi*). The CO_2 production in the first phase is not inhibited, while the gas production in the third phase is.

The lag in CO_2 production before the lyase reaction starts in Expt. a of Fig. 1 is highly significant: no such lag is seen when the cells are given formate only (not shown). It indicates that there is not a simple competition between methylene blue and protons (the final acceptors in the lyase reaction) for the reducing equivalents derived from formate, but that the lyase reaction is blocked until the methylene blue has been reduced completely.

Reduction of nitrate and fumarate

Anaerobically grown cells of *P. mirabilis* are able to catalyse reduction of nitrate or fumarate by formate. Fig. 2 shows the results that are obtained when either of these substrates is allowed to compete with H^+ for the reducing equivalents delivered by formate. The upper left-hand trace shows that in the presence of nitrate only CO_2 is produced in the reaction:

$$NO_3^- + HCOOH \rightarrow NO_2^- + H_2O + CO_2\uparrow \qquad (3)$$

The CO_2 production accounts for reduction of 80% of the nitrate added, is insensitive to formaldehyde (lower left-hand trace) and is not followed by further gas production indicative of reaction 2. The product of nitrate reduction, nitrite, has been shown to inhibit formate hydrogenlyase activity strongly [10], which explains why unlike with methylene blue, after reduction of nitrate there is no subsequent hydrogen evolution.

The right-hand traces in Fig. 2 show that with fumarate as electron acceptor first only CO_2 is produced, immediately followed by 1 : 1 evolution of CO_2 and H_2. The amount of 'extra' CO_2 that is produced in the initial phase is 60% of the amount of fumarate added. Determination of the amount of

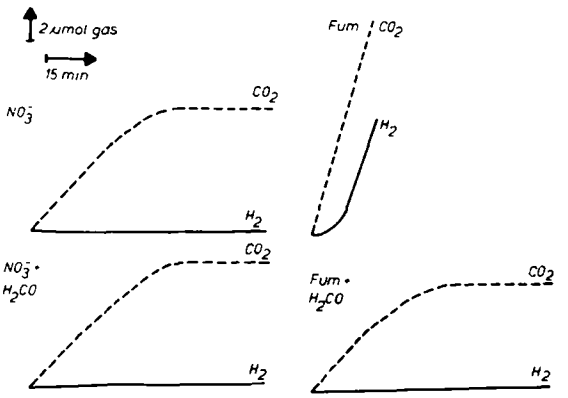

Fig. 2. Reduction of nitrate and fumarate by formate. The reactions were started by addition of 100 µmol formate together with either 10 µmol nitrate (left) or 10 µmol fumarate (right) to the main compartment of the Warburg flasks, containing 4.42 mg protein of pH-auxostat-grown cells. (———) H_2, (- - - - -) CO_2. The gas phase was N_2 and the total volume 2.8 ml. When indicated, 28 µmol formaldehyde was also present. Fum., fumarate.

succinate present at the onset of the H_2 production reveals, however, that all fumarate has been converted into succinate. Moreover, when fumarate is not added simultaneously with formate, but the cells are preincubated with it, the 'shortfall' in CO_2 production increases with preincubation time. Evidently, there occurs extensive reduction of fumarate by (NADH-linked?) endogenous substrates, not associated with CO_2 evolution.

The lower right-hand trace of Fig. 2 shows that whereas CO_2 production due to fumarate reduction is inhibited about 50% by 10 mM formaldehyde, the lyase reaction again is completely inhibited. Addition of succinate has no effect on the course of the reactions (not shown).

The rates of CO_2 production in the first and second phase depend on the preparation. Fig. 3 shows fumarate reduction by a preparation with a comparatively low fumarate reductase activity. The rate of CO_2 evolution in the first phase of Expt. b is considerably less than the rate of CO_2 production either in the second phase of Expt. b or in the absence of fumarate (Expt. a). This again indicates that during fumarate reduction according to:

$$HCOOH + \text{fumarate} \rightarrow CO_2\uparrow + \text{succinate} \qquad (4)$$

the lyase reaction is blocked. Otherwise one would not expect an increase in CO_2 production after exhaustion of the fumarate, as excess capacity of formate dehydrogenase during the first phase would be utilised for hydrogen production.

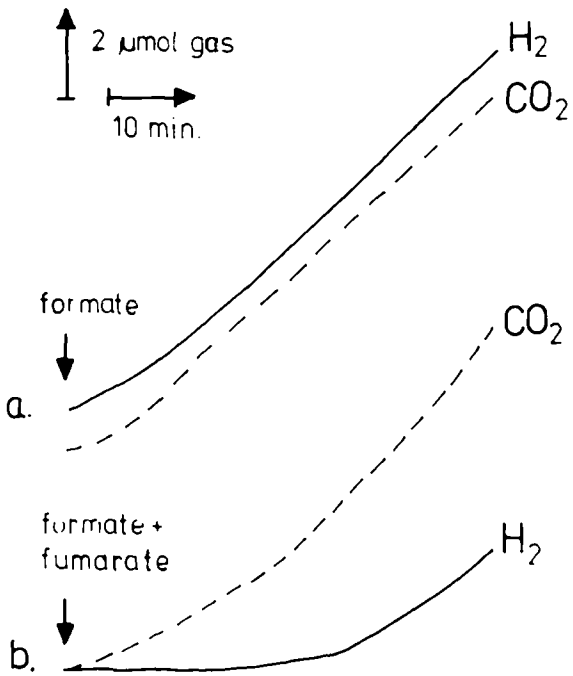

Fig. 3. Kinetics of fumarate reduction by formate. The reaction was started by addition of 50 µmol formate (a) or 50 µmol formate together with 10 µmol fumarate (b) to the main compartment of the Warburg flasks, containing 1.52 mg protein of cells grown as pH-regulated batch culture, and harvested at late linear growth phase. (———) H_2, (- - - - -) CO_2. The gas phase was N_2, and the total volume 2.8 ml.

Reduction of tetrathionate and thiosulphate by H_2

Anaerobically grown *P. mirabilis* possesses tetrathionate reductase (catalysing reduction of both tetrathionate and thiosulphate [1,2,7] as well as a complete formate hydrogenlyase chain and hence should be able to catalyse reduction of tetrathionate and thiosulphate by H_2 or by formate. However, the experiments of Fig. 4 show that cells grown in pH-auxostat culture do not perform these reductions with H_2 as reductant. In Fig. 4a and d it is shown that no H_2 is taken up when cells are incubated with either

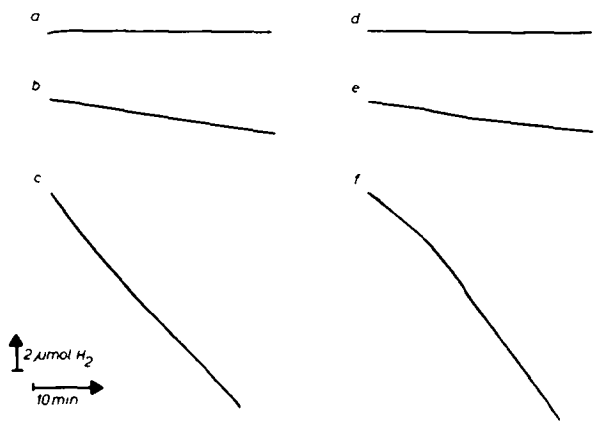

Fig. 4. Reduction of tetrathionate and thiosulphate by H_2. The reactions were initiated by addition of 10 µmol thiosulphate (a–c) or 10 µmol tetrathionate (d–f) to 2.64 mg protein of pH-auxostat-grown cells. The gas phase was H_2 and the total volume 2.8 ml. Any H_2S formed was scavenged by a $CdSO_4$ solution in the center well of the Warburg flasks. In b, c, e and f 1.2 µmol benzyl viologen were present; in c and f hydrogenase was also added.

of these two acceptors. This contrasts with the analogous experiment with nitrate or fumarate, where H_2 uptake is seen until the acceptor is reduced completely (not shown). However, this is not due to lack of tetrathionate reductase, as is shown in Fig. 4b and e, where the presence of the redox mediator benzyl viologen leads to hydrogen uptake. In these experiments benzyl viologen is reduced (by H_2, via the hydrogenase present in the cells) at the onset of the experiments before addition of electron acceptor. When the reaction is started by addition of tetrathionate or thiosulphate, the mediator becomes highly oxidised (colourless) during the ensuing steady state. From this it follows that reduction of benzyl viologen is rate limiting. Increasing the rate of benzyl viologen reduction by addition of a hydrogenase preparation from *D. vulgaris* results in increased H_2 uptake (Fig. 4c and f) during which the mediator is in a more reduced steady state (blue-purple colour). H_2 uptake with tetrathionate as electron acceptor shows the characteristic non-linear kinetics of tetrathionate reductase [2].

These results clearly contrast with the finding of De Groot and Stouthamer [11] that anaerobically grown cells of *P. mirabilis* catalyse reduction of tetrathionate and thiosulphate by H_2 also in the absence of mediators. However, these authors used cells grown in batch culture, without pH regulation. Hence, we studied the growth phase dependency of the coupling between endogenous hydrogenase and tetrathionate reductase.

The result of an experiment carried out with a pH-regulated batch culture is shown in Fig. 5. The rates of total (determined in the presence of benzyl viologen together with added hydrogenase) and coupled (in the absence of these) tetrathionate (A) and thiosulphate (B) reduction are given as a function of growth time. Also shown is the absorbance at 660 nm, as a qualitative measure of the growth phase (C). The figure shows that the total reductase activity remains at a fairly steady level for both substrates, while coupling with the endogenous hydrogenase only ensues after approx. 90 min of growth (at absorbance 0.25) for tetrathionate reduction, and even later (at higher absorbance) for thiosulphate reduction.

This phenomenon is characteristic for this particular reductase, as at all times nitrate and fumarate

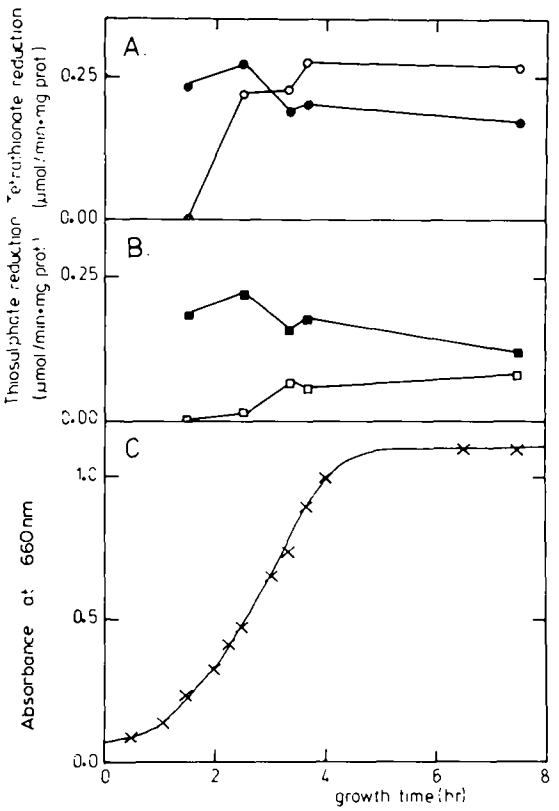

Fig. 5. Reduction of tetrathionate and thiosulphate as a function of growth phase. (A) Reduction of tetrathionate by H_2. (○———○) No additions, (●———●) hydrogenase and 14.4 μmol benzyl viologen were present. Further experimental details as in Fig. 4. The maximal rate of H_2 uptake has been plotted. (B) Reduction of thiosulphate by H_2. (□———□) No additions, (■———■) hydrogenase and benzyl viologen present. (C) Absorbance at 660 nm of the culture as a function of growth time.

could be reduced by H_2 in the absence of mediator (not shown). So in early growth phase (low absorbance of the culture) a result is obtained similar to that with pH-auxostat-grown cells (Fig. 4), while cells harvested at late linear phase (after approx. 3 h growth in Fig. 5) and stationary phase (after approx. 4 h growth in Fig. 5) behave as those grown in a batch culture without pH regulation. We have to conclude that coupling between hydrogenase and tetrathionate reductase depends on the growth conditions.

Interestingly, in cells with a tetrathionate reductase activity fully linked with the endogenous hydrogenase (Fig. 5A after approx. 3 h growth) H_2 uptake

in the presence of added hydrogenase and benzyl viologen is slower than that in the absence of these additions. Also in this type of cell, addition of only benzyl viologen (which then remains virtually oxidised) has an inhibiting effect on the reduction of tetrathionate by H_2 (not shown). These findings indicate that the redox state of the mediator may have some influence on the rate of tetrathionate reduction.

Reduction of tetrathionate and thiosulphate by formate

To test the possibility that the growth-dependent linkage between tetrathionate reductase and hydrogenase is essential also for electron transfer from formate to tetrathionate, we have studied the effect of tetrathionate on formate hydrogenlyase activity. This we have done with pH-auxostat-grown cells and with pH regulated-batch cells harvested both at early growth phase (corresponding to absorbance 0.25 in Fig. 5) and late growth phase (absorbance 0.9 in Fig. 5).

Fig. 6a shows that addition of tetrathionate has no effect on the 1 : 1 ratio of CO_2 and H_2 evolution due to formate hydrogenlyase activity of cells harvested at early growth phase. There is no extra CO_2 produced due to the reduction of tetrathionate by formate. The situation is different with cells harvested at late growth phase (Fig. 6b). Now again two phases are observed: a first phase in which CO_2 is released in the reaction:

$$S_4O_6^{2-} + HCOOH \rightarrow 2S_2O_3^{2-} + 2H^+ + CO_2\uparrow \qquad (5)$$

and a second phase in which there is also H_2 evolution. However, in contrast to the situation with methylene blue or fumarate as acceptor, there still is production of excess CO_2, and also H_2S is produced. This is the result of reduction of the thiosulphate (produced by reaction 5) according to:

$$S_2O_3^{2-} + HCOOH \rightarrow SO_3^{2-} + H_2S\uparrow + CO_2\uparrow \qquad (6)$$

Apparently, reaction 6 may proceed simultaneously with the lyase reaction 2.

To a certain extent this experiment may be duplicated with early growth phase cells, when benzyl viologen is present to mediate between the lyase chain and tetrathionate reductase (Fig. 6c). The difference with Fig. 6b is that there is hydrogen evolution also in the first phase (while benzyl viologen is in a reduced steady state).

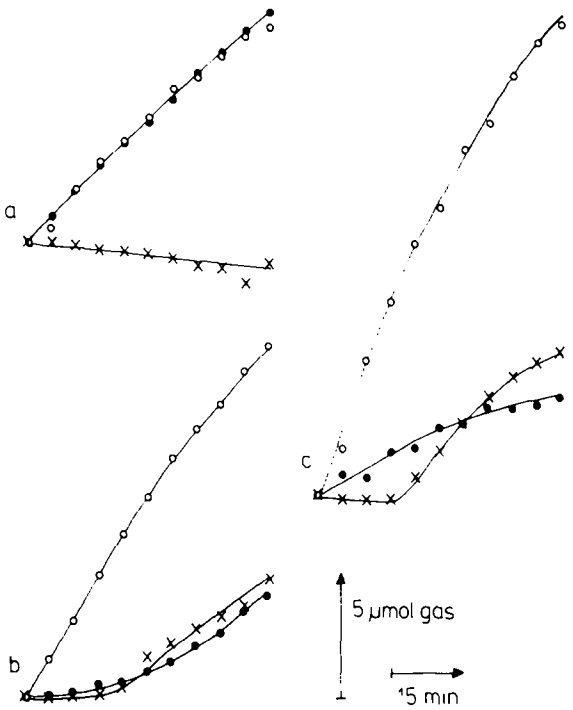

Fig. 6. Reduction of tetrathionate by formate. The reactions were started by addition of 10 µmol tetrathionate together with 50 µmol formate. The gas phase was N_2 and the final volume 2.8 ml. (o——o) CO_2, (•——•) H_2, (X——X) H_2S. The main compartment of the Warburg flasks contained: (a) 1.6 mg protein of cells grown as pH-regulated batch culture, harvested at early growth phase (at absorbance 0.25, cf. Fig. 5C); (b) 3.5 mg protein of cells grown as pH-regulated batch culture, harvested at late linear growth phase (at absorbance 0.9, cf. Fig. 5C); (c) 1.6 mg protein of cells grown in pH-regulated batch culture, harvested at early growth phase, together with 4 µmol benzyl viologen.

Cells grown in the pH auxostat show the same behaviour as early growth phase cells.

These results show that the growth dependency of the linkage between tetrathionate reductase with hydrogenase and formate dehydrogenase, respectively, is the same.

Discussion

When anaerobically grown cells of *P. mirabilis* catalyse the reduction of an oxidant by formate, in

principle it is possible that there is a competition between the oxidant and protons for the reducing equivalents derived from formate. One would expect H_2 evolution and CO_2 evolution equal to the sum of the rates of the two competing pathways.

With fumarate, tetrathionate or the artificial electron acceptor methylene blue as oxidants, there is initially no H_2 evolution. In the cases of fumarate and methylene blue the formate hydrogenlyase reaction starts only after the oxidant has been reduced completely. Before H_2 evolution starts after reduction of methylene blue there is a lag in CO_2 production; also, CO_2 production due to reduction of fumarate by formate may be less than the rate of CO_2 production in the formate hydrogenase reaction. These findings clearly show that the lyase reaction cannot proceed to take the excess electrons produced by formate dehydrogenase before the acceptor is reduced completely. Therefore, there is no simple kinetic competition between pathways, but the lyase reaction is blocked during reduction of the oxidant. This is not due to a direct inhibiting effect of the oxidants, as their structures are quite dissimilar, and moverover tetrathionate does not block formate hydrogenlyase unless tetrathionate reductase is linked to the lyase chain (cf. Fig. 6).

During reduction of thiosulphate (the product of tetrathionate reduction) or of tetrathionate in the presence of the mediator benzyl viologen, there is simultaneous H_2 production. Whereas methylene blue (E'_m(pH 6.5) = 40 mV, calculated from Ref. 26), fumarate (E'_m(pH 6.5) = 60 mV, calculated from Ref. 26) and tetrathionate (E'_m(pH 6.5) = 170 mV [27]) are rather high-potential electron acceptors, thiosulphate (E'_m(pH 6.5) $\approx$ –400 mV [28] and benzyl viologen (E'_m(pH 6.5) = –350 mV [29]) are low-potential electron acceptors. Moreover, benzyl viologen is in a rather reduced steady state while mediating in the reduction of tetrathionate by formate. Reduction of benzyl viologen by formate takes place together with H_2 evolution via the lyase reaction (Ref. 1 and Krab, K., unpublished results).

This suggests that the block on formate hydrogenlyase activity is a redox block. This is supported by the finding that tetrathionate exerts its effect on H_2 evolution only in cells where reduction of tetrathionate by formate (or H_2) is possible.

The difference between the patterns of CO_2 evolution between the fumarate and the methylene blue experiments, notwithstanding the similar redox midpoint potentials of the acceptors, may be due to either a more direct effect of the mediator methylene blue on the redox-operated switch in the lyase reaction, or to kinetic differences in the reduction of the acceptors.

Although premature, it is interesting to speculate on the nature of the redox-operated switch in the lyase chain. It has been suggested that the role of the Fe-S centers omnipresent in hydrogenase is not yet very clear. Van Heerikhuizen et al. [30] have shown that the redox midpoint potential of the EPR detectable Fe-S in hydrogenase from *Chromatium vinosum* (maximally one free spin per molecule) is too high to be directly involved in the generation of H_2. It is possible that this cluster has a regulatory function in the hydrogenase similar to that of the Fe-S present in aconitase, that is not involved directly in catalysis, but has to be reduced for the enzyme to be active [31]. Also hydrogenase from *P. mirabilis* contains 24 Fe atoms and 24 acid-labile S atoms/molecule and exhibits an EPR signal characteristic for an Fe-S center [9], and thus may have a regulating Fe-S cluster.

It is interesting to note that under conditions where electrons can go from formate to tetrathionate, but not from formate to H^+ (see Fig. 6b), it is quite possible to reduce tetrathionate using H_2 (see Fig. 5A). The same phenomenon is seen with fumarate. This suggests that redox control is exerted mainly in one direction, although there may be a redox effect (by, e.g., benzyl viologen on tetrathionate reduction) also on electron transfer in the other direction.

The redox control would immediately explain the lack of hydrogenase and formate hydrogenlyase activities in the presence of O_2: the high redox potential exerted by the O_2/H_2O couple blocks electron transfer to H^+. Also specific loss of H_2-production activity during purification of hydrogenase [9] may be due to the redox state of a putative controlling center in the isolated enzyme.

Coupling between tetrathionate reductase and the lyase chain is dependent on the growth phase of anaerobically grown *P. mirabilis*. H_2 or formate may only reduce tetrathionate or thiosulphate in cells harvested at late linear growth phase or later (cf. Fig. 6). A possibility is that there is a factor needed for mediation of electrons between tetrathionate reduc-

tase and the lyase chain that is absent in early harvested cells and in cells grown in pH-auxostat culture. It is interesting to notice that De Groot and Stouthamer [11] have observed a similar phenomenon in cells grown anaerobically in the presence of thiosulphate.

A scheme summarising the properties of electron transfer in anaerobically grown *P. mirabilis* is given in Fig. 7. Input of reducing equivalents may occur via formate dehydrogenase or hydrogenase; and three of the reductases present (fumarate reductase, tetrathionate reductase and nitrate reductase) catalyse reduction of the different oxidants used. Methylene blue presumably acts directly on formate dehydrogenase. There are indications that in *E. coli* there are two different formate dehydrogenases [32], and this is also a possibility in *P. mirabilis*. However, this would make no difference in the interpretation of our results. The tentative growth phase-dependent factor (Y in the figure) is placed exclusively in the route to tetrathionate reductase: although we have observed some variations in the 'linked' nitrate or fumarate reduction by H_2, these activities were present also under conditions where coupled reduction of tetrathionate was absent. The controlling center in the lyase reaction (Z in the figure) is drawn apart from hydrogenase to emphasise the tentative character of our notion that it is located on the hydrogenase.

We conclude that the use of the formate hydrogenlyase chain of *P. mirabilis* in a biophotolysis system requires the identification of and interference with the redox control of the lyase reaction.

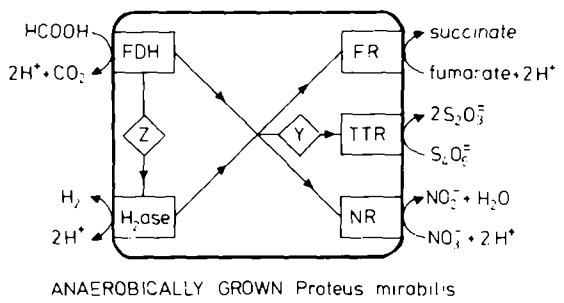

Fig. 7. Schematic representation of reduction of nitrate, fumarate and tetrathionate by formate or H_2 in anaerobically grown *P. mirabilis*. FDH, formate dehydrogenase; TTR, tetrathionate reductase (catalysing also reduction of thiosulphate); H_2ase, hydrogenase; FR, fumarate reductase and NR, nitrate reductase. Straight arrows indicate electron transfer. For further details see the text.

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