PHOSPHORYLATION COUPLED TO OXIDATION OF HYDROGEN WITH FUMARATE IN EXTRACTS OF THE SULFATE REDUCING BACTERIUM, *DESULFOVIBRIO GIGAS*

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The esterification of orthophosphate coupled to the oxidation of molecular hydrogen and concomitant reduction of fumarate to succinate is catalyzed by a particulate preparation from the anaerobic sulfate reducing bacterium, *D. gigas*. The system exhibits a P/H_2 ratio of 0.3-0.4 and does not require the addition of soluble protein. The phosphorylation is uncoupled by gramicidin, pentachlorophenol, dinitrophenol and methyl viologen but not by oligomycin. The added fumarate was quantitatively reduced to succinate. It is suggested that phosphorylation coupled to electron transfer with the reduction of fumarate may be of general occurrence in anaerobic and facultative anaerobic bacteria.

Sulfate reducing bacteria are anaerobes which classically use inorganic sulfate as a terminal electron acceptor (1). Several species including *Desulfovibrio gigas*, grow in a sulfate-free medium in the presence of fumarate (2). In cell-free preparations of *D. gigas* grown on a lactate-sulfate medium, fumarate is reduced to succinate and functions as the acceptor of electrons which are derived from the oxidation of pyruvate (3). It has been suggested that fumarate has an important role in sulfate respiration by participating in a fumarate-succinate cycle which links the electron transfer system between electron donors and sulfate (4).

In cell free preparations, sulfate (5) (6) and fumarate reduction (3) occur with the oxidation of H_2. Since phosphorylation coupled with sulfite reduction and hydrogen oxidation has been demonstrated in extracts from *D. gigas* (7), it seemed possible that phosphorylation is also coupled with the transfer of electrons from H_2 to fumarate. The presence of membrane bound cytochrome (8) and a napthoquinone (9) (10) in *D. gigas* gave further impetus for the examination of ATP production associated with electron transfer during the reduction of fumarate. In this paper we report the coupling of orthophosphate esterification with fumarate reduction by molecular H_2 in cell-free extracts of *D. gigas*.

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METHODS

D. gigas was grown at 37° in the lactate-sulfate medium and extracts were prepared as described earlier (7,11). The cell free extract (ca. 80 mg/ml) was layered over a discontinuous gradient of 1.67 M (1 ml) and 0.96 M (4 ml) sucrose in the buffer of the suspending solution. After centrifugation at 180,000 x G for 2 hr the soluble protein remained in the upper portion of the tube corresponding to the volume of the added extract. The particle protein near the bottom of the 0.96 M sucrose layer was collected and used in the experiments reported here.

Esterification of orthophosphate refers to the difference between the initial phosphate concentration and that remaining after completion of the reaction, as determined by isotopic or chemical procedures (12,13). Molecular H_2 utilization was measured by standard manometric techniques. Krebs cycle acids were separated by celite chromatography and determined as described by Marvel and Rands (14). Bovine serum albumin (BSA) was treated with Norite according to the procedure of Chen (15).

RESULTS

The calculated ΔF° (-20,4 Kcal/mole) for the oxidation of hydrogen with fumarate is more than sufficient to account for the formation of at least one ATP. As shown in Table 1, phosphorylation coupled with the oxidation of hydrogen and reduction of fumarate is observed without added soluble protein in the presence of the particulate fraction. The efficiency of the phosphorylation is indicated in terms of hydrogen oxidation as P/H_2. Routinely P/H_2 ratios of 0.3-0.4 are obtained and ratios as high as 0.9 have been frequently observed.

Minimal levels of $^{32}PO_4^{-3}$ are incorporated in absence of either fumarate or molecular H_2. The requirement of ADP for esterification of phosphate is evident and detection of phosphorylation is not possible when the glucose-hexokinase trap is omitted. Substrate phosphorylation associated with glucose metabolism can be ruled out in these and other reactions because glucose can not be utilized by *D. gigas* and the glucose can be replaced by mannose. No oxidative phosphoryla-

Table 1: Requirements for Phosphorylation Coupled to Hydrogen Oxidation with Fumarate by Particles

Reaction Mixture[1]	Activity (μmoles) H_2 Oxidized	Pi esterified[2]	P/H_2
Complete	15.7	8.35	0.38
minus fumarate	0.0	2.64	-
minus ADP	15.2	3.65	0.09
minus hexokinase and glucose	13.6	2.98	0.05
minus ADP, hexokinase and glucose	14.1	1.89	-
minus H_2, plus N_2	0.0	2.30	-
minus BSA	14.5	6.54	0.29

(1) The reaction mixture (2.2 ml) contained in μ moles: Tris-HCl, 40 (pH 7.3); $MgCl_2$, 40; NaF, 50; $^{32}PO_4^{-3}$, 20 ($3.76x10^5$ cpm/μmole) and as indicated glucose, 100; ADP, 1 and fumarate, 20. Each reaction vessel contained in mg: particle protein, 28; BSA, 5 and hexokinase, 0.1, with exceptions as indicated. The gas phase was H_2, unless specified, and the incubation was at 37° for 20 min.

(2) Calculated from the difference in esterification between each reaction and the one with N_2 as gas phase.

Table 2: Effect of Uncoupling Agents on Phosphorylation Linked to the Hydrogen-Fumarate Reaction

System[1]	H_2 oxidized	Pi esterified	P/H_2
Complete	17.5	5.5	0.31
plus oligomycin	14.6	4.6	0.31
plus gramicidin	15.8	1.2	0.08
plus pentachlorophenol	12.7	0.0	-
plus dinitrophenol	13.4	2.3	0.17
plus methyl viologen	13.4	0.0	-

(1) The contents of the reaction were as described in Table 1 with these exceptions: $^{32}PO_4^{-3}$, 12 μmoles ($6.4x10^4$ CPM/μmole); particle protein, 31 mg and uncoupling agents, $6x10^{-4}$ M.

tion can be demonstrated in the absence of ADP and an ATP trapping system of glucose and hexokinase. An enhancement of the phosphorylation occurs with BSA although this has no effect on H_2 oxidation. Addition of the soluble protein from the sucrose gradient centrifugation or boiled extract has little effect on

either electron transfer or the phosphorylation. Some phosphorylation occurred in the presence of high concentrations of soluble protein but this appears to result from the conversion of fumarate to pyruvate and its subsequent metabolism in the phosphoroclastic reaction (3,16).

The effect of uncoupling agents on the phosphorylation is shown in Table 2. Oligomycin does not uncouple the phosphorylation as evidenced by the P/H_2 ratio. The phosphorylation is uncoupled by gramicidin, pentachlorophenol and dinitrophenol, although the effectiveness of dinitrophenol is difficult to quantitate as it is readily reduced to aminophenols by hydrogenase in extracts of this organism. These results with uncoupling agents are essentially identical to those obtained with the oxidative phosphorylation coupled to sulfite reduction (7). Methyl viologen which acts as an electron carrier between hydrogenase and fumarate reductase has the effect of an uncoupling agent by circumventing the native electron transport system to which phosphorylation is linked. Usually a slight inhibition of H_2 oxidation is observed with the uncouplers, but with some preparations respiration is slightly stimulated. The phosphorylation linked to the reduction of fumarate is relatively stable and the particle can be stored at -20° for several weeks.

As the esterification of orthophosphate is quite high, a difference can be observed chemically in the amount of phosphate present before and after the reaction. The difference in orthophosphate agrees with the radioisotopic measurements but because the incorporation of $^{32}PO_4^{-3}$ has greater sensitivity, it was used routinely to measure phosphorylation.

Examination of the activity of several carboxylic acids in this system reveals that phosphorylation occurs only when fumarate or malate is reduced with hydrogen (Table 3). The phosphorylation occurring with the addition of malate apparently is due to fumarase activity which catalyzes the formation of fumarate from malate. This is indicated by the fact that although the values for H_2 oxidation and the Pi esterification are lower with malate than with fumarate, the P/H_2 ratio with both substrates is essentially identical. The lack of any phosphorylation with

Table 3: Hydrogen Oxidation and Phosphorylation with Various Carboxylic Acids

Substrate[1]	Activity (μmoles) H_2 oxidized	Pi esterified[2]	P/H_2
Fumarate	14.3	4.89	0.34
Malate	4.6	1.60	0.35
Succinate	0.0	0.08	-
Pyruvate	0.0	0.00	-
Lactate	0.0	0.00	-
Formate	0.0	0.07	-

(1) The contents of the reactions are as described for the complete reaction in Table 1 with these exceptions:

$^{32}PO_4^{-3}$, 11 μmoles (3.6×10^4 CPM/μmole) and substrates, 20 μmoles.

(2) Difference in Pi esterified in the coupled and reaction uncoupled with pentachlorophenol (6×10^{-5} M).

Table 4: Partial Stoichiometry of the Hydrogen-Fumarate Reaction with Coupled Phosphorylation

Determination[1]	quantity in μmoles
H_2 oxidized	10.1
PO_4^{-3} esterified[2]	4.4
Fumarate added	16.0
Fumarate remaining	6.9
Succinate formed	9.5
CO_2 formed	0.0
recovery of dicarboxylic acids 102%	

(1) The contents of the reaction were identical to that in the complete reaction, Table 1, with these exceptions: PO_4^{-3}, 11 μmoles; and specific activity of fumarate -1,4-C^{14}, 55,700 cpm/μmole. The center well contained 0.1 ml of 10 N NaOH and, after the reaction was terminated, aliquots were monitored for $^{14}CO_2$.

(2) Determined chemically.

pyruvate indicates that the phosphoroclastic reaction can not be involved in this esterification of phosphate. No activity was observed in the presence of succinate, lactate or formate; however, esterification has been observed in the presence of both lactate and fumarate.

Partial stoichiometry of the hydrogen-fumarate reaction and linked phosphorylation is shown in Table 4. Of the 16 μmoles of fumarate added, over 63% was converted to succinate and a P/H_2 ratio of 0.44 was obtained. Comparisons of the H_2 oxidized and succinate formed reveals that over 94% of the electrons from hydrogen are used for the reduction of fumarate. No CO_2 was evolved during the reaction from carboxyl labeled fumarate and the added fumarate was quantitatively recovered as fumarate and succinate. These results demonstrate that fumarate is exclusively reduced to succinate by this particle and that the phosphorylation can not result from a substrate phosphorylation involved in the metabolism of either fumarate or succinate.

DISCUSSION

Based on a consideration of metabolic pathways (8), it has been postulated that the reduction of sulfate with molecular hydrogen and growth on lactate and sulfate requires phosphorylation coupled with electron transfer. The former concept was substantiated by observations with whole cells (8) and more recently by the report of phosphorylation coupled to the oxidation of hydrogen with sulfite in cell free extracts (7). The fumarate system is simpler and more efficient than the sulfite coupled system and requires only the particle or membrane fraction. Many interferring reactions are thus eliminated and this probably accounts for the higher efficiency of the system; however, the same site may be involved in both phosphorylations.

The phosphorylation appears to be an oxidative phosphorylation as it is dependent upon electron transfer, is inhibited by usual uncoupling agents. Only succinate is formed from fumarate, and substrate phosphorylation as a source of the observed esterification of phosphate is thus eliminated.

It is quite likely that ATP formation coupled with the transfer of electrons from hydrogen, or other substrates or cofactors (18), is not restricted to the sulfate reducing bacteria. Both hydrogenase and fumarate reductase are widely distributed among microorganisms and molar growth yields of a number of bacteria (19, 20,21,22,23,24,25) are greater than that expected on the basis of known substrate

phosphorylations. The properties of the hydrogen-fumarate coupled phosphorylation in *D. gigas* and the occurrence of this anaerobic oxidative phosphorylation in other organisms is currently being pursued.

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