PHOSPHORYLATION COUPLED WITH ELECTRON TRANSFER IN EXTRACTS OF THE SULFATE REDUCING BACTERIUM, DESULFOVIBRIO GIGAS

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The occurrence of an oxidative phosphorylation in the anaerobic respiration of the "sulfate-reducing bacteria" has been postulated from a consideration of the energetics of sulfate respiration (Peck, 1960; Senez, 1962). The reduction of sulfate with molecular hydrogen requires a source of ATP (2 $P/SO_4^=$) for the activation of sulfate (Ishimoto and Fujimoto, 1961; Peck, 1961) that neither of the substrates, $SO_4^=$ or H_2, can obviously provide by means of a substrate phosphorylation. An alternative source of the required ATP could be from oxidative phosphorylation coupled with the oxidation of hydrogen. This idea is further supported by the observation that DNP inhibits the reduction of sulfate (Peck, 1960) but not the reduction of sulfite or thiosulfate that does not directly involve ATP.

With lactate as electron donor, the necessity for oxidative phosphorylation during growth is indicated because, theoretically, there does not appear to be a net production of ATP from substrate phosphorylation

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(Peck, 1960). Furthermore, Senez (1962) has pointed out that the growth yields obtained with lactate and pyruvate can not be explained by summation of the reactions involved in the metabolism of lactate or pyruvate and sulfate.

It has now been possilbe to demonstrate in extracts of one of these anaerobic bacteria, Desulfovibrio gigas, an esterification of orthophosphate dependent upon the oxidation of hydrogen with either sulfite or thiosulfate as electron acceptor. The requirements for this esterification and its inhibition by uncoupling agents indicate that the esterification is due to an oxidative phosphorylation system comparable to that found in aerobic bacteria.

METHODS

D. gigas (Le Gall, 1963) was grown on the medium described by Le Gall et al. (1965) at 30°C. Log phase cells were harvested from 40 liters of medium (30 gm. wet weight) and suspended in 75 ml. of a solution containing 0.25 M sucrose, 0.02 M Tris-HCl (pH 7.8) and 0.01 M $MgCl_2$. The extract is prepared by passing the suspension once through the French pressure cell at 3,000 p.s.i. A few crystals of DNase are added to reduce the viscosity of the extract and, after 5-10 minutes, the preparation centrifuged for 15 minutes in a Spinco Model L at 70,000 x G. The pellet is discarded and the preparation recentrifuged at 140,000 x G for 2 hr. This pellet is suspended in 15 ml. of the previously mentioned sucrose solution and serves as the source of particles. Soluble protein is prepared in the same manner but from a 50% suspension of cells (based on wet weight) from which the soluble cytochrome has been extracted (Le Gall et al, 1965). Hydrogen utilization was determined by means of the conventional Warburg apparatus and P_i esterification by the procedure of Nielson and Lenninger (1955).

RESULTS

Sulfite can function as the terminal electron acceptor in the normal respiration of the "sulfate-reducing bacteria" and the overall reaction for the reduction of sulfite with molecular hydrogen is shown in equation I.

1) $3H_2 + SO_3^{=} \longrightarrow S^{=} + 3H_2O$

As this type of respiration does not involve molecular oxygen, the usual P/O ratio has been expressed as a P/H_2 ratio. The actual number of phosphorylations is unknown; however, $\Delta F^{\bullet}$ calculations (Postgate, 1956) indicate that the maximum number of phosphorylations involved in the reduction of $SO_3^{=}$ (Eq. 1) is 3, or possibly 4, and would result in a theoretical maximum P/H_2 ratio of 1.0 and a minimum P/H_2 ratio of 0.33.

Although centrifuged extracts of D. desulfuricans usually reduce $SO_3^{=}$ with H_2 and activity is stimulated by exogenous cytochrome c_3 (however, see Postgate, 1961), similar extracts from D. gigas do not reduce $SO_3^{=}$ even when supplemented with cytochrome from either organism. In order to obtain the reduction of $SO_3^{=}$ with H_2 by extracts of D. gigas, it is necessary to supplement the soluble protein with the dye, methyl viologen, or with particulate protein as shown in Table I. Similarly, with a glucose-hexokinase trap, the esterification of orthophosphate was observed and shown to be completely dependent upon the presence of both protein fractions. The P/H_2 ratio was routinely found to be between 0.1 and 0.2, but values as high as 0.4 have been observed. The phosphorylation required both H_2 and $SO_3^{=}$ and was destroyed by boiling either the particles or the soluble protein. Thiosulfate would partially substitute for $SO_3^{=}$ in both the oxidation and phosphorylation.

The omission of ADP had little effect upon the phosphorylation but the omission of ADP plus the glucose-hexokinase trap completely eliminated detectable phosphorylation. In the presence of the trap, it was possible to demonstrate a complete dependence of the phosphorylation on ADP by treating the soluble protein with small amounts of norite to remove exo-

TABLE I

REQUIREMENTS FOR PHOSPHORYLATION COUPLED WITH SULFITE REDUCTION

Reaction Mixture	P_i Esterified (μmoles)	H_2 Utilized (μmoles)	P/H_2
Complete	1.77	9.9	0.12[1]
minus soluble protein	0.22	0.0	-
minus particles	0.39	0.0	-
minus H_2	0.58	0.0	-
minus Na_2SO_3	0.60	0.0	-
minus ADP	1.63	12.7	0.08[1]
minus ADP, hexokinase and glucose	0.00	10.0	-
minus hexokinase and glucose	0.89[2]	10.6	0.08
minus glucose, plus mannose	1.05[2]	8.7	0.12
Boiled Particles	0.66	0.0	-
Boiled Soluble Protein	0.37	0.0	-

The complete reaction mixture contained in umoles: Tris-HCl, pH 7.3, 40; $MgCl_2$, 40; glucose (or mannose where indicated) 100; p32 (9.8×10^5 cpm.) pH 7.3, 5; ADP, 5; Na_2SO_3, 20; NaF, 50 and hexokinase, 0.1 mg; soluble protein, 30 mg. ; and particle protein, 39.5 mg. , in a total volume of 3.0 ml. Center wells contained 0.1 ml. of 10% $CdSO_4$ and the gas phase was H_2 or N_2 where indicated. Temperature, 37°, time, 15 min.

1. Calculated from the difference between H_2 and N_2 control indicated above as "minus H_2".
2. Calculated from the difference between H_2 and N_2 control not included in the above data.

genous nucleotides. In the absence of a trapping system, phosphorylation was observed, but the amount of phosphorylation was smaller and more variable than that observed with the hexokinase-glucose trap. In addition, the control or blank values were considerably greater with ADP alone than

with ADP plus glucose and hexokinase. The phosphorylation does not appear to result from the metabolism of glucose because glucose is not fermented by D. gigas, mannose replaces glucose in the trapping system and phosphorylation is observed with ADP alone. These results indicate that both electron transfer and a system for accepting phosphate are required for the phosphorylation.

In Table 2, the effect of uncoupling agents on the phosphorylation is shown. Both gramicidin and pentachlorophenol completely inhibited the esterification of phosphate and generally had little effect upon the amount of H_2 utilized. Although, in this experiment, there appeared to be some inhibition of H_2 utilization by these uncoupling agents, in other experiments, employing these agents at different concentrations, a small stimulation of H_2 utilization was normally observed. DNP also inhibited the phosphorylation but an exact calculation of the effectiveness of this inhibitor is not possible as it is reduced in the presence of H_2 to aminophenols by extracts of this organism. Oligomycin had no inhibitory effect on the system but usually caused by slight stimulation of oxidative activity similar to that described in some bacterial systems (Ishikana and Lehninger, 1962). Both with respect to the dependence of the phosphorylation on electron transfer and the uncoupling of phosphorylation from electron transfer by known uncoupling agents, this system meets the requirements suggested by Chance and Williams (1956), for the demonstration of oxidative phosphorylation.

The soluble protein is stable when stored at -20° and can be fractionated by $(NH_4)_2\ SO_4$ into two protein fractions, both of which are required for phosphorylation as well as electron transfer. Passage of the soluble protein through a short DEAE column (5 x 2.5 cm) renders the protein inactive, but recombination of the dialyzed protein (eluted from the DEAE by 3 M NaCl) with the inactive protein, restores activity for both electron transfer and phosphorylation. The fraction contains

TABLE 2

THE EFFECT OF UNCOUPLING AGENTS ON PHOSPHORYLATION

Additions	Concentration	H_2 Utilized (μmoles)	P_i Esterified (μmoles)	P/H_2
None		13.9	1.82	0.13
Gramicidin	5.2×10^{-5} M	8.05	0.16	0.02
Pentachloro-phenol	3×10^{-5} M	9.12	0.08	0.01
DNP	2×10^{-4} M	8.60	0.58	0.07
Oligomycin	42 γ/ml	13.3	2.34	0.18

Each reaction mixture contained in umoles; Tris-HCl, 40; $MgCl_2$, 20; Na_2SO_3, 20; p^{32} (600,000 cpm) 5; ADP, 5; glucose, 100, NaF, 50; and hexokinase, 1 mg; particle protein, 19.8 mg; soluble protein, 39 mg in a total volume of 3.0 ml. Reaction mixtures were incubated at 37° under H_2 or N_2 and stopped by the addition of 1.0 ml of 10% TCA. The center well contained 0.1 ml of 10% $CdSO_4$. P_i esterified represents the difference between identical flasks incubated under atmospheres of H_2 and N_2. Gramicidin, pentachlorophenol and oligomycin were added in ethanol and the other flasks received an equivalent amount of ethanol (0.025 ml).

large amounts of ferredoxin and the possible role of this electron transfer protein in the system is being investigated.

The particle fraction rapidly looses activity when stored at 0° but does retain activity for several weeks when stored at -20°. Only one cytochrome has been detected on the particles and it is probably identical with the cytochrome isolated from these organisms by Le Gall et al (1965).

Although this system was obtained from a strictly anaerobic bacterium, it appears to be similar to oxidative phosphorylation systems extracted from other bacteria with regard to the effectiveness of uncoupling agents and the requirements of both soluble and particulate components for the oxidation and phosphorylation. Reversal of this oxidative phosphorylation should effect an ATP-dependent evolution of H_2 perhaps similar to that described by Bulen et al (1965) in Azotobacter and Clostridium pasteurianum (Hardy et al, 1965) and implicated in the fixation of nitrogen.

The observation that this organism possesses a single cytochrome suggests the unique possibility that it also carries out a single oxidative phosphorylation.

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REFERENCES

Bulen, W. A., Burns, R. C. and LeComte, J. R. Proc. Natl. Acad. Sci. 53, 532 (1965).
Chance, B. and Williams, G. R. Adv. in Enzymol. 17, 65 (1956).
Hardy, R. W. F., Knight, E., Jr., and D'Eustachio, A. J. Biochem. Biophys. Res. Commun 20, 539 (1965).
Ishikawa, S. and Lehninger, A. I. J. Biol. Chem. 237, 2401 (1962).
Ishimoto, M. and Fujimoto, J. J. J. Biochem. (Tokyo) 50, 229 (1961).
Le Gall, J. J. Bacteriol 86, 1120 (1963).
Le Gall, J., Mazza, G. and Dragoni, N. Biochem. Biophys. Acta 99, 385 (1965).
Nielson, S. O. and Lenninger A. L. J. Biol. Chem. 215, 555 (1955).
Peck, H. D., Jr. J. Biol. Chem. 235, 2734 (1960).
Peck, H. D., Jr. J. Biol. Chem. 273, 198 (1961).
Postgate, J. R. J. Gen. Microbiol. 14, 545 (1956).
Postgate, J. R. in "Symposium on Hematin Enzymes C.J.E. Falk, R. Lemberg and R. K. Morton, eds. Pergamon Press, New York (1961).
Senez, J. D. Bacteriol Rev. 26, 95 (1962).