OXIDATIVE PHOSPHORYLATION AND PROTON TRANSLOCATION IN MEMBRANE VESICLES PREPARED FROM *ESCHERICHIA COLI*

Elliot L. Hertzberg and Peter C. Hinkle

Section of Biochemistry, Molecular and Cell Biology
Cornell University, Ithaca, New York 14850

Received March 8, 1974

SUMMARY

Membrane vesicles have been prepared from *E. coli* which are capable of catalyzing oxidative phosphorylation with several physiological substrates. Phosphorylation efficiencies of 0.62 ± .06 moles ATP formed per gram-atom oxygen consumed have been observed with NADH as substrate. The phosphorylation is sensitive to uncouplers, fulfilling an essential requirement for the study of oxidative phosphorylation in cell-free systems. Since the vesicles also catalyze, either during respiration or ATP hydrolysis, uncoupler sensitive uptake of protons, they are inverted with respect to whole bacteria and allow the study of additional parameters relating to energy coupling.

The discovery of mutants of *Escherichia coli* deficient in oxidative phosphorylation (1) has resulted in a resurgence of interest in the study of energy-linked functions in this bacterium. However, these studies have been confined to secondary energy-linked processes, such as the transhydrogenase (2), reduction of NAD^+ by succinate (3), or substrate transport by the uncoupled mutants (4-8). For the most part, the direct measurement of oxidative phosphorylation has been omitted because of the low efficiencies generally obtained in cell-free systems (9), uncoupled pyridine nucleotide oxidation (10), or lack of sensitivity to uncouplers (11). There is one report of relatively high efficiencies of phosphorylation in the literature, but the authors failed to explore uncoupler sensitivity (12). The possibility of studying proton translocation in an inverted membrane preparation from *E. coli* has also not been pursued.

In this paper we describe the preparation of a particulate fraction from *E. coli* which catalyzes the uncoupler sensitive phosphorylation of ADP with a variety of substrates. In contrast to proton extrusion observed with whole cells (13), the vesicles catalyze the uptake of protons in response to energiza-

tion by either respiration or ATP, as predicted for an inverted preparation.

METHODS

E. coli W1655 was obtained from the Coli Stock Center, Yale University. Cells were grown batch-wise, either in an incubator-shaker, or in a New Brunswick Fermentor, in a defined minimal medium, consisting of 30 mM KPi, 9 mM NH_4Cl, 3 mM $MgCl_2$, 1 μM $FeSO_4$, 1 μM $ZnSO_4$, pH 7.0, supplemented with methionine to 20 μg/ml. The carbon source was glycerol, at a concentration of 15 mM. Cells were grown to mid-exponential phase and harvested by centrifugation in the cold. The cell pellet was resuspended and washed twice with 50 mM KPi, 5 mM $MgCl_2$, pH 7.0, and resuspended to a 20% (wet weight/volume) suspension in 50 mM KPi, 5 mM $MgCl_2$, 10% (v/v) methanol, 1 mM DTT*, pH 7.0. Cells were broken by a single passage through an Aminco French Pressure Cell (Model 43398-A) at 4500±500 pounds total pressure. Debris and unbroken cells were removed by centrifugation at 39,000 x g for ten minutes in a SS-34 rotor of a Sorvall RC-2B centrifuge. Vesicles were sedimented by centrifugation at 210,000 x g for two hours in a 50 Ti rotor of a Beckman L-2 ultracentrifuge. The pellet was resuspended by gentle homogenization to approximately 5 mg protein per ml in 250 mM sucrose, 10 mM morpholinopropane sulfonate, 5 mM $MgCl_2$, 10% (v/v) methanol, 1 mM DTT, pH 7.5, and the centrifugation repeated. The pellet was finally resuspended to approximately 50 mg/ml in the same buffer. Vesicles prepared by this procedure are stable to freezing and thawing, and remain stable for at least two weeks at -70°. Storage in liquid nitrogen stabilizes virtually all activity for at least two months.

Oxidative phosphorylation was measured by adding approximately 1 mg of vesicle protein to a reaction mixture containing 250 mM sucrose, 5 mM Tris-Cl, 2 mM $MgSO_4$, 0.3 mM EDTA, 32 mM glucose, 10.0 units/ml hexokinase, 1 mM ATP, 1 mg/ml fatty acid free BSA, 20 mM [^{32}P]-KPi, pH 7.4, at a specific activity

---

* Abbreviations used: BSA, bovine serum albumin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DTT, dithiothreitol.

of 50-100 cpm per nanomole, in a 1.2 ml oxygraph cell maintained at 25°. With NADH as substrate, the reaction mixture was previously supplemented with 10 units alcohol dehydrogenase (Sigma, Type A-7011) and 10 µl 95% ethanol. After thirty seconds, substrate in amounts indicated in the legends of the tables was added and the reaction was generally allowed to proceed until anaerobiosis occurred, usually in about three minutes. A 0.5 ml aliquot was then withdrawn and rapidly added to 0.5 ml cold 10% trichloroacetic acid. Protein was sedimented by centrifugation, and esterified phosphate analyzed (14).

Respiration and ATP driven proton translocation was measured by the pulse method of Mitchell and Moyle (15,16) using a recording pH meter described by Thayer and Hinkle (17). In respiration experiments 2-5 mg of protein was added to nitrogen-bubbled 150 mM KCl, 5 mM $MgCl_2$ and respiration was initiated after equilibration by injecting microliter quantities of air-saturated 150 mM KCl, 5 mM $MgCl_2$ or oxygen-saturated ethanol. Amounts of proton translocation were estimated by calibration with pulses of standardized, anaerobic HCl. ATP-driven proton translocation was measured in a medium of 150 mM KCl, 5 mM $MgCl_2$ at pH 6.15 in order to prevent net acid or base formation from the hydrolysis of ATP (17).

Protein was measured by the biuret procedure (18). CCCP and valinomycin were obtained from Sigma.

RESULTS AND DISCUSSION

As shown in Table 1, approximately 25% cell breakage was achieved under the conditions described. The bulk of the respiratory activity recovered following removal of unbroken cells and large debris is associated with the particulate fraction. The remainder is decanted with the supernatant fractions, and consists of poorly packed material. This procedure probably yields a fraction contaminated by cell wall debris and ribosomes. However, these are unlikely to interfere with our measurements.

Table 2 shows the respiratory rates and phosphorylation efficiencies with

TABLE 1. Cell Breakage and Fractionation of Respiratory Activity

Fraction	Volume ml	Protein mg/ml	Yield total mg	Specific Activity units/mg protein	Total Activity units
Cell Suspension	34.6	36.4	1259	-	-
29,000 x g Supernatant	19	16.2	308	46.6	14,300
210,000 x g Supernatant	18.8	11.4	214	9.4	2,030
210,000 x g Wash	9.5	3.2	30	23.2	74
Particles	1.2	39	47	248	11,700

Respiration was measured utilizing the NADH regenerating system described in the text in the presence of 167 μM NAD^+. The medium was 250 mM sucrose, 5 mM $MgCl_2$, 20 mM KPi, pH 7.4. Units are expressed in ng atoms oxygen consumed per minute.

TABLE 2. Oxidative Phosphorylation in Respiratory Vesicles

Substrate	Specific Activity units/mg protein	Oxygen Uptake ng atoms O	Pi Esterified nmoles Pi	P/O
NAD^+ (Alcohol dehydrogenase limiting)	66	634	463	.73
+ CCCP	68	658	0	0
NAD^+ (Excess alcohol dehydrogenase)	172	624	353	.57
+ CCCP	202	658	11	.02
α-glycerolphosphate	212	627	239	.38
+ CCCP	230	672	3	0
D-lactate	86	620	278	.45
+ CCCP	66	658	1	0
Succinate	30	277	152	.55
+ CCCP	30	249	14	.06

Oxidative phosphorylation was measured as described in the text. NAD^+ was added to 167 μM. The level of alcohol dehydrogenase required for limitation of the respiratory rate due to the regenerating system was determined empirically. All other substrates were used at 12.5 mM. CCCP was used at 83 μM. Units are expressed in ng atoms oxygen consumed per minute.

various substrates. The esterification of inorganic phosphate has been corrected for low levels of respiration-independent incorporation (2-3 nanomoles per minute per mg protein). Interestingly, in the case of NADH, higher coupling efficiencies were obtained if alcohol dehydrogenase was rate limiting in the NADH regenerating system. Such a system showed a mean P/O of 0.66 ± .06, based on four different preparations. With excess alcohol dehydrogenase, fourteen preparations exhibited a mean P/O of 0.62 ± .06. It is significant that the coupling efficiency of NADH was higher than that of the flavoprotein linked substrates, α-glycerolphosphate, D-lactate and succinate. In all cases, phosphorylation was sensitive to the uncoupler CCCP. If BSA was omitted from the oxidative phosphorylation assay mixture, 4.2 μM CCCP resulted in 94% uncoupling. Omission of BSA resulted in a 15% decrease of the P/O.

Experiments carried out by the method of Hempfling (19) (data not shown) indicate a P/O of 2.5 in whole cells. This technique generally utilizes only the initial levels of reduced pyridine nucleotide for determining total electron flow. Since the levels of reduced quinones, flavoproteins and cytochromes are not considered, somewhat high values are obtained, suggesting that there are actually two sites of coupling in this strain. The data in Table 2 also suggest that there is one coupling site between NADH and the point of entry of reducing equivalents from succinate, D-lactate and α-glycerolphosphate, and another between this point and oxygen.

Figures 1 and 2 demonstrate respiration driven proton translocation in this preparation, with limiting and saturating oxygen pulses, respectively. The uptake of protons indicates that the vesicles are inverted with respect to the whole cell. After correcting for the response time of the electrode by a semi-logarithmic plot of the observed pH change versus time (20) (not shown), a ratio of 1.70 protons translocated per gram-atom of oxygen consumed was observed. The uncorrected value was 1.38. Since a stoichiometry of four protons translocated per oxygen is expected for two coupling sites linked to the oxidation of NADH (15), apparently about half of the respiration is not coupled

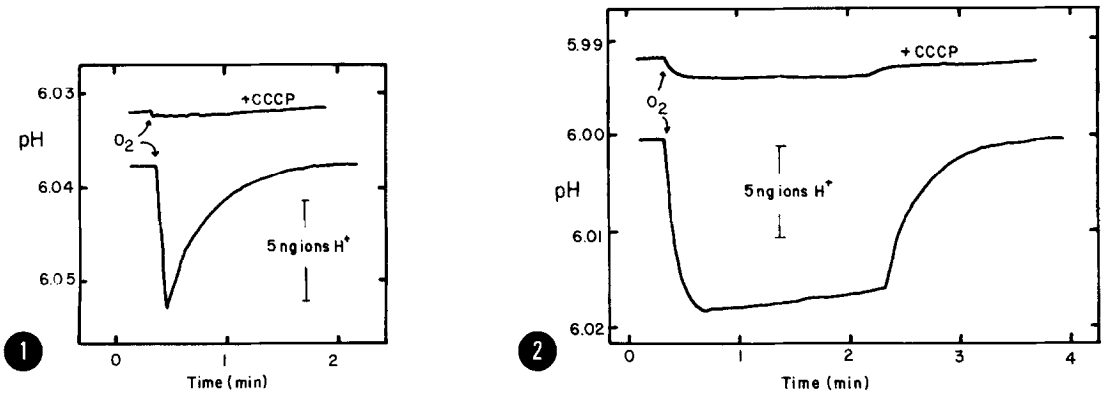

Figure 1. Respiration-driven proton translocation in respiratory vesicles. A 0.6 ml anaerobic pH cell containing 2.2 mg vesicle protein was used. Valinomycin (0.1 µg) was present during the assay. The substrate was 167 µM NAD^+ in the presence of the regenerating system described earlier. At the time indicated by the arrow, 10 µl of air-saturated 150 mM KCl, 5 mM $MgCl_2$, corresponding to 4.7 ng atoms of oxygen, was added. Where indicated, CCCP was present at 16.7 µM.

Figure 2. Respiration-driven proton translocation in respiratory vesicles. The experiment was performed as described in the legend for Figure 1 except that 2.65 mg vesicle protein was used, and the oxygen pulse consisted of 5 µl of oxygen-saturated ethanol.

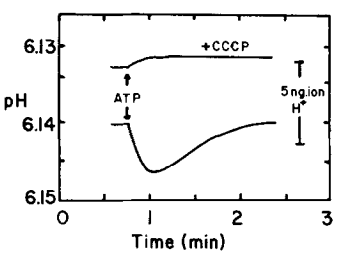

Figure 3. ATP-driven proton translocation in respiratory vesicles. 2.65 mg vesicle protein suspended in 0.6 ml 150 mM KCl, 2 mM $MgCl_2$ was placed in the pH cell. Valinomycin (0.1 µg) was also present during the assay. At the time indicated by the arrow 15 nmoles ATP, pH 6.15 were added. Where indicated, CCCP was present at 16.7 µM.

---

in this preparation. Preliminary measurements of the proton-to-oxygen ratio with flavin-linked substrates indicates a proton-to-oxygen ratio approaching one, consistent with the above interpretation.

Figure 3 demonstrates ATP-driven, uncoupler sensitive uptake of protons

in these vesicles. Since coupling efficiencies were low, the data do not yet warrant a detailed analysis of the proton to ATP stoichiometry (17).

ACKNOWLEDGEMENTS

The authors would like to thank Professors A. Jane Gibson and Efraim Racker for many helpful suggestions. This work was supported by research grant HL 11483 from the National Institutes of Health. P.C.H. is the recipient of Career Development Award GM-22427 and E.L.H. of a National Institutes of Health Predoctoral Traineeship.

REFERENCES

1. Butlin, J. D., Cox, G. B. and Gibson, F. (1971) Biochem. J. 124, 75-81.
2. Bragg, P. D. and Hou, C. (1973) Biochem. Biophys. Res. Comm. 50, 729-736.
3. Sweetman, A. J. and Griffiths, D. E. (1971) Biochem. J. 121, 117-124.
4. Simoni, R. D. and Shallenberger, M. K. (1972) Proc. Nat. Acad. Sci. U.S. 69, 2663-2667.
5. Rosen, B. P. (1973) Biochem. Biophys. Res. Comm. 53, 1289-1296.
6. Or, A., Kanner, B. I. and Gutnick, D. L. (1973) FEBS Letters 35, 217-219.
7. Schairer, H. U. and Haddock, B. A. (1972) Biochem. Biophys. Res. Comm. 48, 544-551.
8. Berger, E. A. (1973) Proc. Nat. Acad. Sci. U.S. 70, 1514-1518.
9. Bragg, P. D. and Hou, C. (1968) Can. J. Biochem. 46, 631-641.
10. Kashket, E. R. and Brodie, A. F. (1963) Biochem. Biophys. Acta 78, 52-65.
11. I-shen, Z. (1965) Acta Biochim. Biophys. Sinica 5, 545-547.
12. Turnock, G., Erickson, S., Ackrell, B. C. and Buck, B. (1972) J. Gen. Microbiol. 70, 507-515.
13. Lawford, H. G. and Haddock, B. H. (1973) Biochem. J. 136, 217-220.
14. Lindberg, O. and Ernster, L. (1956) Methods Biochem. Anal. 3, 1-22.
15. Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147-1162.
16. Mitchell, P. and Moyle, J. (1968) Eur. J. Biochem. 4, 530-539.
17. Thayer, W. S. and Hinkle, P. C. (1973) J. Biol. Chem. 248, 5395-5402.
18. Jacobs, E. E., Jacob, M., Sanadi, D. R. and Bradley, L. B. (1956) J. Biol. Chem. 223, 147-156.
19. Hempfling, W. P. (1970) Biochem. Biophys. Res. Comm. 41, 9-15.
20. Hinkle, P. C. and Horstman, L. L. (1971) J. Biol. Chem. 246, 6024-6028.