Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 46-52

PHOSPHATE TRANSPORT AND THE STOICHEIOMETRY OF RESPIRATORY DRIVEN PROTON TRANSLOCATION IN ESCHERICHIA COLI

John C. Cox & Bruce A. Haddock

Department of Biochemistry, Medical Sciences Institute,

University of Dundee, Dundee DD1 4HN, Scotland, U.K.

Received February 21, 1978

SUMMARY

The stoicheiomety of respiratory driven proton translocation associated with the oxidation of endogenous substrates has been measured in an organic phosphate auxotrophic mutant of Escherichia coli. The results obtained indicate that movements of inorganic phosphate do not result in an experimental underestimation of the observed H^+/site ratio in E. coli, as has previously been suggested for mitochondria.

INTRODUCTION

According to Mitchell's chemiosmotic hypothesis [1,2], the electron transport components of the mitochondrial inner membrane and the bacterial cytoplasmic membrane are functionally organized into an organism-dependent number of 'loops' or 'proton pumps' that serve to generate a trans-membrane proton electrochemical gradient (inside negative) which can be used subsequently to drive reversed electron transport, ATP synthesis or the accumulation of a variety of solutes. Initial work with both mitochondria [e.g. 3,4,5] and E. coli [e.g. 6], suggested a stoicheiometry of 2.0 for the H^+/site ratio; that is, the number of protons translocated per redox 'loop' or 'proton pump'. More recently this value has been questioned for mitochondria and is regarded as an underestimate as a result of investigations on the stoicheiometry of Ca^{++} uptake [7], K^+ uptake [8], the electrogenic nature of the adenine nucleotide translocator [9], and the H^+/site ratio required for synthesis of ATP at maximum phosphorylation potentials [10].

0006-291X/78/0821-0046$01.00/0

Studies performed in Lehninger's laboratory [11-14] have suggested a possible answer to this controversy by showing that when N-ethylmaleimide, (at concentrations sufficient to inhibit specifically the inorganic phosphate translocator) was added to mitochondrial suspensions the observed H^+/site ratios increased significantly to give values of between 3 and 4. Thus it has been proposed that previous results obtained with mitochondria [3-5] gave underestimated values for the H^+/site ratio since adequate precautions had not been taken to preclude the activity of this H^+/inorganic phosphate transporter [14].

Although the need to take into account the activities of the inorganic phosphate and adenine nucleotide translocators when considering mitochondrial oxidative phosphorylation now, perhaps retrospectively, seems obvious, these same considerations do not necessarily apply to bacterial oxidative phosphorylation (Fig. 1). As discussed previously [15] with the exception of certain symbiotic bacteria [16], the bacterial cytoplasmic membrane contains no adenine nucleotide translocator and there is no physiological reason for rapid movements of inorganic phosphate across the membrane. However, we felt it necessary to examine the possible effects of inorganic phosphate transport on the previously reported values for the H^+/site ratio in *E. coli* [6] and demonstrate here that an organic phosphate auxotroph of *E. coli* [17] which does not have the ability to transport inorganic phosphate has the same stoicheiometry of respiratory driven proton translocation associated with the oxidation of endogenous substrates as its parent and other *E. coli* strains.

MATERIALS AND METHODS

E. coli strain 10B5 (K10, Hfr, *pitl*, *pst2*, *glpR2*, *glpD3*, *phoA8*, *relA1*, *tonA22*, T_2^R) was generously provided by Dr B. Bachmann, E. coli Genetic Stock Centre, New Haven, Connecticut as strain CGSC 5506.

Cells were grown aerobically at 37° in a mineral salts medium [18] containing vitamin-free amino acids (0.1% w/v), glucose (0.5% w/v) and *sn*-glycerol-3-phosphate (0.1% w/v). The cells were harvested during early exponential growth, washed twice in mineral medium lacking any carbon source, resuspended in mineral medium containing glucose (0.5% w/v) and incubated for a further 30 min. at 37°. The cells were reharvested by centrifugation, washed

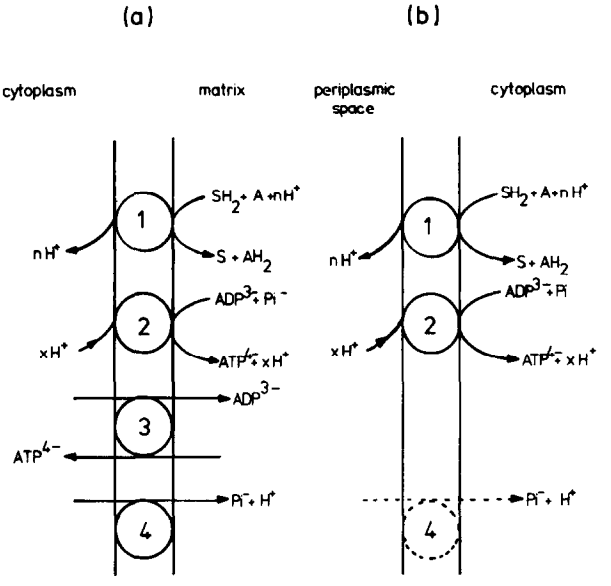

Figure 1: A comparison of vectorial reactions associated with respiratory ATP synthesis in a) the mitochondrial inner membrane and b) the bacterial cytoplasmic membrane. Each membrane is represented by parallel pairs of lines. Circles in the membranes indicate the following vectorial enzymes and translocators:- (1) proton translocating respiratory chain oxidoreductase, (2) proton translocating ATPase, (3) adenine nucleotide translocator and (4) the inorganic phosphate transport system, thought to be a single protein in mitochondria, though in bacteria the phosphate transport system may consist of two proteins. In bacteria, evidence indicates that the number of protons translocated by one site of the respiratory chain (n) is equal to x (the number of protons required by the ATPase for the production of one molecule of ATP).

and resuspended in buffer containing sucrose (300mM), KCl (150mM), $MgCl_2$ (5mM) and 2-(N-2-hydroxyethyl-piperazin N^1-yl) ethanesulphonic acid (HEPES, 10mM; pH 7.0) to a protein concentration of about 20 mg/ml. Sphaeroplasts were prepared by the method of Garland et al [19] and other assays performed as described in previous publications from this laboratory [6].

All chemicals used were of AnalaR grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., with the exception of vitamin-free casamino acids which were purchased from Difco Laboratories, Detroit, Michigan, U.S.A.

RESULTS AND DISCUSSION

The inorganic phosphate transport deficient mutant (strain 10B5) used in this work was originally isolated and characterized by Sprague et al [17]. This strain cannot transport inorganic phosphate (pit and pst

mutant alleles), is constitutive for the sn-glycerol-3-phosphate catabolism operon (glpR) and so can transport but not metabolize sn-glycerol-3-phosphate (glpD) and cannot hydrolyse extracellular sn-glycerol-3-phosphate (phoA$^-$). Thus the only source of intracellular inorganic phosphate in this strain is from the addition of sn-glycerol-3-phosphate to the growth medium and its subsequent hydrolysis within the cell by the action of unspecified phosphatases. It is perhaps pertinent to note that E.coli K10 strains appear to lack the pit-specified transport system for inorganic phosphate unlike the better characterized K12 strains [17,20].

It has been shown previously that the H^+/site ratio for E.coli cells (either K12 [6] or K10 [J. C. Cox, unpublished observations]) respiring different added substrates is approximately 2.0. The stoicheiometry of respiratory-driven proton translocation obtained with sphaeroplasts of the organic phosphate auxotroph of E.coli (strain 10B5) is shown in Fig. 2. For endogenous (unspecified) respiratory substrates the observed $\rightarrow H^+/O$ ratios obtained were about 4.0, corresponding to an H^+/site ratio of 2.0 [6]. This result indicates that, in E.coli, the movement of inorganic phosphate does not contribute to an underestimation of the previously observed H^+/site ratio in other E.coli strains [6]. As expected, with this particular mutant strain, the addition of exogenous inorganic phosphate to the suspension medium used in the 'proton-pulse' experiments made no difference to the observed $\rightarrow H^+/O$ stoicheiometry (Fig. 2).

The possibility that N-ethylmaleimide might exert some effect other than by inhibiting inorganic phosphate transport was also investigated. As indicated in Fig. 2 the addition of N-ethyl maleimide, up to a concentration of 50 nmole/mg protein, had no effect on the observed stoicheiometry of respiratory-driven proton translocation. At higher concentrations, the addition of N-ethylmaleimide resulted in an inhibition of the observed $\rightarrow H^+/O$ ratio that paralleled its inhibitory effect on respiration.

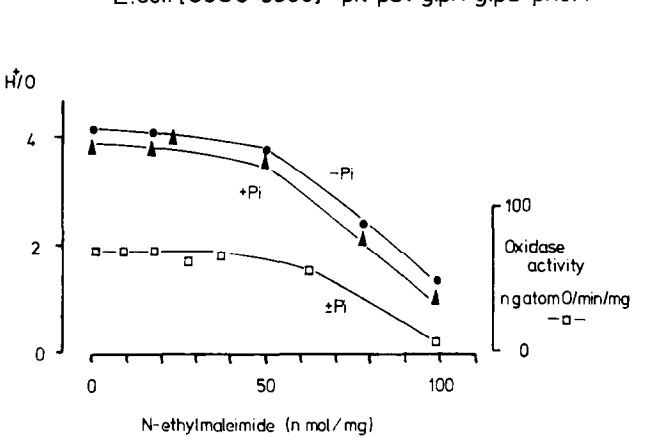

Figure 2: The stoicheiometry of respiration-driven proton translocation in an inorganic phosphate auxotrophic mutant of E. coli. Cells were grown on glucose as carbon source, harvested during early exponential phase and pre-loaded with glucose as described in 'Materials and Methods'. Cells (approx. 4 mg protein) were added to 1.0 ml N_2-saturated KCl (150 mM), sucrose (300 mM), $MgCl_2$ (5 mM) and HEPES (10 mM) buffer, pH 7.0, 25°C containing valinomycin (5 µg/ml). After pre-incubation for 15 min. additions of air saturated KCl (150 mM) were made by injection with a micro-syringe, as described in [6].

The results presented in Fig. 2 suggest that the rate and extent of transport of inorganic phosphate into E.coli do not result in an underestimation of, and hence need to re-evaluate, the observed H^+/site ratio in bacteria as has previously been suggested in equivalent studies with mitochondria [11 - 14]. This observation, together with the absence of an adenine nucleotide translocator in this bacterium, implies that an H^+/site ratio of 2.0 may be sufficient to drive all the energy-dependent reactions of the proton electrochemical gradient in E.coli and related bacteria. Clearly, however, other criteria apply in equivalent studies with symbiotic bacteria like Rickettsia prowazeki that do possess an adenine nucleotide translocator [16].

It should also be noted that although the movement of inorganic phosphate has been shown to have no effect on the magnitude of the observed H^+/site ratio in E.coli, this does not preclude the possibility that the movement of other ion(s), in the experimental system used, may result in

an underestimation of this ratio by a mechanism similar to that proposed for inorganic phosphate transport in mitochondria. Furthermore, it proved difficult to measure the stoicheiometry of proton translocation associated with the respiration of defined exogenously-supplied substrates in this organic phosphate auxotroph using experimental techniques that had previously been applied successfully to similar studies with a prototrophic strain [6]. One possible reason for this difficulty is the apparent need to grow this organic phosphate auxotroph on glucose as a primary carbon source with the concomittant problems resulting from catabolite repression effects on the transport and metabolism of added compounds like L-malate and succinate. Whatever the reason, experiments were limited to measurements of respiratory-driven proton translocation associated with the oxidation of endogenous substrates in strain 10B5 but the results obtained were not significantly different from those observed with other K-10 and K12 E.coli strains that were genetically competent in their ability to transport inorganic phosphate.

CONCLUSIONS

Rapid movements of inorganic phosphate in E.coli are not instrumental in causing an underestimation of the observed H^+/site ratio as has been suggested previously in equivalent studies with mitochondria [11 - 14]. The possibility that the movements of other ions in bacteria cause a similar experimental artefact cannot be excluded. Together with the absence of a functional adenine nucleotide translocator, the results presented here suggest that H^+/site ratios of 2.0 may be sufficient to support the energy requiring processes driven by the proton electrochemical gradient in the majority of bacteria.

ACKNOWLEDGEMENTS

This work was supported by an SRC studentship (JCC). We are indebted to Prof. Peter Garland for many invaluable discussions.

REFERENCES

[1] Mitchell, P. (1966) 'Chemiosimotic coupling in oxidative and photosynthetic phosphorylations'. Glynn Research, Bodmin, U.K.
[2] Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399-430.
[3] Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147-1162
[4] Lawford, H. G. and Garland, P. B. (1972) Biochem. J. 130, 1029-1044.
[5] Lawford, H. G. and Garland, P. B. (1973) Biochem. J. 136, 711-720.
[6] Lawford, H. G. and Haddock, B. A. (1973) Biochem. J. 136, 217-220.
[7] Chance, B. (1965) J. Biol. Chem. 240, 2729-2748.
[8] Massari, S. and Azzone, G. F. (1970) Eur. J. Biochem. 12, 301-318.
[9] Klingenberg, M. and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125-130.
[10] Rottenberg, H. (1970) Eur. J. Biochem. 15, 22-28.
[11] Brand, M. D., Reynafarje, B. and Lehninger, A. L. (1976) J. Biol. Chem. 251, 5670-
[12] Brand, M. D. and Lehninger, A. L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1955-
[13] Alexandre, A. (1977) Fed. Proc. 36, 814.
[14] Lehninger, A. L., Reynafarje, B. and Alexandre, A. (1977) in 'Structure and function of energy transducing membranes', pp. 95-106 Eds. K. van Dam and B. F. van Gelder, Elsevier, North Holland Biomedical Press, Amsterdam.
[15] Garland, P. B. and Haddock, B. A. (1977) Biochem. Soc. Trans. 5 479-484.
[16] Winkler, H. H. (1976) J. Biol. Chem. 251 389-396
[17] Sprague, G. F., Bell, R. M. and Cronan J.E. (1975) Molec. Gen. Genet. 143 71-77
[18] Cohen, G.N. and Rickenberg, M.W. (1956) Ann. Inst. Pasteur, Paris 91 693-720.
[19] Garland, P.B., Downie, J.A. and Haddock, B.A. (1975) Biochem. J. 152 547-559
[20] Medveczky, N. and Rosenberg, H (1971) Biochim. Biophys. Acta. 241, 494-506.