PROTON TRANSLOCATION

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Comparative Aspects of the Energetics of Oxidative Phosphorylation in Bacteria and Mitochondria

STUART J. FERGUSON

Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

and M. CATIA SORGATO

Istituto di Chimica Biologica, Padova 35100, *Italy*

and DOUGLAS B. KELL

Department of Botany and Microbiology, University College, Aberystwyth SY23 3DA, Wales, U.K.

and PHILIP JOHN

Department of Agricultural Botany, University of Reading, Reading RG6 2AS, U.K.

The same fundamental mechanism of coupling between electron transport and ATP synthesis operates in both mitochondria and bacteria, but, with some exceptions (Ferguson, 1977; Garland, 1977; Garland & Haddock, 1977), there has been little enquiry into whether there may be significant differences between the exact energetics of mitochondrial and bacterial oxidative phosphorylation. In particular it has been widely accepted that proton translocation through both the mitochondrial and bacterial ATPases has an identical stoicheiometry $[(H^+/ATP)_{ATPase}]$ of 2 H^+ ions translocated per ATP molecule synthesized or hydrolysed. The experimental evidence for $[(H^+/ATP)_{ATPase}]$ with bacterial ATPases is limited (Garland, 1977; Garland & Haddock 1977) and, as we shall seek to show in the present paper, there are reasons for now reconsidering whether the $[(H^+/ATP)_{ATPase}]$ for the mitochondrial and bacterial enzymes is identical.

There is abundant evidence that respiring mitochondria can maintain an external (cytosolic) phosphorylation potential ($\Delta G_p = \Delta G'_0 + RT\ln[ATP]/[ADP][P_i]$) that is considerably higher than its internal (matrix) counterpart. The simplest explanation for this behaviour is to consider in terms of the chemiosmotic theory that whereas the internal phosphorylation potential is related to the protonmotive force (Δp) by

$$\Delta G_p = -[(H^+/ATP)_{ATPase}] \cdot F \cdot \Delta p \qquad (1)$$

the corresponding relationship for the external phosphorylation potential is

$$\Delta G_p = -[(H^+/ATP)_{ATPase} + x] \cdot F \cdot \Delta p \qquad (2)$$

where x is the number of protons moving down their electrochemical gradient in association with the combined processes of adenine nucleotide translocation and P_i transport. In currently accepted schemes for adenine nucleotide translocation and P_i transport, $x = 1$. Although this interpretation of mitochondrial oxidative phosphorylation is supported by much experimental evidence, it is possible that estimates of the internal (matrix) phosphorylation potential might be underestimates, owing to, for instance, preferential binding of ADP or relatively rapid consumption of ATP by intramitochondrial processes. For these and other reasons it is important that further support for the above scheme has come from recent studies with submitochondrial

particles in which the suspending reaction medium can be regarded as being topologically equivalent to the mitochondrial matrix. Both Ferguson & Sorgato (1977) and Thayer *et al.* (1977) have concluded that submitochondrial particles are able only to generate a phosphorylation potential [43.9–46.0kJ (10.5–11.0kcal/mol)] comparable with that found in the mitochondrial matrix, and a related study by Rottenberg & Gutman (1977) supports this conclusion. It is considered that these results are not a consequence of undue damage to the oxidative-phosphorylation apparatus during preparation of the particles, but rather are a reflection of the characteristics of the mechanism of oxidative phosphorylation in the particles. This view is at variance with the suggestion of Le-Masters & Hackenbrock (1976) that the magnitude of the phosphate potential can be taken as a sensitive parameter of the integrity of phosphorylating membranes; furthermore, the argument developed in the present paper would be refuted if submitochondrial particles able to generate the high values of ΔG_p found outside of mitochondria were to be prepared.

A value of 2 has been obtained for $(H^+/ATP)_{ATPase}$ for the mitochondrial enzyme from experiments with both mitochondria and submitochondrial particles in which H^+ movements associated with the hydrolysis of a pulse of ATP were measured (Mitchell, 1976). Our own work (Sorgato *et al*, 1978) determined the minimum value of $(H^+/ATP)_{ATPase}$ as 3 from measurements of Δp, ΔG_p and application of eqn. (1), although the exact value of $(H^+/ATP)_{ATPase}$ depended on the nature of the suspending reaction medium for reasons that are not yet understood. Also still to be understood are the reasons why, in closely related experiments, Berry & Hinkle (1978) have measured in submitochondrial particles protonmotive forces of up to 260mV, commensurate with $(H^+/ATP)_{ATPase} = 2$. On the other hand, additional evidence for $(H^+/ATP)_{ATPase} = 3$ has come from the work of Rottenberg & Gutman (1977), van Dam *et al.* (1977), Alexandre *et al.* (1978) and Azzone *et al.* (1978), whereas a mitochondrial P/O ratio of 2 for NADH oxidation (Hinkle & Yu, 1979) together with an H^+/O ratio of 9 (Brand, 1977) would also be compatible with $(H^+/ATP)_{ATPase} = 3$. Nevertheless, irrespective of any present uncertainty as to the value for $(H^+/ATP)_{ATPase}$, it is concluded that the protonmotive force generated by the mitochondrial respiratory chain can at most support a ΔG_p of around 46.0kJ (11kcal/mol) on the basis of $(H^+/ATP)_{ATPase} = 2$ or 3, and that the considerably higher values of ΔG_p [63–67kJ (15–16kcal)/mol] can only be accounted for when $x \geqslant 1$ (eqn. 2).

The overall energetics of bacterial oxidative phosphorylation are best studied with membrane vesicles in which the membrane is oriented in the sense that it is inside-out relative to the bacterial cell. Oxidative phosphorylation is catalysed by such vesicles without the participation of an adenine nucleotide translocase (which is believed to be absent from most, if not all, bacteria) or a phosphate-transport system (cf. e.g. Garland & Haddock, 1977), and topologically the vesicles are equivalent to submitochondrial particles. Thus eqn. (1) is expected to describe the process of oxidative phosphorylation (or photophosphorylation) in bacterial vesicles. It has been demonstrated that vesicles from several species of bacteria generate much larger phosphorylation potentials than submitochondrial particles (e.g. Ferguson & Sorgato, 1977; Kell *et al.* 1978*a*,*b*), and this means (eqn. 1) that relative to submitochondrial particles the value of $(H^+/ATP)_{ATPase}$ and/or Δp must be larger for the bacterial systems.

Parallel studies (Table 1) on the magnitude of Δp and ΔG_p in chromatophores from *Rhodospirillum rubrum* (Kell *et al.*, 1978*a*), vesicles from *Paracoccus denitrificans* (Kell *et al.*, 1978*b*) and submitochondrial particles (Sorgato *et al.*, 1978) suggest that $(H^+/ATP)_{ATPase}$ is larger for the two bacterial systems tested. The values of Δp were obtained in each case from measuring, by using the flow-dialysis technique, the uptake of $S^{14}CN^-$ and [^{14}C]methylamine for the respective determination of the respiration- or light-dependent membrane potential and pH gradient. The evaluation of these parameters from the extent of uptake of permeant ions and weak bases (or acids) does entail a number of assumptions [discussed in Kell *et al.* (1978*a*,*b*) and Ferguson *et al.* (1979)], and requires an estimate of the internal volume of the membrane vesicles (particles). These assumptions and estimates are common to all three systems tested, and so even if some

Table 1. *Protonmotive force and phosphorylation potential in submitochondrial particles and bacterial vesicles*

Data were taken from (*a*) Kell *et al.* (1978*a*), (*b*) Sorgato *et al.* (1978), (*c*) Kell *et al.* (1978*b*), where full experimental details can be found. The values of Δp given in parentheses represent upper limits on Δp, obtained by adding the lower limit of detection for the pH gradient to the observed membrane potential.

System	Δp(mV)	ΔG_p kJ/mol	ΔG_p (kcal/mol)
(*a*) *R. rubrum* chromatophores	100 (130)	58.9	14.1
(*b*) Bovine heart submitochondrial particles	145 (175)	43.0	10.3
(*c*) *P. denitrificans* vesicles	145 (175)	53.5	12.8

of the assumptions are not fully justified, the comparison of values of Δp shown in Table 1 would still be valid unless there are any errors that are peculiar to only one of the systems. This possibility is now briefly examined by discussing each of the systems in turn.

Chromatophores from photosynthetic bacteria including *R. rubrum* are unusually small vesicles (60nm diameter) and are generally considered to be homogeneous, oriented inside-out, and to be one of the best available membrane preparations from bacteria. The light-induced $S^{14}CN^-$ uptake by *R. rubrum* chromatophores corresponded to a membrane potential of approx. 100mV under the standard reaction conditions used, and no uptake of [^{14}C] methylamine was detected, so that an upper limit of approx. 30mV was assigned to the pH gradient and 130mV to Δp (Kell *et al.*, 1978*a*). A value in this range for Δp, although very similar to that obtained by Michels & Konings (1978) from similar experiments, is surprisingly small and requires that $(H^+/ATP)_{ATPase}$ be as high as 5. The small size of chromatophores might mean that the sucrose-impermeable-space method used for determining the internal volume is inaccurate, but this possible source of error now seems less likely in view of data from the electron microscope (Packham *et al.*, 1978), which shows that *R. rubrum* chromatophores have an internal volume of approx. 50μl per mg of bacteriochlorophyll. This is the same value as was obtained from the sucrose-impermeable-space method (Kell *et al.*, 1978*a*), and it is noteworthy that in both studies the chromatophores were prepared by sonication of cells. However, these results do not exclude the possibility that the internal volume of the chromatophores that is available to SCN^- is less than the measured volume, but this is a problem common to all three systems.

Bashford *et al.* (1979) have concluded from studies with the membrane-potential-sensitive probe oxonol VI that the light-dependent membrane potential in *R. rubrum* chromatophores is 258mV, and thus large enough to account for the measured ΔG_p with $(H^+/ATP)_{ATPase} = 2$ in marked contrast with the data given in Table 1. The extent of the light-induced oxonol VI reponse was related to the membrane potential by a calibration procedure in which changes in absorbance of oxonol VI were compared with carotenoid band shifts induced in chromatophores from *Rhodopseudomonas sphaeroides Ga* by known potassium-diffusion potentials. The results from experiments with oxonol VI therefore rely on the efficacy of the carotenoid band shift as an indicator of bulk-phase membrane potential. Comparison of the value of membrane potential indicated by $S^{14}CN^-$ uptake or carotenoid band shift under a variety of conditions has shown that these two methods give very different estimates of the membrane potential, with $S^{14}CN^-$ uptake giving the larger value during respiration but the smaller value upon illumination (Ferguson *et al.*, 1979). Possible explanations for this discrepancy between the two methods are discussed elsewhere (Ferguson *et al.*, 1979), but in view of these results it is not surprising that experiments with oxonol VI should also indicate a larger potential

than $S^{14}CN^-$ uptake. A further point to consider is that with the reaction conditions used by Bashford *et al.* (1979) [20mM-Mops (4-morpholine propanesulphonic acid)/100mM-KCl], it is expected, by analogy with previous work under similar conditions (Ferguson *et al.*, 1979), that according to $S^{14}CN^-$ and [^{14}C]methylamine uptake, a substantial fraction of the protonmotive force will be expressed as a pH gradient, whereas the results from experiments with oxonol VI indicate that a membrane potential is the main component of Δp. Thus not only do solute-uptake procedures give lower values for the membrane potential than membrane-bound spectroscopic probes, but they also assign a different weighting to the relative contributions of the membrane potential and pH gradient. These discrepancies need to be resolved by further experiment, but it is concluded that at present there is no physico-chemical basis for doubting that $S^{14}CN^-$ and [^{14}C]methylamine uptake reflect quantitatively the membrane potential and pH gradient in chromatophores.

One of the main problems in determining the magnitude of Δp generated by submitochondrial particles and vesicles from *P. denitrificans* is to assess what proportion of the preparations are oriented with the same polarity as mitochondria or bacterial cells. This information is needed so as not to overestimate the internal volume of the functional inside-out particles or vesicles and thus underestimate Δp. The results shown in Table 1 were obtained by assuming, in line with common practice, that submitochondrial particles are almost exclusively inside-out, while allowance was made for the presence of right-side-out vesicles in preparations of the *P. denitrificans* vesicles (Sorgato *et al.*, 1978; Kell *et al.*, 1978*b*). If submitochondrial particles are contaminated by membranes that have retained the same orientation as mitochondria, then values of membrane potential and the pH gradient given by Sorgato *et al.* (1978) would each need to be increased by 18mV for a 2-fold decrease in the internal volume. Thus unless submitochondrial particles are very heavily contaminated, it is unlikely that Δp has been seriously underestimated, and the same conclusion applies to the *P. denitrificans* vesicles unless the method for assaying the sidedness of the vesicles introduced a substantial error.

Provided that there is not an unsuspected source of error in some of the measurements, the data given in Table 1 require that $(H^+/ATP)_{ATPase}$ is higher for bacterial ATPases than for the mitochondrial ATPase in accordance with eqn. 1. This conclusion can be usefully related to the situation with thylakoids, which, like bacterial membranes, maintain high phosphorylation potentials without the participation of an adenine nucleotide translocator. It is widely held that the value of $(H^+/ATP)_{ATPase}$ for the thylakoid ATPase is higher (3) than for the mitochondrial enzyme (e.g. Junge, 1977), and in this context the proposal of a higher $(H^+/ATP)_{ATPase}$ for bacterial ATPases is reasonable. A serious dilemma is that the mitochondrial, bacterial and thylakoid ATPases are so similar structurally that is it difficult to accept that they differ fundamentally in respect to the stoicheiometry of proton translocation that they catalyse. However, if it is accepted that $(H^+/ATP)_{ATPase}$ is 2 for bacterial ATPases, as suggested by the novel procedure of Petty & Jackson (1979), then either (i) the scheme for mitochondrial oxidative phosphorylation (eqns. 1 and 2) is incorrect, (ii) Δp for bacterial membranes is higher than the corresponding value for mitochondrial membranes, (iii) a simple chemiosmotic mechanism does not provide a complete description of electron-transport-linked ATP synthesis (see e.g. Kell, 1979). With regard to (ii), not only is there the difficulty of demonstrating larger vaules of Δp for bacterial membranes, but also a larger Δp has to be reconciled with the operation of the respiratory chain. It is frequently emphasized that the proton-translocating segment of the electron-transport chain of the Rhodospirillae group of bacteria bears a close resemblance to the coenzyme Q–cytochrome *b*–cytochrome *c* region of the mitochondrial respiratory chain (Prince *et al.*, 1978). If Δp is larger for the bacterial membrane than for mitochondria, then the steady-state redox span would have to be greater in the bacterial membrane in order to drive proton translocation of equivalent stoicheiometry, and thus there would be a significant difference in the modes of operation of the Rhodospirillae and mitochondrial electron-transport chain.

In conclusion it is pointed out that knowledge of the $(H^+/ATP)_{ATPase}$ for bacterial ATPases is very important, since the difficulty of measuring P/O ratios for intact bacteria has meant that experimentally determined H^+/O ratios are often converted into P/O ratios by assuming $(H^+/ATP)_{ATPase} = 2$. We think that the arguments developed in the present paper show that there are grounds for doubting the wisdom of this procedure.

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Stoicheiometry of Charge and Proton Translocation in Mitochondria: Steady-State Measurement of Charge/O and P/O Ratios

MARTIN D. BRAND

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

In the present paper I shall first briefly review the methods that have been used to approach the problem of the stoicheiometry of the mitochondrial redox and hydrolytic proton pumps, and the conclusions that have been drawn from these approaches. I shall then describe an independent steady-state method for investigating movement of charge (and protons) across the inner membrane during electron transport from different substrates to oxygen. Finally I shall present a novel steady-state method of estimating relative P/O ratios from rates of ADP-stimulated (State-3) respiration. The results obtained by using the steady-state approach show that the three energy-conserving 'sites' in the electron-transport chain between NADH and oxygen are not energetically equivalent. These experiments lead to the proposal that NAD-linked substrates are oxidized with an H^+/O ratio of 8 and a P/O of 2.67; succinate is oxidized with an H^+/O of 6 and a P/O of 2; and ascorbate is oxidized with an H^+/O of 4 and a P/O of 1.33.