Novel proteins in oxidative phosphorylation – why and how

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(Note – this was submitted for publication in 2006. It was blocked by a series of referees who clearly did not wish to see this debate reprised. As of October 2014 I have put it on my website for others to follow up.)

Abstract

Genomic and post-genomic analysis of gene expression profiles suggests that many more proteins may be closely involved in oxidative phosphorylation than had previously been recognised. Since it was widely assumed on the basis of the chemiosmotic coupling hypothesis that all the proteins necessary for energy coupling in oxidative phosphorylation had in fact already been identified, what might these 'new' proteins then be doing? A different kind of interpretation of energy coupling explains many observations that chemiosmotic coupling cannot, and has both predicted the existence of such 'extra' coupling proteins and can account for their behaviour.

Introduction

The major finding from genomics was the discovery of many genes encoding proteins whose existence, leave alone function, had previously gone unrecognized. Consequently, a common strategy for post-genomics (e.g. [1]) has been to compare the expression profiles of products (more commonly at the level of the transcript) of genes of 'known' function with those from genes of unknown function and when there is very substantial co-expression over a wide range of conditions to infer that the functions of the 'known' and 'unknown' genes are therefore related. This strategy is sometimes known as 'guilt by association [2], and as with other omics studies is intended as a hypothesis-generating strategy [3]. A study of this type in mammalian mitochondria [4] did exactly this, and found a very strong clustering of gene products known to be involved in oxidative

phosphorylation (oxphos). These genes included those encoding the known electron transport complexes, the ATP synthases, and carriers for ADP/ATP and phosphate. However, this 'OXPHOS' cluster also included a substantial number of other gene products whose functions are either unknown or had not previously been ascribed to the process of oxidative phosphorylation. What then might they do? To answer this, we first need to summarise the textbook view of oxidative and photosynthetic phosphorylation.

The textbook view of oxphos or electron transport-linked phosphorylation (Fig 1A) is that the electron transport-linked protein complexes (known as complexes I to IV) of the inner mitochondrial membrane pump protons from the 'bulk' interior phase of the mitochondrion to the outer aqueous phase, thereby setting up a transmembrane potential difference and pH gradient, the so-called protonmotive force or pmf (Δp). This is considered, according to the chemiosmotic hypothesis of oxphos [5], to act as the energy storage intermediate between the oxidative (electron transport-linked) steps and the phosphorylation activity. The phosphorylation is catalysed by a separate inner membrane complex (complex V), the ATP synthase, with the necessary free energy for this otherwise thermodynamically unfavourable reaction being provided (solely) by the pmf. In this view, no other proteins are necessary, and all that is needed for energy coupling is that they be embedded with the correct polarity in a membrane that is sufficiently impermeable to proteins to allow the maintenance of the pmf. Any ATP synthase can capture free energy from any electron transport chain complex generating a pmf in the same enclosed membrane, and Δp is therefore said to be a 'delocalised' intermediate (see [6; 7]).

Detour: acid bath experiments

While we are here concerned with true energy coupling *in vivo*, i.e. energy coupling solely between primary proton pumps such as electron transport chain complexes and energy consumers such as the ATP synthase it is appropriate to mention ATP synthesis driven by an artificial pmf. As reviewed elsewhere [8; 9], this type of experiment has been performed many times since it was pioneered by Jagendorf and Uribe [10]. In virtually all cases using bacteria, mitochondria and thylakoids, both in purified energy coupling membranes and in proteoliposomes containing only the F_oF_1-H^+-ATP synthase as protein component, the results show (a) that phosphorylation can be driven at something near the *in vivo* rate by an artificial pmf if its magnitude is sufficiently high, but (b) that there is a threshold pmf, usually of some 150 mV, below which no phosphorylation is induced (even if thermodynamically favourable and in the presence of an ATP "trap"). A typical dataset is given in [11] and the general phenomenon is illustrated in Fig 2. Thus [8; 9], whilst the results provide a most useful set of boundary conditions they are not really relevant for the *in vivo* case. In other words, if we are interested in the mechanism of energy coupling *in vivo*, rather than what can be done artificially, we need to know the magnitude of the pmf as generated by electron transport: if less than 150 mV then it is obviously hard to argue that the pmf is an intermediate, since the artificial-pmf experiments show that one cannot for instance take refuge in the idea of an arbitrarily large $\rightarrow H^+$/ATP ratio [12]. Although this is not the focus of the present review, what evidence there is [12] shows that *in vivo* estimates of

the magnitude of the pmf, when performed properly, do not in fact give values above the 150 mV threshold.

Co-reconstitution experiments

Another of the clear predictions of this model (Fig 1A) is therefore that co-reconstitution of one of the protonmotive electron transport chain complexes (or another proton-pumping protein such as bacteriorhodopsin) with complex V in a suitably proton-impermeable proteoliposome and with the correct topologies would thereby be sufficient, alone, to permit the synthesis of ATP at the *in vivo* rates. Such a classical and reductionist strategy, if successful, would indeed allow one to conclude that no other proteins are necessary for energy coupling in oxphos. Many claims have been made that this has in fact been done. However, all of the calculations that have been based on such experiments contain a simple but very important error that shows rather the opposite (i.e. that <u>other</u> 'coupling' proteins are in fact needed to couple the energy released by oxidation to the phosphorylation of ADP to ATP).

Specifically, experiments that attempted to illustrate this were pioneered by Racker and Stoeckenius [13], who showed that such co-reconstituted systems using nominally purified proteins could in fact make ATP, and this has been confirmed in numerous other systems [14]. It was argued (although it is not obvious why any energy coupling observed in these systems might not be going via any routes), that this somehow demonstrated the veracity of chemiosmotic coupling model of Fig 1A, Unfortunately for these claims, the

calculations of the rates observed were expressed in terms of nmol.(min.mg protein)$^{-1}$ (or for photosynthetic systems nmol.(min.mg chlorophyll)$^{-1}$). However, because these were more or less purified preparations, it is the turnover number which is the correct metric, typical values for these being 250-400.s^{-1} [11; 15]. That for the ATP synthases in these early systems was less than 0.01% of the in vivo rate [16], and the "world record" [17] when driven by electron transport or bacteriorhodopsin in these co-reconstituted proteoliposomes is still only approximately 2% of that [9; 12][1]. Thus, especially given that these "purified" preparations, especially of the ATP synthase, are never >98% pure, one could better argue the opposite: that the co-reconstitution experiments show that for successful coupling one requires proteins additional to the generally-recognised primary and secondary proton pumps (Fig 1B), and that chemiosmotic coupling is not therefore accounting for the (limited) phosphorylation observed. Since this turnover number of the ATP synthase is not, beyond a certain level, increased by increasing the proportion of primary proton pump [17-19], it is not reasonable to argue that the cause of the negligible rate of phosphorylation in the in vitro system is simply an energetically insignificant pmf per se. There is also some genetic and other evidence for the existence of these 'extra' proteins that have been termed "protoneural" proteins [9; 12; 16; 20].

[1] If the MW of the ATP synthase complex = 360 kDal, 360 mg = 1 μmol, 1 mg prot = 2.77 nmol and thus 100 nmol/min/mg ATP synthase = 100nmol/min/2.77 nmol synthase = 36 nmol/min/nmol synthase, i.e. a turnover number of 36.min^{-1} or 0.6.s^{-1}.

Novel proteins of the inner mitochondrial membrane

If ‘extra’ energy coupling proteins exist, they should be observed in the proteome of mitochondria, and specifically of the inner membrane of mitochondria. Da Cruz and colleagues [21] carried out a proteomic study of the mouse inner mitochondrial membrane, and determined that 182 different proteins were present, of which 20 were unknown. Taylor and colleagues performed a similar proteomic study in mitochondria [22], and while the coverage of the known proteins of oxphos was 90%, they also noted that 19% of the proteins they determined were ‘unknown’. Mootha and colleagues determined that there must be at least 591 mitochondrial proteins, and discovered 163 proteins not previously associated with mitochondria [4]. A later study [23] detected 297 mitochondrial proteins by mass spectrometry. Ozawa *et al*. [24] performed a genetic study of mitochondrial protein expression using a split-GFP system and determined that 258 proteins had mitochondrial targeting signals (including both inner membrane and matrix components). The most recent estimates suggest that there are some 1500 mitochondrial proteins, of which only half have been identified [25]. These numbers are far in excess of those encoded by the mitochondrial genome [26], indicating that they are nuclear-encoded. Overall, given the existence of numerous novel proteins, a significant number of which are clearly expressed together with known oxphos proteins, there is plenty of scope for additional coupling proteins whose function is of the type described in Fig 1B. These novel oxphos proteins pointed up by the study of Mootha and colleagues [4] included Usmg5, Np15, D10Ertd214e, mito2010100O12Rik, 2610207I16,

Rik1110018B13Rik, 2610205H19Rik, 0610041L09Rik, 0610006O17Rik, 2310005O14Rik, and Gbas.

Other evidence against a delocalized intermediate

Although these studies were generally carried out a considerable time ago, there remain many experimental studies that cannot easily be accommodated within the framework of a chemiosmotic coupling theory as per that in Fig 1A (e.g. [9; 12; 27; 28], and it is important for the modern reader to be aware of these data and the consequent issues. We mention two such approaches in particular: oxygen-pulse experiments and double inhibitor titrations.

Oxygen pulse experiments

Another prediction of Fig 1A is that electron transport should be linked to the appearance of protons in the bulk phase external to mitochondria, with the appropriate kinetics and magnitude. Redox-linked proton translocation experiments were pioneered by Mitchell and Moyle [29], but the appearance of these protons was both slow and their magnitude low in number. This was ascribed to the fact that the mitochondrial membrane capacitance (~ $1\mu F.cm^{-2}$ [30]) was sufficiently small that it was charged up by the passage across the membrane of only a small number of charge-uncompensated protons, and that the bulk transmembrane potential thereby induced either caused the other 'unobserved' protons to flow back before they were detected or inhibited their pumping

('redox slip') in the first place. Certainly allowing charge compensation by the addition of valinomycin in the presence of potassium increases the number of protons observed per O_2 reduced, but then of course there is no coupling. More importantly, the 'membrane potential' explanation fails on two counts: (i) if the first few protons generated such a potential that inhibited further uncompensated proton ejection then no further protons should be seen in the absence of charge-compensating ions on increasing the size of the O_2 pulse – however this is not the case and they are indeed observed [31] (Fig 3); (ii) if the size of the O_2 pulse is made sufficiently small the membrane potential that could be generated is energetically insignificant (<kT, ~ 26 mV) and thus the number of protons observed should be the same whether valinomycin-K^+ or another charge-compensating 'permeant' ion is present or not; again this is not the case [32] and so what is making the energy coupling protons unobserved in the bulk aqueous phase cannot therefore be a bulk, delocalized membrane potential. Indeed attempts to measure an energetically significant transmembrane potential directly in individual mitochondria capable of making ATP also fail to find one [33].

Double inhibitor titrations

According to the chemiosmotic coupling hypothesis, uncouplers act to uncouple oxidation from phosphorylation by acting as protonophores, capturing protons moved across the energy coupling membrane by electron transport and returning them whence they came, thereby lowering the steady-state pmf and in consequence lowering the rate of phosphorylation. Given the data in Fig 2, and the assumption of chemiosmotic coupling,

it is assumed that the relationship between the rate of phosphorylation J_p and Δp is of the general form J_p = [ATP synthase] x Δp. Uncoupler titrations are then assumed to lower the Δp from its starting value in the absence of uncoupler. Decreasing the 'starting' number of ATP synthases by inhibiting some of them with a tight binding inhibitor should not act decrease this starting Δp (indeed it should raise it slightly, as the 'drain' is lowered [34]). Consequently, the titre of uncoupler necessary to effect full uncoupling should remain the same or increase. In fact [35] (Fig 3B) the titre is lowered. No 'delocalised' explanation can accommodate this finding. By contrast, if coupling is 'localised' (Fig 1B) and the uncoupling step is slow relative to the ability of the uncoupler to diffuse between coupling sites, this is exactly what is expected since uncouplers can only uncouple sites of coupling that could be coupled in the first place.

How to test these ideas?

As described above, many of the necessary experiments have already been done in the sense of determining the inadequancy of the magnitude of the pmf during electron transport-linked phosphorylation, the inability of a putative membrane potential to explain O_2 pulse experiments, the inability of any 'delocalised intermediate' to account for the experiments in Fig 3B, and so on. However, and while they bear repeating by new investigators with more modern technology, these are all negative results in that they show that something is 'not' there. The chief 'positive' kinds of experiment that this Opinion article is designed to foster are new kinds of co-reconstitution experiments in which these 'novel' oxphos proteins determined by co-expression analyses are added to

proteoliposomes and thereby increase enormously the rate of electron transport-linked phosphorylation. Modern mass spectrometry-based proteomic techniques can give an accurate account of all the proteins that are present in such experiments (even if they are invisible on gels), and thereby determine all the 'impurity' proteins that may be contributing to the phosphorylation observed and ensure that the book-keeping is done correctly. Such experiments are much more likely to be done if experimenters have confidence that it is worth looking for such activities (Fig 1B), rather than believing the textbook view (Fig 1A) that says they should not exist.

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Legends to Figures

Fig 1. Two models of energy coupling in oxidative (and photosynthetic) phosphorylation. **A**. A classical chemiosmotic type of coupling, in which the proton-pumping activity of an electron transport chain complex lead to the generation of a delocalised, bulk-phase protonmotive force (Δp or Δ**Error! Bookmark not defined.**$\tilde{\mu}_{H^+}$/F) consisting of a membrane potential Δψ and a pH gradient ΔpH, which provides sufficient free energy to drive the synthesis of ATP via an ATP synthase. In this model, no proteins other than the electron transport and ATP synthase complexes are required. **B**. An alternative view based on [28] in which the protonmotive activities of the electron transport chain complexes are indeed expressed as a polarization across the membrane but in which the energy coupling protons are not released into the bulk phase. In this model, other proteins may mediate the transfer of free energy from the electron transport complexes to the ATP synthase.

Fig 2. Typical relationships between the rate or extent of ATP synthesis induced by an artificial pmf and that magnitude of that pmf. Typically there is a threshold value of some 150 mV below which no phosphorylation occurs, while to obtain the *in vivo* rate of phosphorylation at least 180 mV of artificial pmf are necessary.

Fig 3. Two kinds of experiment that suggest that a delocalized pmf is energetically insignificant and not an intermediate in electron transport-linked phosphorylation. **A**. Effect of permeant ions on the apparent stoichiometry of respiration-linked proton

translocation [31]. The number of protons ejected by protoplasts of *Paracoccus denitrificans* is a function of the size of the O_2 pulse. In the absence of a permeant ion such as thiocyanate [34] (○), the apparent stoichiometry is much lower than in its presence (●). However, if this lowered stoichiometry were due to a membrane potential caused by the observable (or indeed unobservable) protons ejected then increasing the size of the O_2 pulse in the absence of SCN^- should cause no further protons to be ejected, in contrast to what is observed. **B**. Uncoupler titrations of phosphorylation at different degrees of inhibition of the ATP synthase (from [35] with permission). Effect of the uncoupler SF 6847 on photophosphorylation by chromatophores of *Rps. capsulata* N22 (●,○,■). In two cases (○,■) the initial number of active ATP synthase molecules, and thus the rate of phosphorylation of the chromatophores, was decreased by preincubating them with appropriate concentrations of the covalent energy transfer (ATP synthase) inhibitor DCCD. The fact that less uncoupler is necessary to inhibit phosphorylation when the drain is lower strongly suggests that the intermediate coupling electron transport with phosphorylation cannot be delocalized.

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