Apoptosis and the laws of thermodynamics

To the editor — The 'chemiosmotic hypothesis', formulated in the 1960s, revolutionized the study of the mitochondrion and made it accessible to thermodynamic analysis. The central aspect of the hypothesis is that the energy from electron transport is transduced into a proton electrochemical gradient (Δp) across the inner membrane, and that protons re-entering the matrix through the F_1F_o ATPase force this proton pump to run in reverse to generate ATP. The magnitude of Δp is given by $\Delta p = \Delta\Psi - 61.5\,\Delta pH$, where ΔΨ is the membrane potential and ΔpH is the transmembrane pH gradient. Although the magnitudes of these components are difficult to estimate with precision, it is firmly established that electron transport is primarily governed by the slight disequilibrium between Δp and the redox spans at complexes I and III and that the net rate (and direction) of the ATP synthase is determined by the disequilibrium between Δp and the Gibbs energy of the matrix ATP/ADP plus phosphate pool (reviewed in ref. 1).

The second mitochondrial 'revolution', detailing the involvement of the organelle in apoptotic and necrotic cell death, is now at its height, as evidenced by the article by Matsuyama *et al.* in *Nature Cell Biology* (**2**, 318–325; 2000). It is, however, unfortunate that the authors have not drawn attention to the fact that key aspects of their paper demand re-evaluation either of the chemiosmotic theory or of the data presented. The central proposal is that staurosporine- and Bax-induced apoptosis increases ΔpH by about –0.9 units. At the same time the 'relative [mitochondrial] membrane potential' increased by some 20%. The consensus in the literature is that intracellular mitochondria maintain a Δp of 160–210 mV, a ΔΨ of 120–180 mV, and a ΔpH of –0.5 units. Staurosporine is therefore proposed to increase Δp by 80 mV, by a process of ATP-synthase reversal.

There are three fundamental problems with this interpretation. First, the respiratory chain is thermodynamically incapable of supporting such an increased Δp without a hypothetical 'change of gear', that is, a reduction in the stoichiometry of proton translocation.

Second, the distribution of Δp between ΔΨ and ΔpH is determined by the balance between the uptake of ions such as Ca^{2+} and the availability of permeant weak acids such as phosphate, bicarbonate and carboxylic acids, which equilibrate across the inner membrane as the first, second or third power of ΔpH depending on the number of acidic groups and buffer against large changes in ΔpH. It is difficult to visualize how a ΔpH of –1.3 units could be generated in the presence of physiological concentrations of weak acids, as the only situation in which mitochondria can maintain a large ΔpH is when permeant weak acids are absent and Ca^{2+} is in excess, in which case ΔΨ is decreased proportionately.

The third, and most fundamental, problem comes from the mechanism advanced to account for these findings. The authors adopt a proposal by Vander Heiden *et al.*[2–4], reviewed in this journal (*Nature Cell Biol.* **1**, E209–E216; 1999), that Bcl-2 related proteins may be required for exchange of adenine nucleotides between the matrix and the cytoplasm. In this controversial hypothesis, pro-apoptotic stimuli would inhibit this exchange, leading to hyperpolarization, accumulation of metabolites, osmotic swelling and release of cytochrome *c*. However, the assay of adenine-nucleotide exchange upon which the Vander Heiden hypothesis is based[3] is invalid as it involves a 10-min exposure of mitochondria to ^{14}C-ADP, rather than the sub-second rapid quenching required to determine initial rates of this extremely active process[5] . Leaving aside further discussion of this hypothesis, Matsuyama *et al.* propose that such inhibition would lead to an increase in the matrix ATP/ADP ratio, 'favouring reverse operation of the F_1F_0-ATPase' and that this proton extrusion would contribute to hyperpolarization and increased ΔpH. However, as the ATPase must function in the direction of ATP synthesis to generate an increased ATP/ADP ratio, it cannot drive the ATP/ADP ratio past thermodynamic equilibrium and then start to hydrolyze it again to increase Δp. The only situation in which ATP-synthase reversal is possible is when Δp is decreased below that required for equilibrium with the matrix ATP/ADP pool, and certainly not in the presence of a supposed 80-mV hyperpolarization.

How do we resolve these discrepancies? One possibility is that the interpretation of the fluorescence changes is inaccurate. Changes in matrix volume could affect the ΔpH signal, and use of fluorescence-activated cell sorting to determine ΔΨ is dependent on the use of low concentrations of probes such as $DiOC6_{(3)}$, as 40 nM $DiOC6_{(3)}$ renders the matrix signal largely ΔΨ-independent and causes severe inhibition of mitochondrial complex I[6].

An understanding of mechanisms that underlie programmed cell death is one of the main goals in mitochondrial physiology. The results presented in this paper are intriguing and are evidently reporting an important event. However, interpretations that contravene the laws of thermodynamics, or at least require re-evaluation of fundamental tenets of bioenergetics, may serve to increase, rather than resolve, the current confusion within the field.

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Response

To the editor — This is a thoughtful critique of our work and we share the belief in the laws of thermodynamics and the chemiosmotic hypothesis, but we do not consider these principles to be inconsistent with our results.

Our main thrust was to test the published hypothesis of a permeability transition pore or massive loss of proton electrochemical gradient as an early triggering event in cytochrome c release and subsequent steps in mitochondria-dependent apoptosis. We found that the membrane potential is at least maintained as the pH gradient increases[1], which conflicts with that hypothesis. Our values for membrane potential and pH gradient, however, are not (and were not claimed to be) calibrated with sufficient absolute accuracy to justify rigorous comparison with the capabilities of the electron-transport chain or phosphorylation potential. We are well aware of the cautions required in using permeant cationic dyes as probes of membrane

potential (and said so in our paper) and we recognize the difficulties in absolute calibration of pH measurements[2,3]. We therefore strongly suggest that our estimates of pH and V_m are most appropriately used as qualitative indications of net changes, rather than for calculating absolute free energies. The originally accepted version of our paper included more discussion of this important point, which was edited out to shorten the document. We are therefore pleased to be able to re-emphasize this caveat here.

To specifically address the chief criticism, we are unaware of measurements of free [ATP], [ADP], and [P_i] in the mitochondrial matrix under parallel conditions of apoptotic stimulation; thus the phosphorylation potential is unknown and should not be assumed to be the same as in unperturbed cells. We therefore do not see any incompatibility between our results and thermodynamics or the chemiosmotic hypothesis. Thus, we beg to differ with the conclusion that we have proposed a 'change in gear' of the stoichiometry of the respiratory chain.

As stated in our paper, we offered our hypothesis of reverse operation of the F_oF_1 ATPase as a speculation, based not on calculations of proton motive force relative to phosphorylation potential, but on the empirical finding that pharmacological inhibition or genetic deletion of the F_oF_1 ATPase attenuates the changes in mitochondrial-matrix and cytosolic pH in response to apoptotic stimuli. Moreover, we felt justified in offering this speculation, as it is currently unknown how large V_m must be before it becomes thermodynamically impossible for the F_oF_1 ATPase to pump protons in the reverse direction, because of the technical limitations of working with submitochondrial particles containing functional F_oF_1 ATPase[4]. Also, as you pointed out (and as we stated in our paper), we do not know the true value of ΔV_m in living cells, due to the limitations of the dyes commonly used. Indeed, it is because of the notorious problems with these dyes[5,6] that we used pH-sensitive green fluorescent protein as an independent way of gaining some insight into the status of the H^+ gradient during apoptosis.

We pointed out (exactly as in the letter above) that "if this hypothesis is correct, then other mechanisms must also be involved, as reverse operation of the F_oF_1/H^+ pump would eventually cause a steady-state condition to be reached, in which H^+ flux and efflux are equalized." Thus, we share the opinion that a firm mechanistic explanation for the changes in regulation of mitochondrial pH that we have observed during apoptosis remains elusive and we acknowledged in our paper the same principles of the chemiosmotic theory are so nicely articulated in the letter. So, we have no argument; we agree and stated as much in our paper.

In our paper, we discussed the recently published hypothesis concerning the adenine-nucleotide exchanger[7], which proposes an apoptosis-associated defect in mitochondrial ATP export/ADP import, and the idea that an aberrant rise in the ATP/ADP ratio could support the reverse operation of the F_oF_1 complex as an ATP-hydrolysing proton extruder. However, we noted, and re-emphasize here, that alternative models could be envisioned — although to date, our unpublished attempts to interrogate this model further have not dissuaded us from it.

We share the concern regarding the possible increased uptake of anions driven by the pH gradient. However, we do not know how the cytosolic concentrations of these anions respond during apoptosis, or that their distributions across the mitochondrial membrane remain in passive equilibrium solely with the pH gradient. It will be important to measure the gradients of such anions, especially that of orthophosphate, which is as important as ATP and ADP in influencing the phosphorylation potential.

Although many details remain to be elucidated, we trust that the contributions made by our paper will serve to resolve some (though certainly not all) of the confusion within the field. We would also point out that our findings are consistent with several reports of cytosolic acidification during apoptosis[8–13], and a report that ectopic expression of Bax in bacteria (the precursors of mitochondria) causes increased efflux of H^+, acidification of the extracellular medium, and alkalinization of the cell interior[14], thus resembling our results obtained for mitochondria in mammalian cells and yeast. Further open-minded experiments are required to reveal the complete mechanisms responsible for these changes in pH regulation.

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