Discussion Letter

UNCOUPLING IN PARTICLES AND INTACT CHLOROPLASTS BY AMINES AND NIGERICIN – A DISCUSSION OF THE ROLE OF SWELLING

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1. Introduction

Amines, including ammonia, uncouple photophosphorylation in envelope-free (stripped) chloroplast preparations by a well-understood mechanism [1], which is formally similar to that of nigericin uncoupling [2]. Neither amine nor nigericin is an effective uncoupler of photophosphorylation in subchloroplast particle preparations [3,4], which have been postulated to be impermeable to chloride in consequence [3]. However nigericin is a powerful uncoupler in intact plastids [5] which have also been suggested to be impermeable to chloride.

This note proposes a new explanation for the failure of these agents to uncouple particles, and shows that there need not be any discrepancy between the particle results and those from intact plastids.

* *Symbols:*

$\triangle E$	electric p.d. across thylakoid membrane ($E_i - E_o$)
C	electric capacitance per unit area of thylakoid membrane (0.01 F m^{-2})
[Osm]	osmolar concentration of all solutes
w	width of intrathylakoid space
r_1	inside radius of spherical particle
r_2	outside radius of spherical particle
$\triangle P$	hydrostatic pressure difference across membrane ($P_i - P_o$)
T	surface tension in membrane
o, i	as subscripts, mean outside and inside (the particle or thylakoid).

2. Experimental

2.1. *Uncoupling in stripped chloroplasts*

In the light, neutral amine molecules enter the interthylakoid space where at the prevailing low pH they are protonated. Given a steady rate of light-driven pumping of protons, we have:

$$H_o^+ \rightarrow H_i^+ \quad (1)$$

$$NH_{3o} \rightleftharpoons NH_{3i} \quad (2)$$

$$NH_{3i} + H_i^+ \rightleftharpoons NH_{4i}^+ \quad (3)$$

Thus accumulation of protonated amine is coupled to proton influx. When the product of external amine concentration and permeability is high enough [6], reaction (2) can proceed to the right at the same rate as (1), while $[NH_3]_i$ remains high and essentially constant and approximately equal to $[NH_3]_o$. In the presence of a permeant ion (Cl^-, NO_3^- etc.) there will also be the reaction:

$$Cl_o^- \rightleftharpoons Cl_i^- \quad (4)$$

This is coupled to the set (1) (2) and (3) by the membrane potential difference (p.d.) which moves (4) to the right. The p.d. can be approximated by:

$$\triangle E = w([H^+]_i + [NH_4^+]_i - [Cl^-]_i)/C \quad (5)$$

The entry of Cl^- and of NH_4^+ explains the swelling [7] of amine-uncoupled plastids as due to osmotic water uptake. If the hydraulic conductivity of the thylakoid

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membrane is high, $[NH_4^+]$ will not rise much above half the external osmolarity. For as swelling proceeds the internal solute will be largely NH_4Cl. So with easy entry of both water and neutral amine:

$$[H^+]_i \simeq [H^+]_o [NH_4^+]_i / [NH_4^+]_o \quad (6)$$

and as swelling proceeds:

$$[H^+]_i \simeq [H^+]_o [Osm]_o / 2[NH_4^+]_o \quad (7)$$

The difference in pH across the thylakoid membrane is then:

$$\triangle pH \simeq \log(2[NH_4^+]_o / [Osm]_o) \quad (8)$$

and uncoupling results from the proton-motive force

$$PMF = \triangle E - \triangle pH \times 58\ mV \quad (9)$$

being too small to drive the ATPase forward [8]. Qualitatively, equation (8) makes the point that amine uncoupling should be relieved by an increase in the external osmolarity, which has been observed [9].

Nigericin acts in a way that can be represented:

$$K_o^+ + H_i^+ \rightleftharpoons K_i^+ + H_o^+ \quad (10)$$

which is in form analogous to the sum of equations (2) and (3), and which leads to:

$$\triangle pH \simeq \log(2[K^+]_o / [Osm]_o) \quad (11)$$

Swelling also occurs in nigericin uncoupling [10] of stripped plastids, although the relief of nigericin uncoupling by an increase in external osmolarity seems not to have been reported.

In each case considered the mechanism of uncoupling involves osmotic swelling which limits the internal concentration of the ion (NH_4^+ or K^+) which is continuously substituted for the H^+ pumped in. This involves also the entry of the anion, in the steady state, at the same rate as that of H^+ pumping: the p.d. will in general take up a value that will ensure this. For putting $\triangle E$ = 100 mV in equation (5) gives $[NH_4^+]_i$ about 1 mM higher than $[Cl^-]_i$ once the intrathylakoid space has swollen to about 20 nm wide, so that a small concentration unbalance will produce an effective $\triangle E$. The value of $\triangle E$ will not be easily predictable until more is known of the mechanism of Cl^- entry, but it might be expected to be comparable with or higher than in a normal steady state of phosphorylation.

In the absence of a penetrating anion, ammonia still uncouples stripped preparations [11], but the mechanism of charge balance in this case remains to be worked out. It does seem of interest that lower ammonia concentrations are effective in the absence of a permeant anion [11].

The evidence quoted appears to justify the following summary: uncoupling by substitution of another cation for the internal protons (substitution uncoupling) can occur in chloroplasts in the presence or in the absence of a permeant anion, but with different mechanisms of charge neutralisation. In the presence of a permeant anion, swelling is essential to uncoupling, which can be relieved by its prevention.

2.2. *Uncoupling in subchloroplast particles*

A subchloroplast particle exposed to a substitution uncoupler should, if it were really impermeable to the anion present, be uncoupled by the (unknown) mechanism operating in the ammonium aspartate experiments [11]; but this is not observed. It can therefore be inferred that particles are permeable to the chloride present in the experiments. This inference is strengthened by the observation that high chloride concentrations increase the uptake of amine by particles [3].

If a particle is exposed to a substitution uncoupler in the presence of a permeant anion, it will swell to its maximum volume and then the internal concentrations will rise as pumping, substitution and anion entry continue. Either (i) the rise in internal osmotic pressure will cause an internal hydrostatic pressure sufficient to burst the particle, effectively uncoupling it, or (ii) the increase in internal concentrations will produce a sufficiently low pH inside for phosphorylation.

It is observed that phosphorylation does continue. That this is reasonable is shown by the following. Consider a spherical particle (fig.1) of inside and outside radii 7.5 and 17.5 nm, supporting a pressure difference ($\triangle P$) by means of the tension T in its fluid wall. Then approximately:

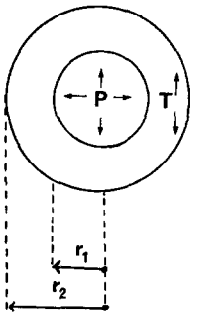

Fig. 1. The forces tending to rupture, and opposing the rupture of, a spherical shell. $\triangle P$ ($= P_i - P_o$) the hydrostatic pressure difference; T the surface tension. The net force on the upper hemisphere is the upward force $\pi r_1^2 \triangle P$ minus the downward force 2π (mean radius) T.

$$\triangle P \pi r_1^2 = 2 T \pi (r_1 + r_2)/2 \quad (12)$$

so $\triangle P$ is given by:

$$\triangle P = (r_1 + r_2)T/r_1^2 \quad (13)$$

The breaking tension of the membrane of subchloroplast particles is not known, but a rough approximation can be got from the known properties of the red cell membrane [12]. The breaking strength is ill-defined, depending on the time of application of the stress, but it seems that the red cell might indefinitely sustain a tension of 10 mN m^{-1}. Substituting this for T in (13) we get an estimate of the maximum $\triangle P$ that could exist in a particle of minimum dimensions as 4.4 MPa (44 atm). This internal pressure would be in equilibrium with an internal osmotic pressure of 4.4 + 1.1 MPa (allowing for the external osmotic pressure). This 5.5 MPa corresponds to 2.28 OsM solute or about 1.2 M NH_4Cl. If this maximum concentration is substituted in equation (6) together with 2 mM for $[NH_4^+]_o$ we get $\triangle pH_{max}$ as 2.8 units. Measurements [9,13] suggest that stripped plastids may phosphorylate with $\triangle pH$ of 2.4 to 2 units and be uncoupled at $\triangle pH$ of about 1 unit.

Thus a particle somewhat larger than the one assumed here or somewhat weaker could remain coupled in the presence of 2 mM NH_4Cl, normally an effective concentration.

Tests of this thesis might include: (i) the required concentration of amine or of K^+ in the presence of nigericin for uncoupling should depend on the particle size and on external osmolarity, (ii) particles should be uncoupled by ammonium aspartate or by nigericin plus K^+ aspartate at the same concentrations as stripped thylakoids are, and (iii) chloride should be detectably taken up by subchloroplast particles in the light.

A test of the conventional view might be its prediction that ammonium chloride should uncouple particles in the presence of a lipid-soluble anion: the latter does happen in submitochondrial particles [14], which therefore differ from subchloroplast particles, on the present thesis, in some aspects of the uncoupling process.

2.3. *Uncoupling in intact chloroplasts*

In view of the potassium content of the stroma, intact plastids should be uncoupled by nigericin, whether the thylakoid membrane in such preparations is or is not permeable to Cl^-. Whether the uncoupling is accompanied by chloride entry and osmotic swelling is not apparently known: since there will be a net uptake of KCl from the stroma the primary effect would be not an increase in chloroplast volume but an exchange of stroma volume for intrathylakoid volume. This could best be detected by freeze-fracture experiments.

In either case the membrane p.d. will be determined by the steady-state requirement of zero net current, and might be expected to be higher than during steady-state phosphorylation. In other words the mechanism producing charge balance will be different in nigericin-uncoupled and in phosphorylating chloroplasts, and one cannot argue simply that nigericin should collapse the $\triangle pH$ leaving the $\triangle E$ unaltered. An examination of the original chart records of Larkum and Boardman [5] shows that in their experiments nigericin did significantly increase the magnitude of the light-induced absorbance change at 518 nm (table 1).

2.4. *Chromatophores compared with chloroplasts*

Bacterial chromatophores are not uncoupled by nigericin [15,16], a fact attributable to their impermeability to chloride [14]. In the terms used in this discussion, the mechanism that produces the zero net current across the membrane in the steady state is not anion entry, nor the unknown that

Table 1
The effect of nigericin on the light-induced change in optical density at 518 nm (data of Larkum and Boardman [5])

Experiment	Wavelength of illuminating beam λ/nm	Change in o.d. at 518 nm relative to control as 1.00, at nigericin concentration: 0.3 μM	5.0 μM
26/2	714	1.09	0.17
	664	1.25	0.29
13/2	714	0.91	
	664	1.24	
ES	720	1.38	
28/2	714	1.91	
	664	1.80	

The change in optical density was estimated by extrapolation of the steady rate at 10 to 30 sec, ignoring the initial transients.

operates in chloroplasts (during ammonium aspartate uncoupling), but simply proton flow through the ATPase.

It is not clear why in chloroplasts treated with nigericin this does not happen – though chloride penetration remains a plausible guess – but the fact that intact plastids are uncoupled by nigericin cannot be used to deduce that 'any $\triangle E$ component does not contribute to the phosphorylation mechanism' [5].

3. Conclusion

Substitution uncoupling reduces the pH difference across a coupling membrane; mitochondrial and chromatophore membranes remain coupled under these conditions, an increase in $\triangle E$ presumably allowing the PMF to rise sufficiently for phosphorylation to continue. In chloroplast membranes and particles it seems probable that a sufficient rise in $\triangle E$ cannot occur, either because anions penetrate or because some other leakage mechanism intervenes. The chloroplast ATPase may require a forward $\triangle$pH to release it from inhibition [17], so that even a large rise in $\triangle E$ might not drive it forward. Finally, uncoupling can only be understood in terms of the kinetics of the competing processes in the steady state. In the establishment of the steady state, the mechanical strength of small membrane vesicles must be considered; they may support a large difference in osmotic pressure.

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