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ON THE FUNCTIONAL UNIT OF ENERGY COUPLING IN PHOTOPHOSPHORYLATION BY BACTERIAL CHROMATOPHORES

G. DUNCAN HITCHENS and DOUGLAS B. KELL *

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

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(1) Putative relationships between the rate of photophosphorylation, the proton-motive force and the concentration of an uncoupling molecule are considered within the framework of the delocalised chemiosmotic coupling hypothesis. The addition of a partially inhibitory titre of a specific, tight-binding H^+-ATP synthase inhibitor is not expected, within the framework of a delocalised coupling model, to alter the form of this relationship. (2) Photophosphorylation in chromatophores from *Rhodopseudomonas capsulata* is potently uncoupled by the protonophore SF6847. Yet the uncoupling potency of this compound is actually further increased when the rate of phosphorylation in the absence of protonophore is decreased by the addition of the energy-transfer inhibitor venturicidin, in contrast to the expectations of a delocalised energy-coupling model. (3) Similarly, valinomycin (in the presence of nigericin) uncouples more potently when the number of active H^+-ATP synthases is decreased by the addition of the energy -transfer inhibitors *N,N'*-dicyclohexylcarbodiimide or venturicidin. (4) The pore-forming ionophore gramicidin D also uncouples photophosphorylation more potently when the number of active H^+-ATP synthases is reduced. (5) These results are discussed in relation to the idea that the functional unit of electrical events and photophosphorylation either is, or is not, the intact membrane vesicle. (6) It is concluded that the unit of energy coupling in bacterial chromatophores is much smaller than the entire coupling membrane vesicle, and that previous analyses of this point, based on titrations with ionophores alone, may need to be re-examined.

Introduction

It is now widely accepted that free energy transfer during the process of electron-transport phosphorylation catalysed by respiratory and photosynthetic membranes is effected by a current of 'energised' protons. Contemporary studies are focussed on the mechanism(s) of proton-motive activity and the pathway(s) of energised proton current flow (e.g., see Refs. 1–8). With regard to the latter question, a variety of experimental approaches have lent apparent support to the chemiosmotic coupling hypothesis, in which it is proposed that, at least in the steady state, the energy-coupling protons come into equilibrium with the electrochemical potential difference of H^+ between the bulk aqueous phases that the coupling membrane serves to separate (e.g., see Refs. 9–12). On the other hand, we recognise that there exist numerous experimental data which are not easily reconciled with the foregoing viewpoint, and which have led several groups to propose modifications to the chemiosmotic coupling idea that are often (and loosely) referred to under the heading 'loca-

* To whom correspondence should be addressed.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; SF6847, 3,5-di(*t*-butyl)-4-hydroxybenzylidenemalononitrile.

lised chemiosmosis' (see, e.g., Refs. 5,7,8 and 13–23).

One often quoted (e.g., see Refs. 12 and 24–29) argument that has led its supporters to favour the former, 'delocalised' chemiosmotic analysis follows from the observation that concentrations of ionophores corresponding to approx. 1 active molecule per chloroplast thylakoid [30,31] or per bacterial chromatophore [32] can exert significant uncoupling of electron transport from phosphorylation, so that the 'unit of energy coupling' is the entire membrane vesicle. Although, as pointed out previously [16], complete uncoupling would be required if this argument alone might properly be used to support the delocalised coupling concept, it is clear that the most potent uncouplers, such as SF6847 [33–35], can effect full uncoupling, under steady-state conditions, at concentrations significantly less than those of the electron-transport chains or H^+-ATP synthases present in the assay.

Following the original approach taken by Baum and colleagues [36], we [35,37] and the Bologna group [38,39] have applied the so-called dual inhibitor titration method to the study of photophosphorylation in bacterial chromatophores, and have concluded that the results are explicable only within the framework of a model in which the free energy released by a particular redox-linked H^+ pump may be utilised only by a particular H^+-ATP synthase, so that a delocalised, bulk-phase proton-motive force cannot be the sole, or even major, intermediate in this process. Similar conclusions have been drawn from experiments of this type using respiratory membrane vesicles [22,23,40].

We have now extended this approach to include dual inhibitor titrations with a variety of uncouplers, ionophores and H^+-ATP synthase inhibitors, the results of which are reported in the present article. It is concluded that the only satisfactory analysis consistent with the data obtained is one in which, at least for bacterial chromatophores, the unit of energy coupling in electron-transport phosphorylation contains only an individual redox-linked H^+ pump complex plus an individual H^+-ATP synthase as its proton-motive constituents, and that the uncoupler and ionophore molecules studied act 'substoicheiometrically' by moving rapidly in the plane of the membrane between the localised energy-coupling sites before, more slowly, uncoupling the localised coupling sites.

Theoretical Approach

One of the principal reasons that we have chosen to exploit the dual inhibitor titration approach is that it is independent of the accuracy of any methods (see, e.g., Ref. 41) deemed to measure the proton-motive force, i.e., the steady-state electrochemical potential difference of protons between the bulk aqueous phases that the coupling membrane separates. It should be obvious that if the dual inhibitor titration approaches demonstrate unambiguously that the unit of energy coupling is not the intact membrane vesicle, then any measurements of the bulk-phase proton-motive force as defined must be regarded as being of obscure relevance to the energy-coupling processes in electron-transport phosphorylation. In any event, there is an enormous amount of uncertainty regarding the quantitative reliability of any available method for measuring the proton-motive force, and particularly the membrane potential component thereof, in bacterial chromatophores (e.g., see Refs. 17 and 42–48).

Notwithstanding, a number of authors have attempted in a number of systems to describe the relationship between the rate of phosphorylation and the proton-motive force in the steady state; most of these workers have proposed that this relationship is of a highly non-linear character, such that both a threshold proton-motive force exists (often given as equivalent to approx. 150 mV) below which the rate of phosphorylation is negligible, and that small increases in the magnitude of the proton-motive force above this threshold value effect large increases in the rate of phosphorylation (see, e.g., Refs. 7,24,25,28,29 and 49–65). We are not aware of any chemiosmotically based models that invoke other than a monotonic relationship between the rate of phosphorylation and the proton-motive force. It is assumed, on a delocalised chemiosmotic coupling analysis, that it is then possible to express the relationship between the steady-state rate of phosphorylation and the proton-motive force (under conditions in which the ADP and phosphate concentrations are large

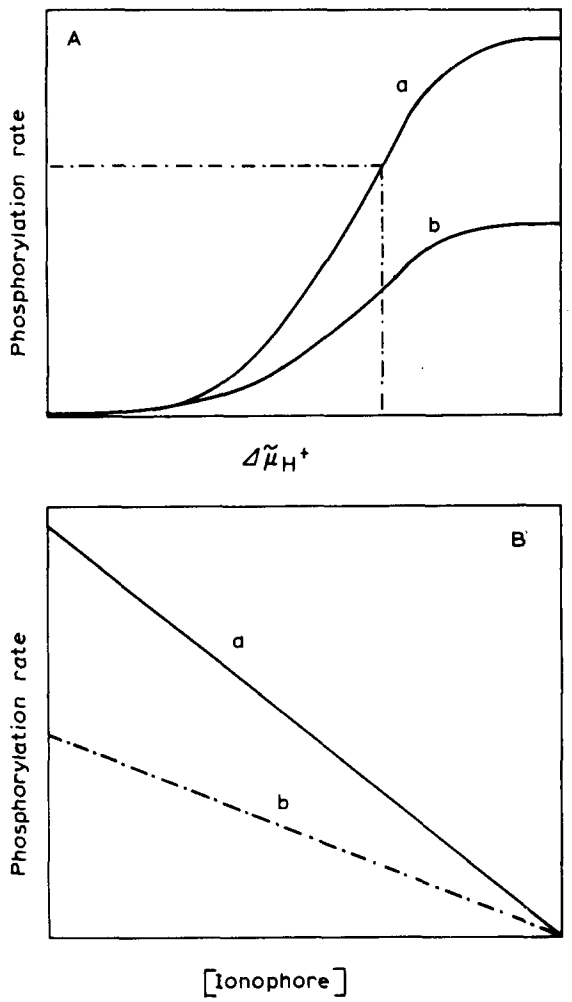

Fig. 1. Putative relationships between the proton-motive force, the rate of phosphorylation and the concentration of an uncoupling ionophore in a delocalised chemiosmotic coupling model. (Panel A(a)) A notional relationship between the rate of phosphorylation and the proton-motive force according to Eqn. 1 (see text). The dotted lines (·–·–·–) indicate that the proton-motive force in the absence of ionophores may not be saturating for the turnover of the H^+-ATP synthases. (Panel A(b)) The same relationship, using a titration with the same ionophore as in a, when the number of H^+-ATP synthases has been reduced through the use of a tight-binding inhibitor. (Panel B(a)) Extremely idealised titration curve of the rate of phosphorylation versus the concentration of a potent ionophore. In a delocalised chemiosmotic coupling model it is assumed that the proton-motive force is decreased by the ionophore in such a fashion that the relationship in A(a) is obeyed (obtained). (Panel B(b)) A similar titration to that given in B(a) except that the number of active H^+-ATP synthases in the system has been reduced by 50% through the addition of a tight-binding H^+-ATP synthase inhibitor. Given the constancy of the proton-motive force in the absence of added ionophore (A), it is to be expected, on a delocalised coupling model, that the form of the titration is unchanged relative to that in B(a), i.e., that the titre of ionophore required for full uncoupling is unchanged. For further discussion, see the text.

with respect to the K_m values of the H^+-ATP synthase and in which the products of the H^+-ATP synthase reaction do not feed back on the H^+-ATP synthase) as:

$$\text{Rate of phosphorylation} = [H^+\text{-ATP synthases}] \times f(\Delta\tilde{\mu}_{H^+}) \quad (1)$$

(see Refs. 59 and 66). We then obtain the type of analysis given in Fig. 1A(a). We may also assume, though this is not important in the present case, that, for whatever reason, the rate of turnover of the H^+-ATP synthases in the absence of added inhibitors is such that each H^+-ATP synthase is incompletely saturated by the proton-motive force [58,67].

Let us now imagine that we decreased the rate of phosphorylation by titrating with an ionophorous type of uncoupler, so as to obtain the curve shown in Fig. 1B(a). If we assume that energy coupling is delocalised in nature, we must assume that the effect of the ionophore (uncoupler) is to decrease the magnitude of the time- and space-averaged proton-motive force. Further, since it has been proposed that uncouplers can cause molecular slipping in redox-linked H^+ pumps [68,69], we may also, by extension of this proposal, permit the uncoupler to change the 'native' relationship between the proton-motive force and the rate of phosphorylation by individual H^+-ATP synthases with which the uncoupler may happen to interact.

Thus, for a titration with a given type of ionophorous uncoupler, we obtain a relationship between the rate of phosphorylation and the putative proton-motive force of the form shown in Fig. 1A(a). We will now repeat the titration of the rate of phosphorylation, using the same ionophorous uncoupler, under conditions in which the rate of phosphorylation in the absence of ionophore is decreased to say 50% of the control rate by the addition of an inhibitor of the H^+-ATP synthases. Although it is possible to imagine that the proton-motive force in the absence of ionophorous uncoupler might now be greater than that found when

the H^+-ATP synthase inhibitor is absent, we will analyse the case in which the proton-motive force in the absence of ionophorous uncouplers is unchanged upon addition of the H^+-ATP synthase inhibitor (Fig. 1A(b)).

It should be evident that each addition of ionophorous uncoupler can neither decrease the proton-motive more effectively in the presence of the H^+-ATP synthase inhibitor than in its absence nor differentially affect the relationship between the proton-motive force and the (coupled) turnover number of each H^+-ATP synthase, given the constancy of the 'starting' proton-motive force. The crucial and, as far as we are aware, novel point about the present dual inhibitor titrations is that a delocalised coupling model cannot possibly expect that a diminution of the output flux of the coupling system occasioned by decreasing the number of active H^+-ATP synthases can in some way decrease the magnitude of the proposed input force (the proton-motive force). Therefore, it is clear that, for the same ionophorous uncoupler, the titre of uncoupler required for full uncoupling should be unchanged, and certainly not decreased, by preincubating the preparation with a partially inhibitory titre of H^+-ATP synthase inhibitor, and this is illustrated in Fig. 1B(b).

Thus (Fig. 1B(b)), the prediction of the delocalised chemiosmotic coupling model is that, for titrations with ionophorous uncouplers, the presence of a partially inhibitory titre of H^+-ATP synthase inhibitor should not decrease the concentration of ionophorous uncoupler required to effect full uncoupling.

Experimental Procedure

The growth and maintenance of *Rhodopseudomonas capsulata* N22, and the preparation of chromatophores therefrom, the estimation of bacteriochlorophyll therein, the measurement of photophosphorylation thereby and the procurement of chemicals and biochemicals were carried out exactly as previously described [35,37].

Results

Fig. 2 shows a titration of photophosphorylation with the potent uncoupler SF6847 under conditions of saturating illumination [35]. As determined previously, the titre of SF6847 required to give complete uncoupling under the stated conditions corresponds to approx. 0.3 molecules per H^+-ATP synthase [35,37]. When the rate of phosphorylation in the absence of SF6847 is decreased approx. 3-fold by preincubation with the energy-transfer inhibitor [70] venturicidin, we should expect, on the basis of a delocalised chemiosmotic coupling model, that the titre of SF6847 required to effect full uncoupling would be unchanged (see above). This is because venturicidin cannot be expected to decrease the stored free energy available to the H^+-ATP synthase enzymes but only to decrease the number of active enzymes. However, the remarkable observation is that the titre of SF6847 required to effect full uncoupling is also decreased approx. 3-fold, roughly in parallel with the remaining number of potentially active H^+-

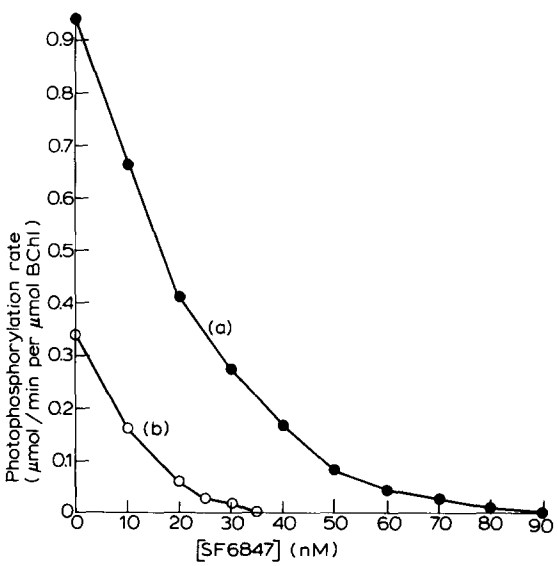

Fig. 2. Effect of SF6847 and venturicidin on photophosphorylation by chromatophores of *Rps. capsulata*. Photophosphorylation was measured as described previously [35] in a 6 ml reaction vessel containing, at 25°C and pH 7.8, 3 mM KH_2PO_4, 10 mM magnesium acetate, 30 mM potassium acetate, 0.2 mM sodium succinate, 1.5 mM sodium ADP, 5 μM P^1,P^5-bis(5′-adenosyl)pentaphosphate, 800 μg carbonic anhydrase and chromatophores corresponding to a bacteriochlorophyll (BChl) concentration of 20 μM. SF6847 was added to the concentrations indicated (●——●, ○——○) and where indicated (○——○) the chromatophores were preincubated for 25 min with venturicidin (0.01 μg/ml).

ATP synthases. This result complements the earlier observation in chromatophores that the inhibition of a proportion of the H^+-ATP synthases in the system prevents the utilisation of a comparable fraction of the electron-transport-derived free energy by the remaining active H^+-ATP synthases, under both transient [38,39] and steady-state [35,37] conditions.

Fig. 3 shows a comparable experiment in which the phosphorylation rate was titrated, in the presence of a saturating concentration of nigericin, with the potassium ionophore valinomycin. Venturicidin was either absent (Fig. 3a) or present at a concentration sufficient to reduce the rate of phosphorylation in the absence of valinomycin approx. 3-fold (Fig. 3b). A similar result to that observed with SF6847 is obtained: the ionophore acts more efficiently (potently) as the number of active H^+-ATP syntheses is decreased.

Comparable data (Fig. 4) to those of Fig. 3 for valinomycin titrations are obtained when the H^+-ATP synthases are titrated with the covalent modifier DCCD, which at the concentrations used,

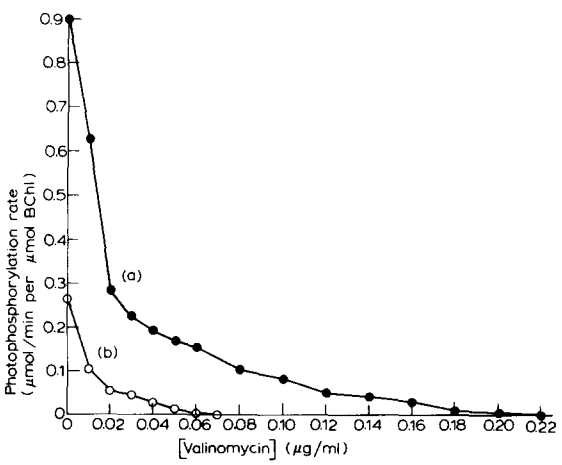

Fig. 3. Effect of valinomycin and venturicidin on photophosphorylation by chromatophores of *Rps. capsulata*. Photophosphorylation was measured as described in the legend to Fig. 2, except that nigericin was also present at a concentration of 2 μg/ml. In the absence of valinomycin, this concentration of nigericin had no effect on the rate of phosphorylation, probably due to the high concentration of acetate present in the reaction medium to inhibit the formation of any pH gradient. Valinomycin was added to the concentrations indicated (●——●, ○——○) and where indicated (○——○) the chromatophores were preincubated for 25 minutes with venturicin (0.01 μg/ml).

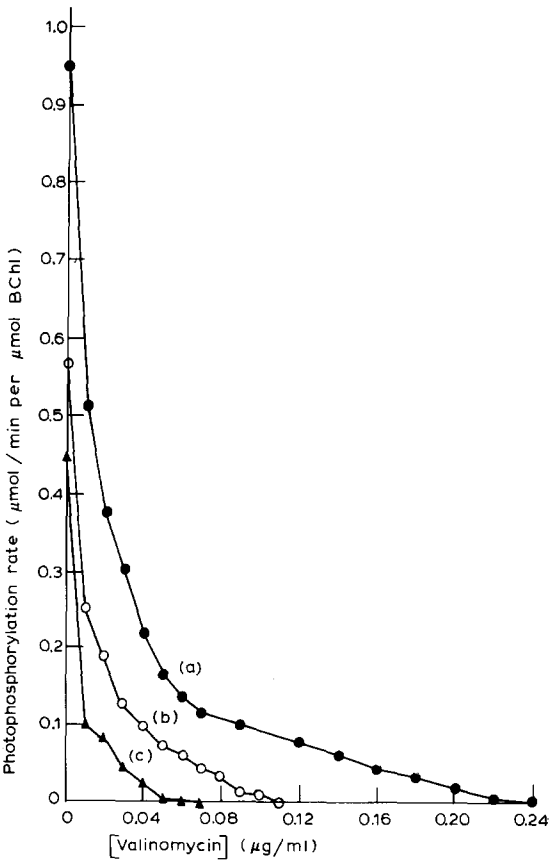

Fig. 4. Effect of valinomycin and DCCD on photophosphorylation by chromatophores of *Rps. capsulata*. Photophosphorylation was measured as described in the legend to Fig. 3. Valinomycin was added to the concentrations indicated (●——●, ○——○, ▲——▲) and where indicated the chromatophores were preincubated for 25 min with DCCD at concentrations of 20 μM (○——○) or 30 μM (▲——▲).

behaves as an orthodox energy-transfer inhibitor in chromatophores [38].

In contrast to SF6847 and valinomycin, which are well known to be carrier-type ionophores (e.g., see Refs. 71 and 72), gramicidin D, a mixture of gramicidins A, B and C, forms a transmembrane pore or channel [73,74]. It is of interest, therefore, not least because of the historical pre-eminence of gramicidin in the 'unit of coupling' debate, to see how this type of ionophore behaves in dual inhibitor titrations with an H^+-ATP synthase inhibitor. As is evident from the data in Fig. 5, a similar phenomenon to that found with the carrier-type ionophores is observed: gramicidin uncouples

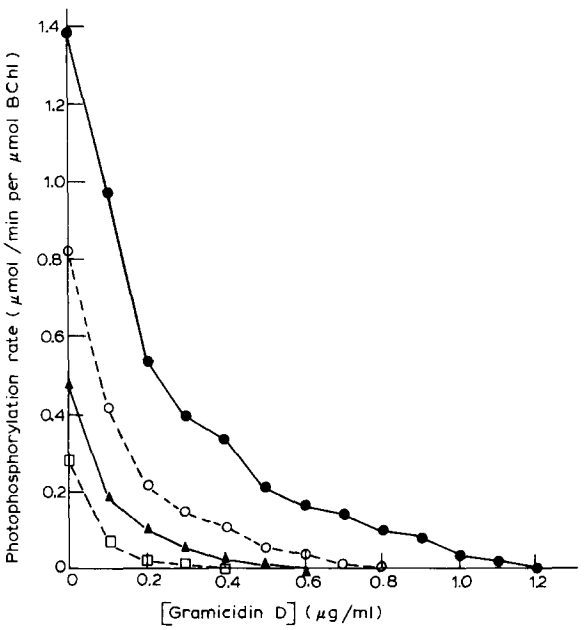

Fig. 5. Effect of gramicidin D and DCCD on photophosphorylation by chromatophores of *Rps. capsulata.* Photophosphorylation was measured as described in the legend to Fig. 2. Gramicidin was added to the concentrations indicated (●———●, ○———○, ▲———▲, □———□). Where indicated, the chromatophores were preincubated with DCCD at concentrations of 20 μM (○———○), 30 μM (▲———▲) or 40 μM (□———□).

phosphorylation more potently when the rate of phosphorylation in its absence is decreased by preincubation of the chromatophores with an H^+-ATP synthase inhibitor.

Discussion

A historically important finding during the evolution of our understanding of the behaviour of proton-motive systems was the observation that a single gramicidin dimer was sufficient to effect a significant degree of uncoupling in a whole thylakoid vesicle [30,31], an extent of uncoupling which roughly paralleled the rate of decay of the electrochromic '515 nm' spectroscopic signal (but, cf. Refs. 21 and 75). Comparable data were obtained using valinomycin in the 100-fold smaller chromatophore vesicles from *Rps. sphaeroides* [32] and *Rsp. capsulata* [76]. These observations were taken to indicate that the functional unit of electrical events and photophosphorylation was the intact vesicle, as proposed by the delocalised chemiosmotic coupling hypothesis, so that the energised protons pumped by a particular electron-transport chain could be used to an equal extent, on a time scale corresponding to the average turnover time of an H^+-ATP synthase, by any of the H^+-ATP synthases in the vesicle membrane. Equally, the proposed delocalised membrane potential could be reduced by an ionophorous molecule acting anywhere in the bilayer portions of the energy coupling membrane.

Now there also exists an important alternative class of explanation to that given in the foregoing analysis, and this is that the electrical events of electron-transport phosphorylation are in fact more localised than is implied therein. Since the movement of ionophore molecules in the plane of the fluid coupling membrane is likely to be rather fast (see later), a single ionophore might be expected to be able to uncouple several localised energy-coupling sites with the possible number of coupling sites that might be uncoupled in a given time (i.e., the uncoupler's potency) being determined (kinetically) by the ionophoric activity driven in (by) the localised areas of proton-motivated membrane polarisation.

In contrast to experimental approaches in which only titrations with ionophores are performed, the present dual inhibitor titration approach using ionophores plus H^+-ATP synthase inhibitors can effect a distinction between the above two possibilities. This is because the concept of localised coupling implies that there is no usable membrane energisation transferable from a unit containing one redox-linked H^+ pump and one H^+-ATP synthase if the H^+-ATP synthase is inhibited [39]. Since an uncoupler can evidently, by definition, only uncouple a unit that may potentially be coupled, the concept of localised coupling implies that restricting the number of such potential coupling units with an H^+-ATP synthase inhibitor decreases the number of coupling units that a given uncoupler molecule has to uncouple in a given time, assuming that the rate-limiting step is the uncoupling step itself and not diffusion of uncoupler molecules to their site of action. The finding (Fig. 2–5) that a variety of known protonophores and ionophores uncouple more efficiently under

conditions in which some of the H^+-ATP synthase enzymes present have been inhibited is consistent only with the 'localised' coupling scheme (see also Refs. 83 and 84).

We may point out here that the finding, discussed elsewhere [35,84], that the $P/2e^-$ ratio in the absence of added inhibitors is independent of the rate of electron transport over a wide range militates against problems of heterogeneity in the energy-coupling properties of bacterial chromatophores. This finding also truncates the virtues of 'parallel coupling' models that are sometimes discussed in the present context (see Ref. 20).

Interestingly, Schmid and Junge [77] did find that only 1% of valinomycin molecules bound to the thylakoid membrane were active in net ion transport following flash illumination, but did not consider the possibility that the transmembrane field to which the ionophores might respond was also localised in nature, proposing instead that the majority of the thylakoid phospholipids were in the gel state. This latter is not now thought to be the case, as evidenced, inter alia, by the great lateral mobility of a variety of molecules in the plane of the membrane at temperatures above say 10°C (e.g., see Refs. 78 and 79). In photosynthetic bacteria, the gel-to-liquid phase transition occurs at temperatures below 0°C [80], in line with our presumption that the lateral mobility of ionophores in chromatophores is rather rapid. Thus, the data with valinomycin would seem to indicate unambiguously (Figs. 3 and 4) that these molecules must act preferentially at localised sites of membrane energisation and, their ionophoric activity completed at one site, move rapidly in the plane of the membrane to another site. Although gramicidin did not in fact affect the decay of the carotenoid band shift in *Rps. sphaeroides* chromatophores [32], it was nevertheless very efficient at decreasing the rate of phosphorylation, its effectiveness roughly paralleling the number of active H^+-ATP syntheases (Fig. 5). The simplest analysis of the behaviour of this molecule, and of that of SF6847, is equivalent to that given above for valinomycin.

It has been proposed, within the framework of the delocalised chemiosmotic coupling concept, that uncoupler molecules may induce molecular slip in proton-motive complexes [68,69], and there certainly exists rather suggestive evidence that specific protein-uncoupler interactions may be of significance in the uncoupling process (see Refs. 81 and 82, but cf. Ref. 72). The present data are quite consistent with the view that uncouplers, and indeed ionophores generally, must interact in some way with one or more of the proton-motive complexes in these membranes, but are definitely not consistent with the concept of delocalised coupling, since the degree of uncoupling, at a given concentration of added uncoupler, depends upon the number of potentially active H^+-ATP synthases.

When taken together with previous observations [37,83], we have now found that three entirely different types of ionophore (anionic protonophore, cationic potassium ionophore and pore-forming ionophore), titrated in the presence and absence of three distinct H^+-ATP synthase inhibitors (DCCD, oligomycin and venturicidin), uncouple more effectively (potently) in the presence of the H^+-ATP synthase inhibitors than in their absence. Comparable observations have been made by the Amsterdam group in submitochondrial particles [22,23].

We have recently checked (Hitchens, G.D. and Kell, D.B., unpublished observations), using the system described in Ref. 84, that DCCD does not inhibit H^+ translocation linked to electron transport following trains of single-turnover flashes, and is thus acting, in conformation of other findings [38,39], as an orthodox energy-transfer inhibitor.

We conclude that the present data, based on titrations with ionophores plus H^+-ATP synthase inhibitors, provide compelling evidence that the functional unit of electrical events and photophosphorylation in bacterial chromatophores is significantly smaller than that implied in the delocalised chemiosmotic coupling concept, and that earlier analyses of this point, based on single-ionophore titrations, may require re-examination.

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