Photosynthesis Research **80:** 245–263, 2004.

Minireview

On why thylakoids energize ATP formation using either delocalized or localized proton gradients – a Ca^{2+} mediated role in thylakoid stress responses

Richard A. Dilley
Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA (e-mail: ddilley@bilbo.bio.purdue.edu; fax: +1-765-496-1496)

Received 20 March 2003; accepted in revised form 18 June 2003

Key words: ATP formation, Mordhay Avron, calcium gating, Yaroslav de Kouchkovsky, Richard Dilley, energy coupling, Peter Gräber, light stress response, localized and delocalized proton gradients, Andrea Melandri, Peter Mitchell, Evangalos Moudrianakis, Donald Ort, Uri Pick, photoprotection, proton gradients, thylakoid membranes, Alexander Tikhonov, Hans Westerhof, Robert Williams

Abstract

By the early 1970s, the chemiosmotic hypothesis of Peter Mitchell was widely accepted by bioenergetics researchers as the best conceptual scheme to explain how ATP is formed in oxidative and photosynthetic phosphorylation. At about the same time, however, work from a few laboratories suggested that some aspects of that elegant, relatively simple hypothesis required revision – not abandonment, but refinement to accommodate more complex movements of protons in the ATP formation mechanism than originally envisioned by Peter Mitchell. In some situations it appeared that protons were constrained to localized domains rather than always delocalized within an enclosed vesicle as envisioned by chemiosmosis. This minireview tells that story from my perspective, as one of the researchers involved in the experimental approaches that revealed more complex energy coupling proton flux patterns. Ionic conditions during isolated thylakoid storage were found to reversibly switch the $\Delta\tilde{\mu}_{H^+}$ gradient driving ATP formation between delocalized and localized energy coupling modes. Thylakoid accessible Ca^{2+} ions proved to be the switching factor that was responding to the ionic conditions in the storage treatment. The mechanism of Ca^{2+} was at least partially demystified when it was shown that the reversible switching between $\Delta\tilde{\mu}_{H^+}$ energy coupling modes involved Ca^{2+} interactions with the 8 kDa CF_0 (the H^+ channel) subunit in a type of H^+ flux gating action. Other experiments showed that the Ca^{2+} gating of H^+ flux into the lumen may be a critical regulatory factor in controlling the lumen pH and thereby help regulate the activity of the violaxanthin de-epoxidase enzyme, a key part of the chloroplast photoprotective response to over-energization (excess light) stress.

Ion gradient-coupled membrane bioenergetics – emergence of a new paradigm

A new era in cell membrane bioenergetics began in August 1960 at a meeting in Prague, Czechoslovakia – the International Conference on Membrane Transport and Metabolism – where Robert K. Crane presented his concept that glucose transport into intestinal cells was powered by a Na^+-glucose co-transport. This is told in an interesting historical article by Robert Crane about this topic (Crane 1983). He was the first to propose (diagrammed in Figure 4 of Crane's article) that the energy contained in the Na^+ concentration gradient acted on a *carrier protein*, not directly on the *glucose molecule*. Other models being proposed and studied often held that the glucose was somehow covalently changed, that is, activated. Peter Mitchell was at that Prague meeting and spoke with Robert Crane, voicing enthusiasm about the new notion of ion flow coupled to powering glucose active transport. It may well be that Crane's new concept stimulated and encouraged Peter Mitchell's thinking about oxidative

phosphorylation being coupled, not to covalent 'high energy intermediates' (the X ~ I hypothesis) so popular in the 1950s research, but to the flow of H^+ ions that somehow interacted with the coupling proteins.

By the 1950s, a variety of independent researchers had published ideas concerning possible vectorial charge separation across membranes, including H^+ transport; so the stage was being set for the important developments relating to proton gradients and proton fluxes driving ATP formation. Williams (1993) discusses the various early contributors to those concepts, carried forward principally by himself (Williams 1961) and Mitchell (1961). By 1966, Peter Mitchell had formulated and published his concepts about energetically downhill H^+ movement through the 'reversible H^+-ATPases' present in mitochondria, chloroplasts and bacterial chromatophores as the driving force for ATP formation (Mitchell 1966). Those concepts relate to Crane's 1960 innovative suggestion of ion flows linked to metabolite transport, and a new era in cellular bioenergetics was in full swing. See Larkum (2003) for discussion of closely related contributions by Lundegardh.

Protons and thylakoid membrane conformational changes: work in the 1960s

I had an interest in proton–thylakoid interactions starting in 1963 while a post-doc with Leo Vernon at the C.F. Kettering Research Laboratory, Yellow Springs, Ohio (see Leo's commentary on the Kettering Lab in this series: Vernon 2003). He was a fine colleague and mentor, giving me freedom to follow an interesting observation I made about thylakoid electron transport-induced light-scattering changes. This is an example of serendipity in research and it led me directly into studying ion transport in thylakoids. I was setting up a dual-beam Beckman spectrophotometer to study the kinetics of $NADP^+$, ferricyanide and dichlorophenol–indophenol reduction in thylakoids and happened to notice absorbance changes unrelated to the electron acceptor absorption spectrum. The light scattering changes (turbidity changes giving an apparent absorbance change) were later explained as the thylakoid volume changes (shrinkage) driven by H^+/K^+ and H^+/Mg^{2+} exchanges that caused water loss and thylakoid shrinkage (Dilley 1964; Dilley and Vernon 1965; Dilley et al. 1967). It was great fun working out that this first order, reversible light-scattering change was directly associated with conditions related to energization of thylakoid photophosphorylation (Dilley and Vernon 1964). I started work in April 1963 and by the late summer I was thinking about the conformational changes that caused the light-scattering changes as being associated with the 'high energy intermediate' involved in the process of ATP formation that Geof Hind and André Jagendorf were working on (Hind and Jagendorf 1963; Jagendorf 2002). The pH dependence and the kinetics of the conformational change I was studying were similar to that of the 'high energy intermediate' of ATP formation reported in May 1963 by Hind and Jagendorf (1963). Uncouplers and membrane leakiness-inducers inhibited the light-scattering change and it clearly was stimulated under basal (-ADP) conditions and greatly attenuated under ATP-forming conditions (Dilley and Vernon 1964). Those characteristics were in my mind when, sometime around June, 1963, I read Mitchell's 1961 Nature paper (my first introduction to the possibilities of H^+ ion gradients being involved in ATP formation) and it occurred to me that thylakoid redox-linked H^+ accumulation may be the underlying cause of the thylakoid conformational changes that I was working on. However, being young and inexperienced in the competitive research game, I put off trying to set up a pH measurement system while I finished the series of experiments I was involved with and then took a long summer holiday. When October rolled around I attended the symposium on 'Photosynthetic Mechanisms of Green Plants' held 14–18 October 1963 at Airlie House, Warrenton, Virginia; organized by André Jagendorf and Bessel Kok (see Hans Rurainski, this issue); where André presented the preliminary light-induced pH measurements with thylakoids he and Geof Hind had recently carried out. I was crushed and I cursed my stupidity and slowness! There was the experiment I had contemplated doing the previous June but had put off! (A note to young researchers who may read this: do not delay doing a new experiment your intuition tells you might be a good one.)

I returned to the Kettering Lab psychically wounded but a wiser person and within a few months discovered the predictable and necessary counter ion fluxes (K^+ and Mg^{2+}) driven by the beautiful light-induced H^+ uptake activity (Dilley 1964; Dilley and Vernon 1965). I was convinced the H^+ pumping and cation movements were important bioenergetic phenomena and I began aiming my research activities toward that area. One thing I felt I needed to do was another post doc stint with a membrane physiologist

to get more background in that area. While talking with André Jagendorf at a meeting in 1964, he suggested I contact Aser Rothstein, a well-known yeast and red blood cell membrane physiologist. I did that and had a wonderful year (1965) with Aser learning membrane research techniques. He was enthusiastic about thylakoid membranes, but very little was known at that time regarding their ion permeabilities and osmotic characteristics. I proceeded to study thylakoid osmotic properties as a beginning point in determining what the permeabilities were toward various cations and anions, as well as collecting information about the fixed charge arrays of the membrane (Dilley and Rothstein 1967).

In January of 1966 I returned to the Kettering Lab as a Staff Scientist and worked on various aspects of H^+ ion transport and photophosphorylation (Dilley 1969, 1970). Noun Shavit joined Tony San Pietro as a post doctoral colleague in 1967 and we collaborated on several projects related to ATP formation, becoming good friends as well as research colleagues. That was the period when ion transport antibiotics such as nigericin were being actively studied and some of our results can be found in Shavit et al. (1968) and Dilley and Shavit (1968).

During the period 1966–1973, I thought a good deal about pH effects on thylakoids and the proteins in thylakoid membranes and about possibilities for linking acidity-induced conformational changes of thylakoids to mechanochemical energy transduction. But nothing really dramatic came of those efforts except the demonstration that Aser Rothstein and I published (Dilley and Rothstein 1967) showing that the thylakoid H^+ pump-induced (or dark pH-induced) thylakoid shrinkage was greater than could be explained solely by osmotic-induced shrinkage (cf. Figures 9 and 10 of the above reference). It seemed clear that the H^+ uptake was causing dramatic conformation changes in the thylakoid membrane during energization, so the membrane was responding to protons in a more complex way than just as an osmotic barrier. I kept toying with the (admittedly rather vague) idea that H^+-membrane interactions may be an important part of the energy-coupling story perhaps along the lines of Robert Williams' concepts about 'in-the-membrane' proton movements (Williams 1961; see below). However, during those years Mitchell's ideas were much in vogue and gaining many proponents. Conformational changes would come back into importance later with Paul Boyer's conformational coupling concepts (Boyer 1993) and Peter Horton and colleagues' work on proton-induced conformational changes in the light harvesting Chl complex (Horton et al. 1996).

It was only when Don Ort began his excellent work in my laboratory (Ort and Dilley 1976; Ort et al. 1976) that we focused on making a clear attempt to test a basic assumption of Peter Mitchell's chemiosmotic hypothesis; namely that purely transmembrane proton gradients were the key to energy coupling. As will unfold below, considerable excitement and a lot of perplexing data came into the picture suggesting something more complex than the straight forward Mitchell view. It took about 15 years for Don's experiments to fit into a sensible (to me at least) mechanistic and physiological pattern, but I believe there now is such a pattern and I will tell that story below.

Proton gradients and ATP formation

By the early 1970s, there was general agreement that photosynthetic and oxidative phosphorylation produce ATP driven by a proton electrochemical potential gradient ($\Delta\tilde{\mu}_{H^+}$) as proposed by Peter Mitchell and known as the *Chemiosmotic Energy Coupling Mechanism* (Mitchell 1996). In Mitchell's view, only delocalized proton gradients were hypothesized; that is, protons on the acidic, high protonic potential side of the coupling membrane are in the bulk aqueous phase, not at all constrained to any membrane domain. The contentious issue of possible membrane-localized proton gradients rather than only a bulk phase delocalized $\Delta\tilde{\mu}_{H^+}$, was present right from the start, owing mainly to Williams (1961) (see Figure 1 for an informal

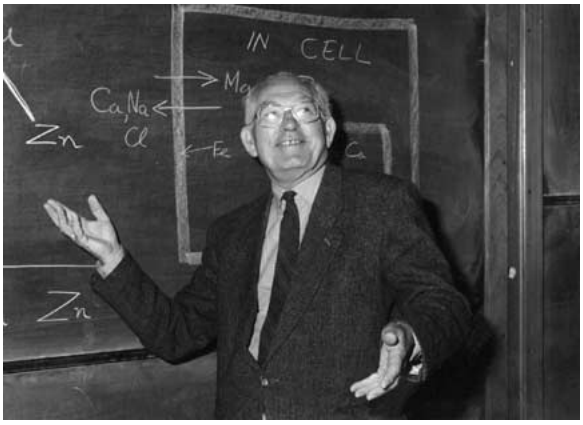

Figure 1. Robert (Bob) J.P. Williams, Emeritus Professor of Chemistry, Wadham College, Oxford University. Bob was a key contributor to developing concepts concerning localized proton gradients in energy transducing membrane systems.

photograph), who suggested in-the-membrane-proton diffusion as a mechanism for coupling high potential protons to the energy-requiring ATP synthesis.

In fact, it now seems clear, based on a thoughtful, fair and clear exposition by Williams (1993) that his ideas and published papers, while very much on the main topic of proton-driven ATP formation, were generally ignored by Mitchell in his writings. Why this was the case is something of a puzzle (discussed poignantly by Williams (1993) in an historical account). Mitchell's unfair treatment of Williams' contributions is a regrettable part of the story concerning the development of concepts in proton gradient-linked membrane bioenergetics, because had there been a more balanced, fair-minded and open exchange between the two seminal thinkers in this area, the field would have benefited. It must be said that Williams was always fair to Mitchell in his writings (Williams 1988, 1993).

Mitchell and colleagues did lots of experimental work, mostly using mitochondria in the early 1960s testing his hypothesis and this effort was joined by many laboratories, some using chloroplasts or bacterial chromatophores, during the 1960s. Peter Mitchell (1920–1992) was awarded the Nobel Prize for Chemistry in 1978 for his contributions in bioenergetics. An obituary for Mitchell was written by Crofts (1993), and an interesting analysis of discourse of scientists of that time was published by Gilbert and Mulkay (1984). A photograph of Peter Mitchell can be found in Jagendorf (2002).

Delocalized and localized proton gradient energy coupling

The question my colleagues and I have spent many years experimentally probing is: 'where are the protons when they form the proton battery in chloroplast thylakoids'? An excellent review of work on this topic up to about 1985 can be found in Ferguson (1985); he presents a balanced discussion of delocalized and localized energy coupling based on the somewhat confusing literature reported up to that time. As discussed below, new developments after 1985 gave new, clearer insights. As pointed out in Ferguson's review, much of the experimental data fit Mitchell's concept of delocalized $\Delta\tilde{\mu}_{H^+}$-driven ATP formation, with a few groups reporting evidence having anomalies that made the data inconsistent with a delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling mechanism as the only way the system works. Such anomalies called for some type of localized $\Delta\tilde{\mu}_{H^+}$ energy coupling, at least under certain conditions (see Ort and Melandri 1982; Westerhoff et al. 1984; and Rottenberg 1985 for additional reviews). Such alternatives to the beautifully simple Mitchellian view of a single delocalized $\Delta\tilde{\mu}_{H^+}$ gradient mode as the driving force for ATP formation by the F_0F_1 complex were quite unwelcome or viewed with skepticism by many workers in the field.

Being skeptical about new hypotheses in science is important and it helps a field mount critical thinking and critical tests for any new development that purports to alter the conventional, generally accepted conceptual schemes. After all, Mitchell's first suggestions in the early 1960s about chemiosmotic coupling as an alternative to the 'high energy intermediate' or X ~ I view of energy transduction were also viewed with great skepticism by the energy coupling community (Gilbert and Mulkay 1984).

Acid–base jump experiments support delocalized $\Delta\tilde{\mu}_{H^+}$ coupling

After André Jagendorf and his colleagues presented the so-called acid–base jump experiments of artifically imposed pH gradients in chloroplast thylakoids (Jagendorf and Uribe 1966; Jagendorf 2002, see Figure 2 for André's photo), Mitchell's ideas rapidly

Figure 2. The author (left) and André Jagendorf in 1977 at the 4th International Congress on Photosynthesis Research at Reading, UK.

gained broad attention and within a few years the chemiosmotic coupling concept replaced the high energy chemical intermediate concept as the prevailing paradigm for oxidative and photosynthetic phosphorylation.

The exciting data from André Jagendorf's laboratory fit very well with the delocalized $\Delta\tilde{\mu}_{H^+}$ proton gradient ideas proposed in the chemiosmotic hypothesis as presented by Mitchell. Some years later, Peter Gräber's group did extensive, elegant experiments using acid–base jump, electric field jump and light-induced generation of transthylakoid $\Delta\tilde{\mu}_{H^+}$ gradients for driving ATP formation (cf. Gräber 1982, for a review of that work), leaving little room for doubt that de-localized $\Delta\tilde{\mu}_{H^+}$ gradients were the driving force for thylakoid ATP formation, *under the conditions of their experiments.*

As other laboratories began testing the concepts, the database broadened, and certain results (such as those of Ort et al. 1976) required a more complex hypothesis, namely a consideration that under some conditions some type of localized proton fluxes energize the F_0F_1 coupling mechanism. Such ideas were never welcomed by Peter Mitchell, even though the essential concept, that of a proton $\Delta\tilde{\mu}_{H^+}$ as the energy mediator between electron transport reactions and the terminal steps of ATP formation, was there as the central feature.

In my opinion, there are three problems that pose logical reasons for researchers (either neutral on the issue or for those strongly favoring the delocalized view) to question or have doubts about the possibilities for there being localized $\Delta\tilde{\mu}_{H^+}$ gradients coupled to the F_0F_1 for ATP formation:

(1) It is so clearly shown experimentally (as in the acid–base jump or post-illumination ATP formation experiments, some that will be discussed below) that delocalized protons – such as those loaded into the thylakoid lumen as succinic acid in the acid–base jump experiment – clearly are the energy source for ATP formation in the relevant experiments. That being so, is it necessary to posit any other locus for the $\Delta\tilde{\mu}_{H^+}$?

(2) There is no clear understanding of how any proposed localized domain may be structured. The absence of such structural understanding is a huge problem, notwithstanding the – in my opinion – compelling data pointing to some type of physical barrier that can, under certain conditions, constrain H^+ ions to a localized domain connected to the F_0F_1 coupling complex.

(3) In most of the work prior to about 1985 (with the exception of the work by Don Ort in my lab in the mid-1970s, that showed *both* types of energy coupling gradients could occur) the published work suggesting a localized $\Delta\tilde{\mu}_{H^+}$ model was such that (usually) only a localized $\Delta\tilde{\mu}_{H^+}$ mode was considered; that is, the experiments were often indirect enough to obscure what was actually being measured. I will not discuss those early experiments herein.

In the following, I will present some of the main lines of work supporting the concept that thylakoids quite clearly carry out both delocalized $\Delta\tilde{\mu}_{H^+}$ and localized $\Delta\tilde{\mu}_{H^+}$ energy coupling. Ultimately, what I found most compelling for supporting the dual proton coupling mode concept was our finding that we could, with the same batch of thylakoids, demonstrate both types of $\Delta\tilde{\mu}_{H^+}$ coupling modes and a switching between them, mainly depending on how we manipulated the ionic conditions, particularly Ca^{2+} ions associated with the CF_0 H^+ channel.

ATP formation in short light flashes – permeable buffer effects

Probably the most compelling early data supporting localized energy coupling in thylakoids was the mid-1970s work of Don Ort in the author's lab (Ort and Dilley 1976; Ort et al. 1976), wherein thylakoids would show delocalized $\Delta\tilde{\mu}_{H^+}$ coupling in a post-illumination ATP formation experiment, but localized behavior in a short illumination light pulse mode. The labor-intensive but very sensitive [^{32}P] ATP assay was used in those experiments. The primary method used short illumination times with or without permeable hydrogen ion buffers (i.e., buffers such as imidazole, Tris and pyridine that readily enter the lumen space as the neutral species) to test the prediction that for a delocalized $\Delta\tilde{\mu}_{H^+}$ mechanism, there should be a lag in the time required to reach the minimum threshold ΔpH needed to energize ATP formation (the electrical potential component of $\Delta\tilde{\mu}_{H^+}$ being suppressed by valinomycin and K^+). In a later study, Graan et al. (1981) used Tricine to buffer the lumen, overcoming any possible criticisms about using the other buffers, which at sufficiently high concentrations (much higher than used in the Ort et al. 1976 studies) can cause uncoupling. Using [^{14}C] pyridine, we validated (Beard and Dilley 1988; Renganathan et al. 1991) that the uncharged amine (pKa = 5.4) is

readily permeable and completely equilibrates into the lumen bulk phase in 30–40 s. The presence of exogenous buffer in the thylakoid lumen not only delays the formation of a delocalized ΔpH, it hugely increases the extent of total proton uptake in the light. This in turn will allow more H^+ loading as more neutral pyridine moves in to replace that which has been titrated (Nelson et al. 1971). At pH 8.0 outside, we commonly observe that steady state H^+ uptake values increase from near 200 nmol H^+ (mg Ch1)$^{-1}$ up to 320 nmol H^+ (mg Ch1)$^{-1}$ with 5 mM pyridine. The existence of this large stored reservoir is evident in increased post-illumination phosphorylation ATP (PIP) yield by as much as two-fold (Ort et al. 1976; Vinkler et al. 1980).

To sum up what is accepted by workers in this field: when the protons used for ATP formation use the lumen bulk phase as the $\Delta\tilde{\mu}_{H^+}$ source (i.e., delocalized $\Delta\tilde{\mu}_{H^+}$ gradient coupling), and when $\Delta\Psi$ is absent, permeable buffers: (1) delay the time to onset of ATP formation and (2) increase the yield of post-illumination ATP. If, on the other hand, protons driving ATP formation can be constrained to some non-lumen, membrane-localized space and from there directly enter the F_0F_1 energy coupling complex (i.e., localized $\Delta\tilde{\mu}_{H^+}$ coupling), and if that localized space is not accessible to significant amounts of aqueous phase permeable buffers, then one *should not detect a time lag* (or detect a much shorter lag) in the light-driven ATP formation onset or an increase in the PIP ATP yield. This was the thinking that informed the work of Ort in my laboratory in the 1970s and later work, also in my laboratory, using somewhat different techniques (see Figures 3A and B for two photographs of Don Ort, one when he was a post doc in my laboratory, and the other when he was President of the American Society of Plant Physiologists).

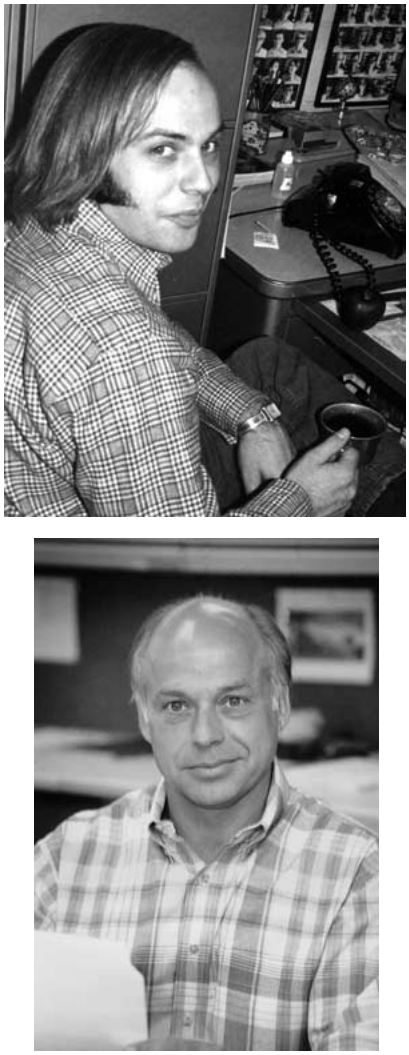

Figure 3. Donald R. Ort, a key figure in the work on thylakoid bioenergetics. *Top*: A photograph taken by David Krogmann in David's office sometime during Don Ort's post-doctoral time in the author's laboratory (≈1974). *Bottom*: Don Ort, photograph taken at the offices of the American Society of Plant Physiology (sometime in the 1990s) when he served as its President.

Permeable buffer experiments supported both delocalized and localized $\Delta\tilde{\mu}_{H^+}$ energy coupling

When Don Ort set about measuring the ATP onset lag in short light flashes, with and without permeable buffers (including Tris, pyridine, imidazole and phosphate) we were quite surprised to see no, or very little, lag in the ATP onset time (Ort and Dilley 1976; Ort et al. 1976). That looked as predicted for localized $\Delta\tilde{\mu}_{H^+}$ coupling, and contrary to the predictions for a delocalized $\Delta\tilde{\mu}_{H^+}$ coupling. We calculated, based on the amount of hydrogen ion buffer that we measured within the thylakoid lumen, that lags in ATP formation onset of near 1 s should have occurred, but we measured only very short delays in the onset of ATP (∼50 ms). Yet, with the same thylakoid preparations, when a light period of 5 s or longer was used and then ADP added in the dark for a post-illumination ATP formation protocol, there was a clear increase in ATP

yield when pyridine or imidazole was present during the illumination period. That response was clearly as predicted for a delocalized $\Delta\tilde{\mu}_{H^+}$ coupling. In that report (Ort et al. 1976), we concluded that 'different (proton) reservoirs must be involved in the two different reactions...'; either the bulk phase where permeant buffers could add to the buffering power, or some not-understood localized space where the added buffers could not contribute to the buffering power.

However, other workers reported that permeable amines *did extend* the lag time needed to reach the threshold ΔpH (Davenport and McCarty 1980; Vinkler et al. 1980). Vinkler et al. (1980) found ATP formation onset lags caused by pyridine to be near 500 ms, with comparable lags in the onset of post-illumination ATP formation, precisely what would be expected for a delocalized $\Delta\tilde{\mu}_{H^+}$ gradient coupling mode. Those data were very different from the data of Ort et al. (1976), who found little or no permeable buffer effect on the lag in the time for energization of light-dependent ATP formation, but a large effect of the buffer on post-illumination ATP formation. For the post-illumination ATP formation protocol, ADP was not present during the light and protons pumped were not utilized by the coupling mechanism and would be expected to flood any localized domains far beyond their buffering capacity, leading to H^+ accumulation into the lumen. The implication is that any postulated localized H^+ diffusion pathway would have a finite buffering power and when the localized H^+ ions are not effluxed out through the coupling mechanism, the filling of the local domains leads to H^+ ions dumping into the lumen. The later experiments from my research group directly measured such effects and they will be discussed below.

Clearly, both camps had made competent and highly repeatable measurements, so why the large discrepancy in the results? Both sets of workers on the two sides of the issue were confident of their data – as we will see below, justifiably so – and there did not at first appear to be a conceptual scheme that would resolve the contradiction.

Resolving the contradiction in the permeable buffer effects

Horner and Moudrianakis (1983, 1986), de Kouchkovsky et al. (1984) and Sigalat et al. (1985) resolved this contradiction by showing that the expression of the localized coupling response required maintaining a fairly low concentration of NaCl or KCl during the thylakoid preparation and assay steps. (A photograph of de Kouchkovsky appears in de Kouchkovsky 2002.) In the work of Vinkler et al. (1980) and Davenport and McCarty (1980) higher NaCl concentrations were used, readily accounting for their results. Stimulated by such data my research group began an extensive study of the effects of mono- and divalent chloride salts in the thylakoid storage buffer (the ATP formation assay buffer always had a single composition, only slightly altered by the salts coming with the thylakoids) on the expression of localized or delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling (Beard and Dilley 1986, 1988; Chiang and Dilley 1987, 1989; cf. review articles, Dilley et al. 1987; Dilley 1991). We used the more convenient and very sensitive luciferin–luciferase ATP detection method, which allows immediate detection of ATP formed by a sequence of single-turnover light flashes (Schreiber and Del Valle-Tascon 1982) as well as clearly detecting a component of post-illumination ATP formation. As mentioned above, the latter being a key parameter for judging if the $\Delta\tilde{\mu}_{H^+}$ driving the observed ATP formation was a delocalized or a localized $\Delta\tilde{\mu}_{H^+}$ gradient. Figure 4 (from Chiang and Dilley 1989) compares the low salt versus the 100 mM KCl thylakoid storage treatments showing different effects of pyridine on both the number of flashes needed to reach the threshold ΔpH for energizing ATP formation, and on the post-illumination ATP yield.

Figure 5A is a photo from about 1985, showing some of my research group. Figure 5B shows the author with colleagues Noun Shavit and Heinrich Strotmann at the European Bioenergetics Congress, held in 1977 in Hannover, Germany.

The results from the three independent groups agreed that by avoiding near 100 mM levels of KCl or NaCl in the thylakoid storage buffer or in the ATP formation assay buffer, thylakoids could energize ATP formation with a proton gradient that apparently did not equilibrate rapidly enough with the lumen bulk phase to drop the pH into the buffering range of the permeable amine (see Notes 1 and 2).

High salt thylakoid storage or a low-salt storage medium with the Ca^{2+} chelator ethylene glycol tetra acetic acid, EGTA (Chiang and Dilley 1987; Dilley 1991) or low-salt stored thylakoids with a low concentration of the uncoupler nigericin (Allnutt et al. 1991) resulted in the proton gradient rapidly equilibrating

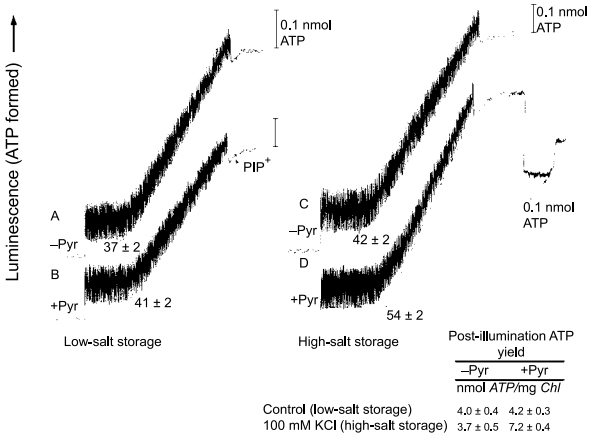

Figure 4. Effect of pyridine on flash-initiated phosphorylation using intact chloroplasts stored in the absence and presence of 100 mM KCl and freshly osmotically broken prior to the assay, from Chiang and Dilley (1989). Saturating single-turnover flashes were delivered at a 5 Hz repetition rate after 3.5 min dark incubation. The energization lag parameter was determined from the back extrapolation of the steady-state rise in signal to the base line (see trace D). The number by the bottom of each trace gives the number of flashes required to reach the intersection of the two lines. The post-illumination ATP yield (PIP^+) is indicated for trace B. Top traces A and C were from osmotically broken samples without pyridine. Traces B and D were from osmotically broken samples with 5 mM pyridine. Storage media for A and B was low salt (control) and for C and D, high salt media as specified in 'Materials and methods' in Chiang and Dilley (1989). Calibration of the ATP yield was by addition of 0.1 nmol ATP to the cuvette after the flash train (see trace D). The calibration signals for each reaction are indicated by the vertical line to the right of each trace. Standard deviations given with each number were determined from three separate trials. *Inset*: The PIP ATP yield is given for each situation (average of three determinations). Note that for the high-salt stored sample the effect of pyridine was to significantly increase the PIP ATP yield, as predicted for de-localized $\Delta\tilde{\mu}_{H^+}$ gradient coupling, in contrast to no effect on the PIP ATP yield by pyridine in the low-salt storage case (see text).

Figure 5. Top: The author and some of the students and post doctoral colleagues from the period of the middle 1980s; from left to right: Emmanual Atta-Asafo-Adjei, Gisela Chiang, Steve Theg, Yoon-Hi Federspiel (laboratory technician), Bill Beard, the author, Ahmed Mawgood and Tom Allnutt. *Bottom*: The author with from left to right: Heinrich Strotmann (University of Düsseldorf, Germany), the late Noun Shavit (University of the Negev, Israel; deceased; 1930–1997, see Aflalo et al. (1997) for an obituary) and Mrs. Strotmann, at the occasion of the 1977 European Bioenergetics Congress (Hannover, Germany). Noun Shavit and I became good friends while coworkers and collaborators in bioenergetics research in 1967–1968 at the Charles F. Kettering Research Labs, Yellow Springs, Ohio (see Vernon 2003).

with the lumen (i.e., showing the predicted permeable amine effects on increasing the number of flashes needed to reach the energetic threshold for ATP formation, as well as the amine increasing the post-illumination ATP yield). So we saw that it was not the high or low salts *per se* that controlled the expression of one or the other coupling mode, but it had something to do with Ca^{2+} ions.

Horner and Mondrianakis (1986) obtained flash-driven ATP formation and amine effects similar to the Ort et al. (1976) data and they suggested a type of H^+ gating at the lumen side of the CF_0 H^+ channel to explain the apparent localized or delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling patterns. My research group's work on the Ca^{2+} effects in switching between localized and delocalized $\Delta\tilde{\mu}_{H^+}$ modes fit this notion well and our data indicated that Ca^{2+} bound to the 8 kDa CF_0 subunits (subunit III or *c*, the dicyclohexylcarbodiimide (DCCD) binding protein) seemed to be acting as the gating factor (Chiang and Dilley 1987; Dilley 1991; Chiang et al. 1992).

For readers not familiar with the fairly complex data and experimental set-up used for such work, an explanatory model may help, along with a few clarifying comments. Figure 6 from Dilley (1991) is a cartoon-style model drawn up by my post doctoral colleague Tom Allnutt and used with subsequent modifications in several publications (Chiang and Dilley

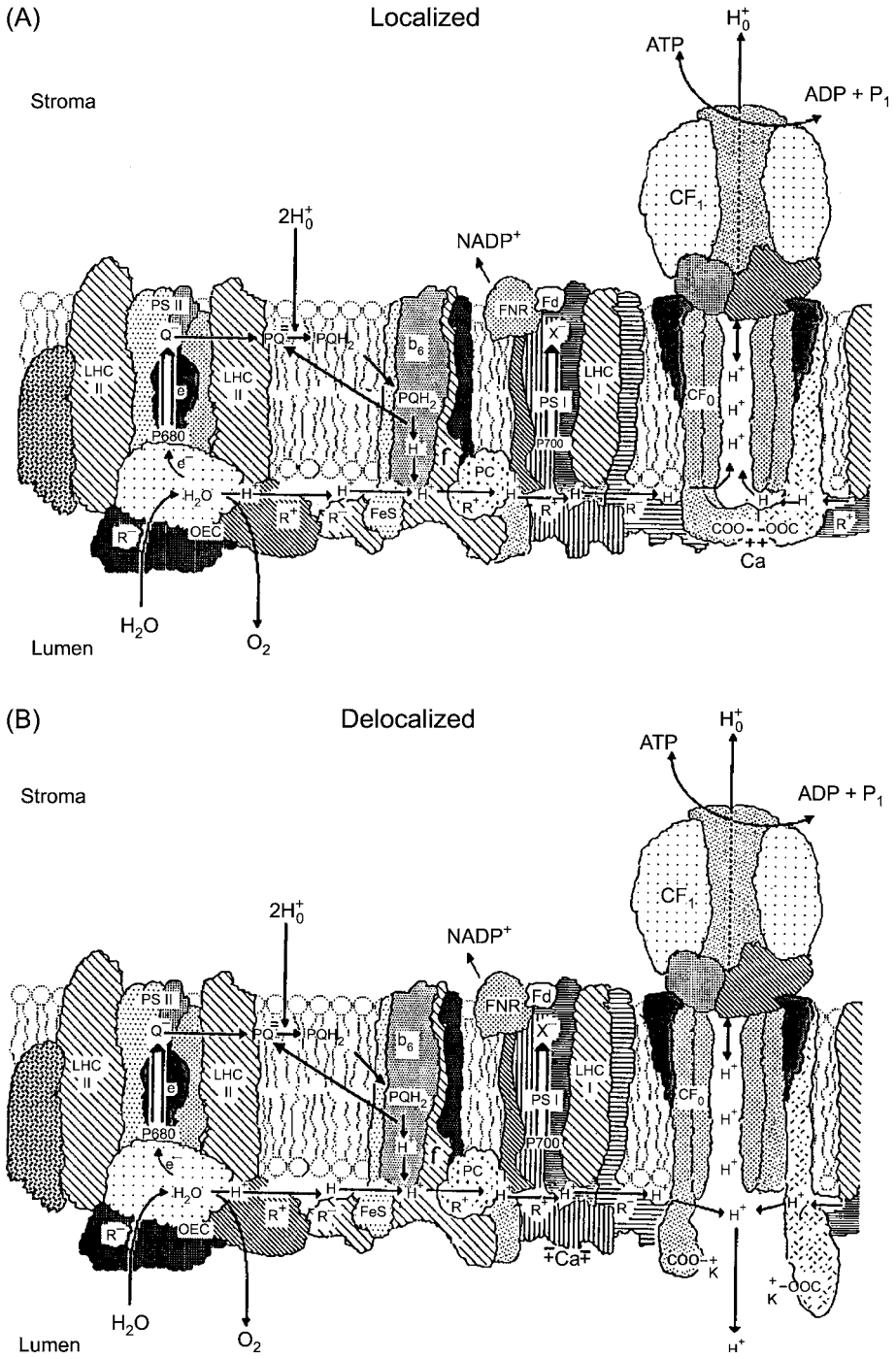

Figure 6. Cartoon-style model for a possible gating of proton fluxes between localized (A) or delocalized (B) energy-coupling gradients. (A) A portion of a thylakoid membrane is shown, depicting in a generalized and as yet speculative way, several intrinsic membrane proteins participating to form a localized proton diffusion domain from the proton-releasing reactions in water oxidation and plastoquinol oxidation into the CF_0 channel (see Dilley 1991). Ca^{2+} ions are hypothesized to interact with the 8 kDa subunit III (called subunit *c* in mitochondria and bacteria) polypeptides of the CF_0 which form the 'ring of c' (cf. Rastogi and Girvin 1999) part of the CF_0 H^+ channel. The Ca^{2+} ions bound to the lumen-facing parts of the 8 kDa subunit III proteins are postulated as forming a closed-gate configuration of the lumen opening of the H^+ channel, blocking access of lumen protons into the H^+ channel and blocking flow of membrane domain protons out of the CF_0 channel into the lumen. (B) An open H^+ gate occurs owing to the putative Ca^{2+} ligands on the 8 kDa subunit III proteins being displaced by K^+ producing an H^+ equilibration pathway between the localized domains and the lumen. The Ca^{2+} chelators such as ethylene glycol tetra acetic acid, under acidic conditions, also result in the open-gate mode, without the need for the 100 mM KCl treatment (Chiang and Dilley 1987).

1987; Dilley et al. 1987; Dilley 1991; Zakharov et al. 1995). The figure depicts in part B a delocalized $\Delta\tilde{\mu}_{H^+}$ with the protons from the redox proton source reactions (water oxidation in Photosystem II and plastoquinol (PQH_2) oxidation by the cytochrome $b_{6/f}$ complex and Photosystem I) equilibrating freely with the lumen. Figure 6A shows the hypothesis for keeping the H^+ gradient localized in a still poorly understood localized domain, but connected to an input into the CF_0 H^+ channel (perhaps involving the CF_0 subunit *a* in association with subunit *c*, but no clear explanation is available, Vik et al. 2000).

As discussed in more detail below, the mechanism for maintaining the localized $\Delta\tilde{\mu}_{H^+}$ is hypothesized to be a Ca^{2+}- controlled closure of the CF_0 H^+ channel at the subunit III (or *c*) 8 kDa 'ring of *c*' (Rastogi and Girvin 1999; Seelert et al. 2000) at the lumenal side of the channel. For the localized $\Delta\tilde{\mu}_{H^+}$ case we proposed a H^+ relay or diffusion pathway from the H^+ sources to the CF_0 H^+ channel that is kept occluded by the masses of membrane proteins at the lumenal surface (this possible occluded pathway is discussed in reviews, cf. Dilley et al. 1987; Dilley 1991).

Figures 7A and B show a differently stylized version of Figure 6, drawn by Rodney McPhail of the Biological Sciences Department Scientific Illustration unit, that exaggerates the proposed occluded domains. Of course, we understand that the CF_0 8 kDa subunit III (or *c*) probably exists as a ring of 10–14, 8 kDa subunits that interact with the CF_0 subunit *a* on the H^+ input side and with the γ subunit of the CF_1 on the opposite side of the membrane (Capaldi et al. 1994; Rastogi and Girvin 1999). However, we know virtually nothing about how protons enter the 'ring of *c*,' so I leave that part of cartoon models unspecified. Equally, a problem in our lack of understanding is any detailed structural information about the putative occluded domains that I suggest form the localized H^+ relay pathway.

Later in this Minireview I describe a variety of experiments that give support to the concept of a sequestered H^+ relay pathway, but unambiguous structural insights are yet to be developed. I clearly recall where the idea occurred to me that the localized domain we postulated could be between the membrane bilayer and the lumen-exposed parts of thylakoid proteins. It was at a Gordon Conference, probably the one in 1984 on the 'Biochemical Aspects of Photosynthesis' at Kimball Union Academy in Meriden, New Hampshire, August 1984. In 1982 (cf. Dilley et al. 1982) we were drawing models of pathways in the lipid bilayer, and I recall some of my friends telling me to 'get rid of that model' (good advice). It was not a good model, resembling somewhat a string of sausage links embedded in the lipid bilayer. I never meant it to be taken all that seriously (I surely did not) but it was a way to begin thinking about constraining protons to local domains. By 1984 there were new analyses of membrane proteins in many cases showing α-helical, probably transmembrane, portions connected by more hydrophilic, exposed parts. I saw several reports at that Gordon Conference, showing many membrane proteins in thylakoids and photosynthetic bacteria with quite extensive hydrophilic loops connecting multiple (predicted) transmembrane α-helical segments, and it struck me as though a light went on, that such structures could occlude considerable space along the surface of the membrane bilayer. Obviously, we still have this as a hypothesis for explaining the possible local domains and it needs further testing, but it is the best hypothesis I can think of up to this day. Anyway, by 1986 and 1987, we were drawing the model along the lines of Figure 6 and we took the (probably) good advice of our colleagues who objected to the 1982 'sausage' version.

The absence of clear structural data about such a H^+ relay pathway is one reason, I believe, for the difficulty on the part of some workers in the field in accepting the localized $\Delta\tilde{\mu}_{H^+}$ model as a working hypothesis. Rather, for a few of us, the quite large and varied array of experimental results that are inconsistent with a single delocalized $\Delta\tilde{\mu}_{H^+}$ gradient coupling mode is what drives us to posit the more radical dual, switchable, $\Delta\tilde{\mu}_{H^+}$ coupling modes.

Before getting deeper into the details relevant to clarifying what is known about the existence of membrane sequestered proton buffering domains, let me list the key points supporting the concept that two distinct $\Delta\tilde{\mu}_{H^+}$ coupling modes can be experimentally observed:

(1) Lumenally located protons drive ATP formation in both the acid–base jump and the PIP ATP formation events (Jagendorf and Uribe 1966; Nelson et al. 1971; Ort et al. 1976; Vinkler et al. 1980).

As a corollary to this,

(2) Under *basal electron transport* conditions (no ADP) the proton accumulation quickly exceeds the roughly 60–100 nmol H^+ $(mg\,Chl)^{-1}$ H^+ uptake threshold known to occur prior to the beginning of ATP formation when energization is by *coupled electron transport* (ADP is present, Hangarter and

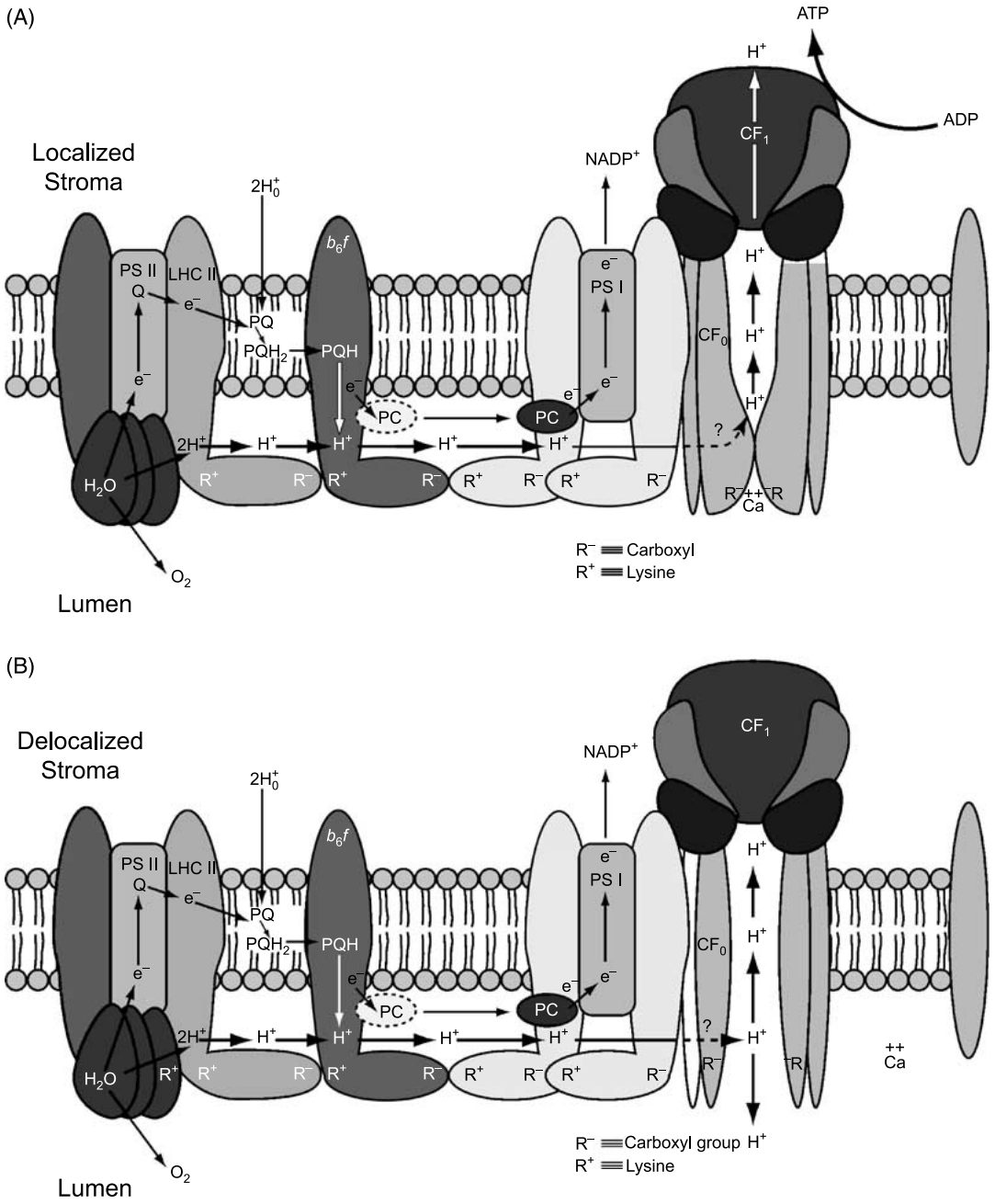

Figure 7. A cartoon-style model depicting the hypothetical occluded space, for localized H^+ diffusion, in a more exaggerated way than shown in Figure 6, that emphasizes the role of the lumen-exposed portions of some intrinsic thylakoid proteins. (A) the localized $\Delta\tilde{\mu}_{H^+}$ gradient; (B) the delocalized mode.

Ort 1985). Under the basal conditions, H^+ uptake into the lumen is readily observed using the permeable buffer-dependent increased H^+ uptake criterion (Nelson et al. 1971; Beard and Dilley 1988; Renganathan et al. 1991; Chiang et al. 1992). It is this permeable amine-dependent additional H^+ uptake into the lumen that is the source of the additional ATP yield in the post-illumination ATP formation assay, when ADP is added after the illumination.

(3) Lumenal protons also drive ATP formation using light-driven coupled conditions provided that

thylakoids are stored in buffer with 100 mM KCl or NaCl (Beard and Dilley 1986, 1988) [or using low-salt stored thylakoids with 2 mM EGTA to draw Ca^{2+} away from the 8 kDa CF_0 subunit III (Chiang and Dilley 1987; Chiang et al. 1992)].

(4) Localized domain protons were deduced to drive ATP formation in thylakoids stored in low salt medium (or with high-salt stored thylakoids with 1 mM $CaCl_2$ in the KCl buffer) because the presence of a permeable buffer did not cause a significant increase in the flash length (Ort et al. 1976) or the number of single-turnover flashes needed to start ATP formation (Graan et al. 1981; Horner and Moudrianakis 1983, 1986; Beard and Dilley 1986, 1988; Chiang and Dilley 1987, 1989). Typical *flash-numbers-to-ATP-formation-onset* are shown in Figure 4, taken from Chiang and Dilley (1989), as noted earlier. Low salt-stored thylakoids gave only a 4 ± 2 flash increase to ATP formation onset caused by pyridine whereas high salt-stored thylakoids gave a 12 ± 2 flash pyridine-dependent increase. The inset table in Figure 4 gives the (PIP) ATP yield data (indicated by PIP^+ in trace B) showing that the PIP ATP yield was not increased by pyridine in the low-salt stored case but was increased by 3.5 nmol ATP $(mg\,Chl)^{-1}$ in the high-salt thylakoids. Thus, by both the length of the onset to ATP formation and the PIP ATP yield criteria, the response to pyridine in the low-salt stored thylakoids indicated localized $\Delta\tilde{\mu}_{H^+}$ energy coupling but the high-salt stored thylakoids indicated de-localized $\Delta\tilde{\mu}_{H^+}$ coupling.

The models shown in Figures 6 and 7 allow visualization of the working hypothesis we adopted following the Horner and Moudrianakis (1986) suggestion for a H^+ gating function at the lumenal side of the CF_0 H^+ channel and are based on the detailed studies by eight PhD and post doctoral colleague 'lifetimes' in my lab (see 'Acknowledgments'). In the remainder of this essay, I will explain other results that give flesh to the bones of the working hypothesis, and equally importantly, provide a biological rationale for why chloroplasts have dual proton flux pathways. But the reader can visualize the conceptual scheme we have in mind from the points made above and the cartoon models. Namely, when the H^+ gradient is not excessive (moderate light intensities giving H^+ uptake levels near or less than 200 nmol H^+ $(mg\,Chl)^{-1}$) and ATP formation conditions are favorable, protons driving ATP formation do not equilibrate with the lumen, the pH of which remains above pH 6. The extensive studies we made of Ca^{2+}–CF_0 H^+ gating activity led us to hypothesize that Ca^{2+} ions bound to the 8 kDa CF_0 subunit III (*c*) are intimately involved in keeping the H^+ gradient confined to the sequestered pathway, until the local acidic pH reaches sufficient acidity to displace the Ca^{2+} from the CF_0 gating site, allowing free access of the H^+ ions to the lumen, producing the delocalized $\Delta\tilde{\mu}_{H^+}$ gradient mode. As I will discuss below, the acidic lumen pH is involved with activating the violaxanthin de-epoxidase enzyme in a photoprotective response to excess energization.

Ca^{2+}–CF_0 interactions in H^+ gating between localized and delocalized H^+ gradients

The detailed pharmacological studies by the late 1980s available on Ca^{2+}- protein (e.g., calmodulin, troponin *c*) interactions gave us very nice tools with which to study the Ca^{2+} effect on switching the energy-coupling mode between the localized and delocalized modes. Gisela Chiang's PhD thesis work very clearly showed that a calmodulin antagonist, [^{3}H] chlorpromazine, bound tightly (covalently after a photoaffinity UV light exposure), in a Ca^{2+}-specific way, to the 8 kDa subunit III (*c*) of the CF_0 H^+ channel (Chiang et al. 1992). Our colleague Stas Zakharov joined us from Pushchino, Russia and extensively studied Ca^{2+} binding to the purified 8 kDa subunit III (*c*) using a well-known $^{45}Ca^{2+}$ binding assay (Zakharov et al. 1993, 1995, 1996). The pattern that emerged showed: (1) tight Ca^{2+} binding to the 8 kDa subunit III (*c*), but not to subunits *a* or *b* of the CF_0; (2) reversibility of the Ca^{2+} binding upon switching from localized to delocalized energy coupling; (3) when Ca^{2+} is bound at the CF_0, H^+ ion flux from either direction (domain $\longrightarrow$ lumen or lumen into the CF_0) is blocked, and (4) thylakoid membrane Ca^{2+} channels allow efflux of the Ca^{2+} displaced from the CF_0 (Ewy 1997, and references therein). These data support the notion of a Ca^{2+} regulated, proton flux gating function as part of the CF_0 H^+ channel activity (Chiang et al. 1992; Wooten and Dilley 1993; Van Walraven et al. 2002).

The key point concerning the amine effects on the lag in the onset of ATP formation (or the lack of the amine effect for low salt-stored thylakoids) was that for the situation we called localized $\Delta\tilde{\mu}_{H^+}$ coupling, the lumen pH must have been kept above pH 6 during the ATP formation; but when high salt-stored

thylakoids were used, the lumen pH dropped below pH 6, consistent with a delocalized $\Delta\tilde{\mu}_{H^+}$ during ATP formation. That point was tested directly using a pH-sensitive fluorescent dye, pyranine (Renganathan et al. 1993). Although absolute pH calibration of the pH-dependent fluorescence quenching is open to some debate, the pattern of a significantly more acidic lumen in high salt-stored thylakoids under ATP coupling conditions compared to that in low salt-stored thylakoids, was clear. The ΔpH values estimated from the pyranine fluorescence signals with the external pH at 8.9 were: low salt-stored thylakoids (coupling conditions), $\Delta pH = 1.6$; high salt-stored thylakoids (coupling conditions), $\Delta pH = 2.9$.

Later work using the activity of the violaxanthin de-epoxidase activity as an intrinsic lumen pH indicator, very clearly supported the lumen pH limits mentioned above. In Pan and Dilley (2000) we showed that the localized $\Delta\tilde{\mu}_{H^+}$ coupling condition (low salt-stored thylakoids) occurred with no or very little violaxanthin de-epoxidase activity, that is, lumen pH remained above 6. In the delocalized mode the de-epoxidase was very active indicating a lumen below 6.

For the Ca^{2+} gating concept discussed above to make sense, the pH of the Ca^{2+} de-binding from the closed-gate CF_0 should be logically related to the threshold ΔpH for driving ATP formation (near pH 6, when the stroma phase is near pH 7.8–8.0). Titrating the Ca^{2+} de-binding pH was most easily done using thylakoid vesicles of the cyanobacterium *Synechocystis* sp. PCC 6803 with the acid–base jump ATP formation assay as a means of carefully controlling the acid stage pH. Van Walraven et al. (2002) reported such experiments, finding that a pH near or somewhat above pH 5.5 is the range for H^+ displacement of bound Ca^{2+} from the Ca^{2+}–CF_0 binding site at the H^+ channel. As will be seen below, this is a very relevant pH for the H^+ gating events to be involved in important chloroplast physiological responses to over-energization.

Other research groups provided evidence showing a pattern consistent with a localized $\Delta\tilde{\mu}_{H^+}$ energy coupling gradient under suitable conditions, but space permits only listing, not discussing any details of those publications: Graan et al. (1981), Tikhonov et al. (1981), de Kouchkovsky et al. (1984), Van Walraven et al. (1984), Westerhoff et al. (1984), Hangarter and Ort (1985), Tikhonov and Timoshin (1985), Pick et al. (1987), Pick and Weiss (1988) and Laasch et al. (1993).

Identifying the localized domains: proteins interacting with sequestered protons

Hypothesizing about localized H^+ gradients would always have an element of mystery and vagueness unless research could identify sequestered protein or lipid functional groups that can be shown to interact with the energy coupling proton gradients. Serendipity led us precisely to such data at roughly the same time we were struggling to understand the meaning of Don Ort's seminal work (Ort and Dilley 1976; Ort et al. 1976; Graan et al. 1981) that strongly supported the concept of localized $\Delta\tilde{\mu}_{H^+}$ energy coupling.

An important finding in developing ideas as to the thylakoid structural aspects involved in defining the local domains was identifying an unusual array of sequestered lysine amino groups, traceable to roughly six or seven thylakoid membrane proteins (Prochaska and Dilley 1978; Baker et al. 1981, 1982; Tandy et al. 1982; Laszlo et al. 1984a, b; Theg et al. 1988; Allnutt et al. 1989; Renganathan and Dilley 1994). The sequestered lysine group array (roughly 30–40 nmol per mg Chl) show anomalously low p*K*a values (around pH 7 or so), whose protonation state was correlated with the loading of protons into the localized, sequestered domains. The characteristics and possible significance of the sequestered domain lysine buffering groups in energy coupling was reviewed in Dilley et al. (1987).

The model (cf. Figures 6 and 7) coming out of those studies proposed that the localized protons are sequestered in spaces between the lipid bilayer and the overlaying lumen-exposed parts of those several thylakoid proteins (Laszlo et al. 1984a; Dilley et al. 1987; Allnutt et al. 1989). It should be emphasized that the model we suggested for the sequestered domain posits *not a space at the interface* between the membrane and the lumen (Nagle and Dilley 1986), but *a space behind a barrier* (such as could be formed by the overlying protein masses on the lumen side).

Such a barrier, to allow a metastable sequestered proton pool to exist at a higher proton activity than the external bulk phase or the lumen bulk phase, was independently studied in some depth by Peter Homann and his students and similar conclusions were reached (Theg and Homann 1982; Theg et al. 1982; Pfister and Homann 1986). [A photograph of Homann appears in Homann (2002).] The lumen-exposed parts of membrane-associated extrinsic (e.g., the PS II-associated polypeptides with M_r of 18, 22 and 33 kDa) or intrinsic (e.g., the light-harvesting

complex (LHC II) and the 8 kDa CF_0 polypeptides) proteins could play that role and provide a H^+ diffusion pathway shielded from the lumen and leading into the CF_0 H^+ channel, although structural details for this remain to be elucidated.

The buried proton buffering groups contributing to the localized domains also include carboxyl groups of Glu and Asp, and such groups help to account for the observations concerning the magnitude (roughly 150 nmol H^+ (mg Chl)$^{-1}$) of the sequestered H^+ pools (cf. reviews, Dilley et al. 1987; and Dilley 1991). Buried carboxyl groups (having higher p*K*a values and dicyclohexylcarbodiimide (DCCD) reactivity owing to the hydrophobic effect) associated with the light harvesting complex proteins (LHC II) have been identified from [^{14}C] DCCD chemical modification, sequence analysis, likely protein α-helix and folding patterns (Jahns and Junge 1990; Walters et al. 1994; Jahns et al. 1998) and most clearly by recent electron crystallography structure determinations (Kühlbrandt et al. 1994). The LHC II proteins make up roughly 50% of the thylakoid protein and they contribute buried lysine amine groups and buried carboxylic groups. This is consistent with the LHC II proteins being candidates for providing both some of the shielding and the pathway for the proposed H^+-relay into the CF_0–CF_1 complex via a sequestered pathway. An extremely important set of findings consistent with this view was that blocking buried carboxyl groups with DCCD (Jahns et al. 1988; Jahns and Junge 1990) or blocking the sequestered lysine groups by acetic anhydride (Ewy and Dilley 2000), *blocked the delivery of H^+ ions into the lumen.*

Other support for the possibility of the LHC II proteins being part of the local H^+ diffusion pathways comes from Renganathan and Dilley (1994) where they showed that in a barley mutant (chlorina f_2) lacking a major amount of the LHC II proteins, the thylakoids displayed only the delocalized $\Delta\tilde{\mu}_{H^+}$ coupling mode for thylakoids suspended in *either* a low salt or a high salt medium, whereas the wild type barley thylakoids gave either the localized or the delocalized $\Delta\tilde{\mu}_{H^+}$ coupling modes (depending on low- or high-salt thylakoid storage, respectively) just as for spinach or pea thylakoids. The absence of the LHC II proteins appears to allow a type of direct leakage of H^+ ions into the lumen, akin to having a hole in a pipe; admittedly this is a crude analogy but it seems to fit. That result seems to me a strong supporting evidence for the model we proposed for a sequestered H^+ relay pathway operating in localized H^+ gradient coupling.

The existence of sequestered domain buffering groups having the properties summarized above and whose deprotonation delayed, by 12–15 flashes, the onset of ATP formation energized by a train of, say, 5 Hz single-turnover flashes (Dilley and Schreiber 1984; Theg et al. 1988) greatly strengthened my belief that localized $\Delta\tilde{\mu}_{H^+}$ energy coupling played some key role in chloroplast physiology. But what was that role? Until 1988 we had the phenomena described above, but we had no larger vision of what physiological purpose the dual, switchable, coupling modes might serve.

The missing link – a physiological rationale for there being two $\Delta\tilde{\mu}_{H^+}$ gradient coupling modes

Being able to detect the two $\Delta\tilde{\mu}_{H^+}$ gradient modes in driving ATP formation, the facile and reversible Ca^{2+}-dependent switching between them, and having identified thylakoid proteins with sequestered H^+ buffering groups that provide the structure for the localized domains all pointed to some sort of regulated physiological system. A new way of viewing the situation came as a result of a paradigm shift that occurred in the related field of photoinhibition/photoprotection, brought about by the work of Barbara Demmig and colleagues at the University of Würzburg Botanical Institute (Demmig et al. 1987) dealing with light stress-dependent zeaxanthin formation (see Demmig-Adams 2003, for her perspective).

Lumen-pH-dependent violaxanthin de-epoxidase and photoprotection against excess energization

The xanthophyll cycle chemistry provided a new way of thinking about our emerging data on Ca^{2+} gating of the CF_0 H^+ channel, an action that obviously can influence H^+ delivery to the lumen and therefore lumen pH. The key point in the xanthophyll cycle/photoprotection paradigm is that overenergization of chloroplasts, having the potential to cause photoinhibition of Photosystem II, triggers the enzymatic conversion of violaxanthin to the de-epoxidized forms, antheraxanthin and zeaxanthin. Those pigments together with acidic pH conditions act to dissipate, by nonphotochemical quenching, the

excited state energy of some of the antennae chlorophyll pigments (Demmig-Adams 1990; Gilmore 1997; Govindjee 2002). The violaxanthin de-expoxidase enzyme is: (A) contained in the lumen space; and (B) has an acidic pH optimum (Gilmore et al. 1998; cf. reviews by Demmig-Adams 1990; also see Gilmore 1997). The sharp pH curve for the violaxanthin de-epoxidase activity decreases from a maximum activity at pH 5.9 to near zero activity at pH 6.3 and above (Pfündel and Dilley 1993). Given the acidic pH dependence and the fact that the de-epoxidase is less active under non-stress conditions or when rates of ATP formation are such as to efficiently utilize the proton gradient, we see that a contradiction presents itself when the 'standard' (delocalized $\Delta\tilde{\mu}_{H^+}$) Mitchell chemiosomotic view is taken as the only way proton gradients are coupled to ATP formation.

The contradiction is that when delocalized $\Delta\tilde{\mu}_{H^+}$ conditions energize ATP formation, the lumen is expected to be at a pH less than 6. If that occurs, the violaxanthin de-epoxidase enzyme would be activated and non-photochemical quenching (NPQ) would be stimulated, meaning less absorbed light energy would be available for doing photosynthesis. That expectation contradicts the abundant observations that under non-stressful light intensities, good rates of photosynthesis occur with quite low rates of NPQ (Demmig-Adams et al. 1986). However, if our hypothesis is correct about localized or delocalized $\Delta\tilde{\mu}_{H^+}$ gradients being used to drive ATP formation, and the switching between the two modes controlled by a pH-sensitive Ca^{2+} gate, then the contradiction is resolved.

At low-to-moderate rates of ATP formation and with Ca^{2+} ions occupying the CF_0 sites, our data show that the lumen is not acidified to below pH 6 (a conclusion based on the early work described above primarily using the criterion of the permeable amine effects). Therefore, the violaxanthin de-epoxidase (VDE) enzyme is predicted to be not active. When Ca^{2+} is not present at the CF_0 site (high light/high levels of H^+ accumulation in the local domains, or in our experimental procedures when we displace the Ca^{2+}) the lumen is expected to be acidified to below pH 6 and the violaxanthin de-eposidase enzyme would be activated.

Fortunately, the VDE enzyme action being contained in the lumen provides an endogenous lumen pH indicator (Pfündel and Dilley 1993; Günther et al. 1994). As the VDE enzyme converts violaxanthin to antheraxanthin and zeaxanthin, the action can be easily measured using the 505 nm absorbance increase of the two de-epoxidized pigments (Yamamoto et al. 1972; Pfündel and Dilley 1993).

Switching between localized and delocalized H^+ gradients controls the violaxanthin de-epoxidase enzyme

Sometime in 1988 after the Demmig et al. (1987) paper appeared, Harry Yamamoto (the discoverer of the Xanthophyll cycle) and I discussed this and he agreed with my suggestion that one resolution of this contradiction could be in adopting the localized/delocalized $\Delta\tilde{\mu}_{H^+}$ energy coupling model as the motif, with the switching between the two modes constituting a stress response signal. (For a photograph of Yamamoto, see Govindjee and Seufferheld 2002.) In this model, the initiation of the stress response signal would be the Ca^{2+} de-binding from the CF_0 8 kDa subunit H^+ gating site, allowing protons from the local domains to freely enter the lumen, acidifying it to below pH 6. The lumen acidity dropping below pH 6 would stimulate the violaxanthin de-epoxidase enzyme, leading to the increased non-photochemical quenching, the key event in the photoprotective mechanism used by green plants.

Another reason for talking with Harry about this was that he had a very nice dual wavelength spectrophotometer assay for the VDE enzyme activity available in his laboratory that his group had developed in 1972 (Yamamoto et al. 1972). Adam Gilmore, a student in Harry's laboratory in 1988, and I did a preliminary set of experiments comparing the rate of the violaxanthin de-epoxidase enzyme activity in thylakoids prepared so as to favor either the localized or the delocalized coupling mode. The initial results were gratifying inasmuch as the de-epoxidase rate was much less in localized conditions compared to delocalized conditions, even though a similar ATP formation rate occurs in both cases. The work was followed up in my laboratory by Erhard Pfündel (Pfündel and Dilley 1993; Pfündel et al. 1994) and Run Sun Pan (Pan and Dilley 2000). The picture that emerges is completely consistent with the hypothesis discussed above for Ca^{2+} de-binding in the switching from localized to de-localized $\Delta\tilde{\mu}_{H^+}$ gradient coupling being a stress response signal.

Pan and Dilley (2000) tested the hypothesis outlined above in an extension of the earlier work in my laboratory (Pfündel et al. 1994). The low-salt stored thylakoids capable of giving the localized

$\Delta\tilde{\mu}_{H^+}$ energy coupling response showed close to zero rate of de-epoxidation; that is, the lumen pH must have remained above 6 for the up to 8 min of illumination and ATP formation in those experiments. High salt-stored thylakoids – giving the expected de-localized $\Delta\tilde{\mu}_{H^+}$ coupling mode and having essentially the same ATP formation rates as the low salt-stored sample – gave an immediate and large rate of violaxanthin de-epoxidation, indicating the lumen pH must have been below 6. Removing Ca^{2+} from the low salt-stored thylakoids with an EGTA treatment also resulted in a prompt, large de-epoxidation rate similar to the high salt-stored sample. Those experiments show the value of using the pH dependence of the lumenally located violaxanthin de-epoxidase as an intrinsic lumen pH indicator (Pfündel and Dilley 1993; Günther et al. 1994). Moreover, the results are consistent with our earlier, less direct methods using the permeable amine approach to estimate the relative lumenal acidification during ATP formation in the localized or the de-localized $\Delta\tilde{\mu}_{H^+}$ gradient coupling responses (cf. Dilley 1991 for a review of that work). The results are entirely consistent with the hypothesis that Ca^{2+} interaction with the 'ring of subunit III (*c*)' subunits of the CF_0 (Fillingame 1999; Rastogi and Girvin 1999; Seelert et al. 2000) acts as a H^+ flux gate which in turn functions as a lumen pH-regulating event, providing a stress response signaling system to up-or down-regulate the violaxanthin de-epoxidase activity and through that to help regulate the chloroplast photoprotective response.

Summing up

This short historical review could not do justice to many of the important papers which have been published in this field; however, many of them have at least been cited. I apologize to my friends and colleagues for not being able to go into more details about their work.

The role suggested herein of switchable, dual $\Delta\tilde{\mu}_{H^+}$ gradients in chloroplast physiology seems quite logical and useful for plant function. It remains for future work to: (1) establish better understanding of the still-mysterious localized proton domains (particularly the structural constraints); (2) to define more explicitly the molecular parameters important in the Ca^{2+} binding to the CF_0 H^+ channel; and (3) to test more thoroughly the suggested role of Ca^{2+} gating of H^+ flux at the CF_0 H^+ channel as a part of a stress-signaling, stress-alleviation mechanism in photoprotection of plants.

Acknowledgments

I want to thank Govindjee and Howard Gest for inviting me to write this brief historical piece, and also to thank Govindjee for his very helpful editorial comments. Special mention and deep gratitude is warranted for the students and post doctoral associates who worked in my laboratory for their excellent work and superb contributions. A list of those wonderful friends and companions who journeyed with me on this scientific quest follows: *Post doctoral colleagues* in approximate chronological order are: Charles Arntzen, Robert Keck, Gerald Peters, Bruce Selman, Donna Smith, Robert Giaquinta, Donald Ort, Larry Prochaska, Roberto Galli, Paul Millner, Deepak Bhatnagar, Joe Laszlo, Steve Theg, Tom Allnutt, M. Renganathan, Stas Zakharov, Taya Red'ko Zakharova and Erhard Pfündel. *Graduate students* (chronologically listed) are: Faye Schwelitz, Larry Bering, Gary Johnson, Kay Voegeli, David Sadawasser, Gary Baker, Marilyn Abbott, Norman Tandy, Joe Farchaus, Dan O'Keefe, Emmanual Atta-Asafo-Adjei, Bill Beard, Gisela Chiang, Ahmed Mawgood, Dennis Wooten, Robert Ewy and Run Sun Pan. The excellent assistance of Wanitta Thompson in manuscript preparation is gratefully acknowledged. I thank Don Ort for his suggestions in preparing the revised version of this paper.

Notes

[1] For readers not having experience with the measurements of H^+ gradient formation and ATP formation in thylakoids, it is worthwhile to point out that, thanks primarily to the work of Nelson et al. (1971) and Mordhay Avron in the late 1960s and early 1970s, the effect of permeable weak amines on increasing the extent of H^+ uptake and the predictable delay caused by such amines on thylakoid proton uptake is well understood. As shown in Vinkler et al. (1980) the amine effects provide a very robust indicator for when a delocalized ΔpH drives ATP formation; that is, the amine causes an easily measured increase in H^+ uptake in the light, a delay in the time-in-the-light needed to reach the threshold energization of ATP formation and in a post-illumination ATP formation measurement, the amine effect results in an increase in the ATP yield. The first work on amine (ammonium ion) uptake effect on H^+ uptake was that of Crofts (1967), followed by a study on pyridine effects by Nelson et al. (1971).

[2] Some acidification of the lumen always was observed in our work, even in the best of 'localized' coupling conditions, possibly indicating that the putative local domains were not all com-

pletely sealed, or that some entry of the uncharged amine into the local spaces was possible. But, knowing that there is a significant background H^+ permeability of the thylakoid membrane, I have calculated that such a background H^+ leak could keep the lumen pH from going below about pH 6 provided the rate of H^+ deposition into the lumen under localized coupling conditions occurred at rates near or below about 30 μmol H^+ (mg Chl h)$^{-1}$. This rate is close to the observed H^+ uptake rate in spinach thylakoids at the threshold light intensity where ATP formation is just beginning [that is, below that rate of H^+ uptake where no ATP formation occurred, as shown in Figure 2 of Dilley (1971)]. The H^+ permeability values for thylakoids used in my calculation (background membrane permeability, not that through the CF_0CF_1) were those given by Schönfeld and Schickler (1984) of 2×10^{-5} cm s^{-1} (roughly similar to the value given by Fuks and Homble (1996) of $5.5 \pm 1.1 \times 10^{-4}$ cm s^{-1} for solvent-free bilayers made from thylakoid lipids). More experimental work is necessary to test this idea.

References

Aflalo C, Baum H, Chipman DM, McCarty RE and Strotmann H (1997) Obituary for Noun Shavit. Photosynth Res 54: 165–167

Allnutt FCT, Atta-Asafo-Adjei E and Dilley RA (1989) Chloroplast thylakoid proteins associated with sequestered proton-buffering domains. Plastocyanin contributes buffering groups to localized proton domains. J Bioenerg Biomembr 21: 535–551

Allnutt FCT, Ewy R, Renganathan M, Pan RS and Dilley RA (1991) Nigericin and hexylamine effects on localized proton gradients in thylakoids. Biochim Biophys Acta 1059: 28–36

Baker GM, Bhatnagar D and Dilley RA (1981) Proton release in photosynthetic water oxidation. Evidence for proton movement in a restricted domain. Biochemistry 20: 2307–2315

Baker GM, Bhatnagar D and Dilley RA (1982) Site-specific interaction of ATPase-pumped protons with Photosystem II in chloroplast thylakoid membranes. J Bioenerg Biomembr 14: 249–264

Beard WA and Dilley RA (1986) A shift in chloroplast energy coupling by KC1 from localized to bulk phase delocalized proton gradients. FEBS Lett 201: 57–62

Beard WA and Dilley RA (1988) ATP formation onset lag and post-illumination phosphorylation initiated with single-turnover flashes. III. Characterization of the ATP formation onset lag and post-illumination phosphorylation for thylakoids exhibiting localized or bulk-phase delocalized energy coupling. J Bioenerg Biomembr 20: 129–154

Borchard A and Junge W (1990) The effect of pyridine on the onset lag of photophosphorylation: no evidence for 'localized coupling.' In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol. III, pp 97–100. Kluwer Academic Publishers, Dordrecht, The Netherlands

Boyer PD (1993) The binding change mechanism for ATP synthase – Some probabilities and possibilities. Biochim Biophys Acta 1140: 215–250

Capaldi RA, Aggeler R, Turina P and Wilkens A (1994) Coupling between catalytic sites and the proton channel in the F_0F_1-type ATPases. Trends Biochem Sci 19: 284–289

Chiang GG and Dilley RA (1987) Evidence for Ca^{2+} gated proton fluxes in chloroplast thylakoid membranes: Ca^{++} controls a localized to delocalized proton gradient switch. Biochemistry 26: 4911–1916

Chiang GG and Dilley RA (1989) Intact chloroplasts show Ca^{2+} gated switching between localized and delocalized proton gradient energy coupling (ATP formation). Plant Physiol 90: 1513–1523

Chiang GG, Wooten DC and Dilley RA (1992) Calcium-dependent interaction of chlorpromazine with the chloroplast 8-kilodalton CF_0 protein and calcium gating of H^+ fluxes between thylakoid membrane domains and the lumen. Biochemistry 31: 5808–5819

Crane RK (1983) The road to ion-coupled membrane processes. Compr Biochem 35: 43–67

Crofts AR (1967) Amine uncoupling of energy transfer in chloroplasts. J Biol Chem 242: 3352–3359

Crofts AR (1993) Obituary for Peter Mitchell. Photosynth Res 35: 1–4

Davenport JW and McCarty RE (1980) The onset of photophosphorylation correlates with the rise in transmembrane electrochemical proton gradients. Biochim Biophys Acta 589: 353–357

de Kouchkobsky Y (2002) The Laboratory of Photosynthesis and its successors at Gif-sur-Yvette, France. Photosynth Res 73: 295–303

de Kouchkovsky Y, Haraux F and Sigalat C (1984) A microchemiosmotic interpretation of energy-dependent processes in biomembranes based on the photosynthetic behaviour of thylakoids. Bioelectrochem Bioenerg 13: 143–162 [A section of J Electroanal Chem 174: Elsevier Sequoia, Lausanne]

Demmig-Adams B (1990) Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. Biochim Biophys Acta 1020: 1–24

Demmig-Adams B (2003) Energy dissipation and the xanthophyll cycle: the beginnings. Photosynth Res 76: 73–80

Demmig B, Winter K, Krüger A and Czygan FC (1987) Photoinhibition and zeaxanthin formation in intact leaves. Plant Physiol 84: 218–224

Demmig-Adams B, Gilmore AM and Adams III WW (1996) *In vivo* functions of carotenoids in higher plants. FASEB J 10: 403–412

Dilley RA (1964) Light-induced potassium efflux from spinach chloroplasts. Biochem Biophys Res Commun 17: 716–722

Dilley RA (1969) Evidence for the requirement of H^+ ion transport in photophosphorylation by spinach chloroplasts. Prog Photosynth Res III: 1354–1360

Dilley RA (1970) The effect of various energy conversion states on chloroplast proton and electron transport. Arch Biochem Biophys 137: 270–283

Dilley RA (1971) Coupling of ion and electron transport in chloroplasts. Curr Topics Bioenerg 4: 237–271

Dilley RA (1991) Energy coupling in chloroplasts: a calcium-gated switch controls proton fluxes between localized and delocalized proton gradients. Curr Topics Bioenerg 16: 265–318

Dilley RA and Rothstein A (1967) Chloroplast membrane characteristics. Biochim Biophys Acta 135: 427–443

Dilley RA and Schreiber U (1984) Correlation between membrane-localized protons and flash-driven ATP formation in chloroplast thylakoids. J Bioenerg Biomembr 16: 173–193

Dilley RA and Shavit N (1968) On the relationship of H^+ transport to photophosphorylation in spinach chloroplasts. Biochim Biophys Acta 162: 86–96

Dilley RA and Vernon LP (1964) Changes in light absorption and light-scattering properties of spinach chloroplasts upon illumination: relationship to photophosphorylation. Biochemistry 3: 817–824

Dilley RA and Vernon LP (1965) Ion and water transport processes related to the light-dependent shrinkage of spinach chloroplasts. Arch Biochem Biophys 11: 365–373

Dilley RA, Park RB and Branton D (1967) Ultrastructural studies of the light-induced chloroplast shrinkage. Photochem Photobiol 66: 407–412

Dilley RA, Prochaska LJ, Baker GM, Tandy NE and Millner PA (1982) Proton-membrane interactions in chloroplast bioenergetics. Curr Topics Membr Transp 16: 345–369

Dilley RA, Theg SM and Beard WA (1987) Membrane–proton interactions in chloroplast bioenergetics: localized proton domains. Annu Rev Plant Physiol 37: 348–389

Ewy RG (1997) Delineation between sequestered domain and lumen buffering in the chloroplast thylakoid membrane, pp 74–87. PhD thesis. Purdue University

Ewy RG and Dilley RA (2000) Distinguishing between luminal and localized proton buffering pools in thylakoid membranes. Plant Physiol 122: 583–595

Ferguson SJ (1985) Fully delocalized chemiosomotic or localized proton flow pathways in energy coupling? A scrutiny of experimental evidence. Biochim Biophys Acta 811: 47–95

Fillingame RH (1999) Molecular rotary motors. Science 286: 1687–1688

Fuks B and Homble F (1996) Mechanism of proton permeation through chloroplast lipid membranes. Plant Physiol 112: 759–766

Gilbert GN and Mulkay M (1984) Opening Pandora's Box. Cambridge University Press, Cambridge, UK

Gilmore AM (1997) Mechanistic aspects of xanthophyll cycle-dependent photoprotection in higher plant chloroplasts and leaves. Physiol Plant 99: 197–209

Gilmore AM, Shinkarev VP, Hazlett TL and Govindjee (1998) Quantitative analysis of the effects of intrathylakoid pH and xanthophylls cycle pigments on chlorophyll a lifetime distributions and intensity in thylakoids. Biochemistry 37: 13532–13593

Govindjee (2002) A role for a light-harvesting antenna complex of Photosystem II in photoprotection. Plant Cell 8: 1663–1668

Govindjee and Seufferheld MJ (2002) Non-photochemical quenching of chlorophyll *a* fluorescence: Early history and characterization of two xanthophyll cycle mutants of Chlamydomonas reinhardtii. Funct Plant Biol 29: 1141–1155

Graan T, Flores S and Ort DR (1981) The nature of ATP formation associated with single turnovers of the electron transport carriers in chloroplasts. In: Selman B and Selman-Reimer S (eds) Energy Coupling in Photosynthesis, pp 25–34. Elsevier, North-Holland, New York

Gräber P (1982) Phosphorylation in chloroplasts: ATP synthesis driven by $\Delta\Psi$ and by ΔpH of artificial or light-generated origin. Curr Topics Membr Transp 16: 215–245

Günther G, Thiele A and Laasch H (1994) A new method for the determination of the transthylakoid pH gradient in isolated chloroplasts: the pH-dependent activity of violaxanthin depoxidase. Plant Sci 102: 19–30

Gutman M and Nachiel E (1990) The dynamic aspects of proton transfer processes. Biochim Biophys Acta 1015: 391–414

Hangarter R and Ort DR (1985) Cooperation among electron-transfer complexes in ATP synthesis in chloroplasts. Eur J Biochem 149: 503–510

Homann PH (2002) Chloride and calcium in Photosystem II; from effects to enigma. Photosynth Res 73: 169–175

Horner TD and Moudrianakis EN (1983) The effect of permeant buffers on initial ATP synthesis by chloroplasts using rapid mix-quench techniques. J Biol Chem 258: 11643–11647

Horner TD and Moudrianakis EN (1986) Effects of permeant buffers on the initiation of photosynchronous phosphorylation and post-illumination phosphorylation in chloroplasts. J Biol Chem 261: 13408–13414

Horton P, Ruban AV and Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Plant Mol Biol 47: 655–684

Jagendorf AT (2002) Photophosphorylation and the chemiosmotic perspective. Photosynth Res 73: 233–241

Jagendorf AT and Uribe E (1966) ATP formation caused by acid–base transition of spinach chloroplasts. Proc Natl Acad Sci USA 55: 170–177

Jahns P and Junge W (1990) Dicyclohexylcarbodiimide-binding proteins related to the short-circuit of the proton-pumping activity of Photosystem II. J Biochem (Tokyo) 193: 731–736

Jahns P, Polle A and Junge W (1988) The photosynthetic water oxidase: its proton pumping activity is short-circuited within the protein by DCCD. EMBO J 7: 589–594

Kühlbrandt W, Wang DW and Fujiyoshi Y (1994) Atomic model of plant light-harvesting complex by electron crystallography. Nature 367: 614–621

Laasch H, Ihle C and Günther G (1993) Detecting localized proton currents in photophosphorylation by procaine inhibition of the transthylakoid pH-gradient. Biochim Biophys Acta 1140: 251–261

Larkum AWD (2003) Contributions of Henrik Lundegardh to photosynthesis. Photosynth Res 76: 105–110

Laszlo JA, Baker GM and Dilley RA (1984a) Chloroplast thylakoid proteins having buried amine buffering groups. Biochim Biophys Acta 764: 160–169

Laszlo JA, Baker GM and Dilley RA (1984b) Nonequilibrium of membrane-associated protons with the internal aqueous space in dark-maintained chloroplast thylakoids. J Bioenerg Biomembr 16: 37–51

Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191: 144–148

Mitchell P (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Cambridge Phil Soc 41: 445–540

Nagle JF and Dilley R (1986) Models of localized energy coupling. J Bioenerg Biomembr 18: 55–64

Nelson N, Nelson H, Naim Y and Neumann J (1971) Effect of pyridine on the light-induced pH rise and post-illumination ATP synthesis in chloroplasts. Arch Biochem Biophys 145: 263–267

Ort DR and Dilley RA (1976) Photophosphorylation as a function of illumination time. I. Effects of permeant cations and permeant anions. Biochim Biophys Acta 449: 95–107

Ort DR and Melandri BA (1982) Mechanism of ATP synthesis in Photosynthesis. In: Govindjee (ed) Photosynthesis: Energy Conversion by Plants and Bacteria, Vol 1, pp 537–587. Academic Press, New York

Ort DR, Dilley RA and Good NE (1976) Photophosphorylation as a function of illumination time. II. Effects of permeant buffers. Biochim Biophys Acta 449: 108–124

Pan RS and Dilley RA (2000) Influence of Ca^{2+} on the thylakoid lumen violaxanthin de-epoxidase activity through Ca^{2+} gating of H^+ flux at the CF_0 H^+ channel. Photosynth Res 65: 141–154

Pfister VR and Homann PH (1986) Intrinsic and artifactual pH buffering in chloroplast thylakoids. Arch Biochem Biophys 246: 525–530

Pfündel E and Dilley RA (1993) The pH dependence of violaxanthin deepoxidation in isolated pea chloroplasts. Plant Physiol 101: 65–71

Pfündel EE, Renganathan M, Gilmore A, Yamamoto H and Dilley RA (1994) Intrathylakoid pH in isolated pea chloroplasts as probed by violaxanthin de-epoxidation. Plant Physiol 106: 1647–1658

Pick U and Weiss M (1988) The mechanism of stimulation of photophosphorylation by amines and by nigericin. Biochim Biophys Acta 934: 22–31

Pick U, Weiss M and Rottenberg H (1987) Anomalous uncoupling of photophosphorylation by palmtic acid and by gramicidin D. Biochemistry 26: 8396–8403

Prochaska LJ and Dilley RA (1978) Chloroplast membrane conformational changes measured by chemical modification. Arch Biochem Biophys 187: 61–71

Rastogi VK and Girvin ME (1999) Structural changes linked to proton translocation by subunit *c* of the ATP synthase. Nature 402: 263–268

Renganathan M and Dilley RA (1994) Evidence that the intrinsic membrane protein LHCII in thylakoids is necessary in maintaining localized $\Delta\tilde{\mu}_{H^+}$ energy coupling. J Bioenerg Biomembr 26: 117–125

Renganathan M, Pan RS, Ewy RG, Theg SM, Allnutt FCT and Dilley RA (1991) Evidence that localized energy coupling in thylakoids can continue beyond the energetic threshold onset into steady illumination. Biochim Biophys Acta 1059: 16–27

Renganathan M, Pfündel E and Dilley RA (1993) Thylakoid lumen pH determination using a fluorescent dye: correlation of lumen pH and gating between localized and delocalized energy coupling. Biochim Biophys Acta 1142: 277–252

Schönfeld M and Schickler H (1984) The permeability of the thylakoid membrane for protons. FEBS Lett 167: 231–234

Schreiber U and Del Valle-Tascon S (1982) ATP synthesis with single turnover flashes in spinach chloroplasts. FEBS Lett 150: 32–37

Seelert H, Poetsch A, Dencher NA, Engel A, Stahlberg H and Müller DJ (2000) Proton-powered turbine of a plant motor. Nature 405: 418–419

Siefermann D and Yamamoto HY (1975) Properties of NADPH and oxygen-dependent zeaxanthin epoxidation in isolated chloroplasts. Arch Biochem Biophys 171: 70–77

Sigalat C, Haraux F, De Kouchkovsky F, Hung S and De Kouchkovsky Y (1985) Adjustable microchemiosmotic character of the proton gradient generated by systems I and II for photosynthetic phosphorylation in thylakoids. Biochim Biophys Acta 809: 403–413

Shavit N, Dilley RA and San Pietro A (1968) Ion translocation in isolated chloroplasts. Uncoupling of photophosphorylation and translocation of K^+ and H^+ ion by nigericin. Biochemistry 7: 2356–2363

Tandy N, Bhatnagar D, Hermodson MA and Dilley RA (1982) Evidence for an interaction between protons released in chloroplast Photosystem II water oxidation and the 8000 M_r hydrophobic subunit of the energy-coupling complex. J Biol Chem 257: 4301–4307

Theg SM and Homann PH (1982) Light, pH and uncoupler-dependent association of chloride with chloroplast thylakoids. Biochim Biophys Acta 679: 221–234

Theg SM, Johnson JD and Homann PH (1982) Proton efflux from thylakoids induced in darkness and its effect on Photosystem II. FEBS Lett 145: 25–29

Theg SM, Chiang G and Dilley RA (1988) Protons in the thylakoid membrane-sequestered domains can directly pass through the coupling factor during ATP synthesis in flashing light. J Biol Chem 263: 673–681

Tikhonov AN and Timoshin AA (1985) Electron transport, proton translocation and their relation to photophosphorylation in chloroplasts. Biol Membr 2: 608–622

Tikhonov AN, Khomutov GB, Ruuge EK and Blumenfeld LA (1981) Electron transport control in chloroplasts. Effects of photosynthetic control monitored by the intrathylakoid pH. Biochim Biophys Acta 637: 321–333

Van Walraven HS, Marvin HJP, Koppenaal E and Kraayenhof R (1984) Proton movements and electric potential generation in reconstituted ATPase proteoliposomes from the thermophilic cyanobacterium *Synechococcus* 6716. Eur J Biochem 144: 555–561

Van Walraven HS, Scholts MJC, Zakharov SD, Kraayenhof R and Dilley RA (2002) pH-dependent Ca^{2+} binding to the F_0 *c*-subunit affects proton translocation of the ATP synthase from *Synechocystis* 6803. J Bioenerg Biomembr 34: 455–465

Vernon LP (2003) Photosynthesis and the Charles F. Kettering research laboratory. Photosynth Res 76: 379–388

Vik SB, Long JC, Wada T and Zhang D (2000) A model for the structure of subunit *a* of the *E. coli* ATP synthase and its role in proton translocation. Biochim Biophys Acta 1458: 457–466

Vinkler C, Avron M and Boyer PD (1980) Effects of permeable buffers on the initial time course of photophosphorylation and post-illumination phosphorylation. J Biol Chem 255: 2263–2266

Walters RG, Ruban AV and Horton P (1994) Higher plant light-harvesting complexes $LHCII_a$ and $LHCII_c$ are bound by dicyclohexylcarboiimide during inhibition of energy dissipation. Eur J Biochem 226: 1063–1069

Westerhoff HV, Melandri BA, Venturoli G, Azzone GF and Kell DB (1984) A minimal hypothesis for membrane-linked free-energy transduction. Biochim Biophys Acta 768: 257–292

Williams RJP (1961) Possible functions of chains of catalysts. J Theor Biol 1: 1–17

Williams RJP (1988) Proton circuits in biological energy interconversions. Ann Rev Biophys Chem 17: 71–97

Williams RJP (1993) The history of proton-driven ATP formation. Biosci Rep 13: 191–211

Wooten DC and Dilley RA (1993) Calcium gating of H^+ fluxes in chloroplasts affects acid–base-driven ATP formation. J Bioenerg Biomembr 25: 557–567

Yamamoto HY, Kamite L and Wang YY (1972) An ascorbate-induced absorbance change in chloroplasts from violaxanthin de-epoxidation. Plant Physiol 49: 224–228

Zakharov SD, Ewy RG and Dilley RA (1993) Subunit III of the chloroplast ATP-synthase can form a Ca^{2+} binding site on the lumenal side of the thylakoid membrane. FEBS Lett 336: 95–99

Zakharov SD, Ewy RG and Dilley RA (1995) Calcium binding to the chloroplast and *E. coli* (CF_0) F_0 subunit (III) *c* of the ATP-synthase. Protoplasma 184: 42–49

Zakharov SD, Li X, Red'ko TP and Dilley RA (1996) Calcium binding to the subunit *c* of *E. coli* ATP-synthase and possible functional implications in energy coupling. J Bioenerg Biomembr 28: 483–493