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Minireview

Protons, proteins and ATP

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Abstract

The machinery of life has been disclosed in the second half of the 20th century to a degree not in the least envisioned previously by even the most daring players in this field. It has been extremely rewarding to start out from the fogs and to enjoy the brightness at the end of one's active career. Perhaps the most astounding lesson to learn is how conservative and modular is the construction of key devices. Oxidative and photophosphorylation are carried out by ATP synthase, which is unique in converting electrochemical, mechanical and chemical forms of energy within one nano-machine. This complex protein consists of more than 20 polypeptides of at least eight different kinds. Still, its activity survives in engineered chimerical constructs joining parts from organisms that underwent billions of years of separate evolution. The path of discovery of its structure and function is sketched here from a personal viewpoint. It has been a long way from before-structure-bioenergetics to the post-structural one (which now dominates the biology textbooks), but there is still a long way to go for a rigorous physical understanding. The author has been privileged to enjoy the friendship, cooperation and competition of excellent scientists from widely different backgrounds and expertise.

The beginning

I joined photosynthesis research through the Max-Volmer-Institute of Physical Chemistry at the Technical University of Berlin, Germany. It was in 1966, right after I obtained my diploma in physics. Though in retrospective a lucky move, at the time the transition was loaded with grief: I had been relegated from physics because of a musical that four of us students were commissioned by the director to write and perform at the Institute's 1964 Christmas party. Somehow ahead of its time, the plot was conceived in the spirit of 1968, and not appreciated by the director, Hans Boersch, an otherwise impressive scientist and teacher. Searching for another job, I happened to contact Horst Witt (Figure 1). He charmed me with challenging ideas, was

anything but embarrassed by my previous 'misconduct'; and, even better, he offered a salary. Above all, his lifelong theme, photosynthesis, then seemed, and has remained, an interesting and wide field to explore.

In 1966 the Max-Volmer Institute was running high on photosynthetic electron transport, energy transfer, photoprotection, and water oxidation. Bernd Rumberg had just begun to tackle ATP-synthesis. The early version of Peter Mitchell's chemiosmotic hypothesis had been published (Mitchell 1961) and was up for reformulation in order to include the electric portion of the protonmotive force (Mitchell 1966). The classical Jagendorf experiments had been performed and were in its favor [see (Jagendorf 2002) in this series]. For a newcomer from physics, the hypothesis was easy to grasp. If the hydrolysis of ATP electrifies

Figure 1. Horst Tobias Witt and his wife, Ingrid Witt. Photo taken in 1966, in the library of the Max-Volmer-Institute, TU-Berlin.

nerve membranes, then why should electrochemical force not drive the synthesis of ATP? The dominating beliefs among biochemists were the 'direct' coupling of ATP synthesis with the electron transport by an enigmatic phosphorylated intermediate (coined the 'squiggle'), or the then dubious conformational coupling schemes, and both appeared more complicated. During the decade-long battle over chemiosmotic and squiggle phosphorylation which followed, it seemed often to be ignored that Peter Mitchell had described the energy flow only phenomenologically, merely scratching the surface of a molecular mechanism. At that time the alternatives were chemiosmosis, conformational coupling, and squiggle. It took three decades to appreciate fully nature's wonderful blend of the first two. In 1966, the structure and action of proteins was not the common knowledge it is today – the pivotal book by R.E. Dickerson and I. Geis appeared only in 1969 (Dickerson and Geis 1969). Furthermore, membranes were still conceived of as solid devices, sclerotized by a dense pack of proteins at their surface. In Witt's institute Werner Kreutz was advocating and wrongly detailing this concept for the thylakoid membrane (Kreutz 1972). For us young physicists and chemists he was the much appreciated source of basic knowledge in protein biochemistry, and the senior partner in stimulating discussions on life as an historical event, ruled by 'Le hazard et la nécessité' (Monod 1970). Later, Kreutz moved to Freiburg where he hatched a flock of young infra-red spectroscopists (Fritz Siebert, Werner Mäntele, and Klaus Gerwert)

now well-known in photosynthesis research. In 1973 I was lucky to inherit Werner Kreutz's position at TU-Berlin as associate professor of physical and biophysical chemistry. The same position was inherited by Gernot Renger when I left Berlin for Osnabrück in 1979.

A molecular voltmeter

Back to 1966, and to an environment of enthusiasm, physical skills and biochemical ignorance. I was commissioned to solve the riddles of certain light-flash-induced absorption transients at the wavelengths of 515 nm, 475 and 648 nm, which had been discovered by Horst Witt (Witt and Moraw 1959). Observable in suspensions of purple bacteria, chloroplasts of green plants, and algae, they were attributed to chlorophyll *b* by some (Rumberg 1964) and mainly to carotenoids by others. For the time being they were 'spectroscopic signals,' as marginalized by Otto Warburg, who, at the first, unofficial photosynthesis congress, in France 1962, pretty single-mindedly asked Horst Witt: 'Could you tell us how the chemical mechanism of photosynthesis can be described on the basis of your spectroscopic observations?' Again it took decades to fully appreciate the great potential of spectroscopists, kineticists, biochemists, geneticists and structural biologists joining forces.

Upon starting, I learned that the rapid rise of absorption at 515 nm after firing of a flash of light was linked to the activity of both photosystems, and that the subsequent decay was at least biphasic, fast and slow, with the slow phase being not only related in some way to photophosphorylation (Rumberg et al. 1966), but also sensitive to several electron acceptors for Photosystem II. This multi-faceted signal seemed to present, in an unknown way, the 'missing link' between electron transport and ATP synthesis. I found that the relative extent of the slow decay phase varied between different preparations of fragmented chloroplasts. By leaving a diluted suspension of chloroplasts in the photometer beam over lunch-break, and re-inspecting the aged sample, I found that the slow decay phase was converted into the rapid one, while the total extent of the rise after the flash was unaffected. If simple aging accelerated the decay, what about other mistreatments? I recalled reading about a permeabilizing effect of ethanol on biomembranes. Indeed, a sip of booze drastically accelerated the signal decay, and this broke the ice. Exposure to hypo- or

hyperosmolar media or to high temperature, and addition of ionophoric antibiotics (Horst Witt had obtained gramicidin from Bert Pressman) all accelerated the decay. Increasing the concentration of several, chemically different cations accelerated it further, and, with the ionophore gramicidin, the cation specificity was the same as that known from model studies. The spectroscopic signal seemed related to ion transport across the thylakoid membrane. Titration of the accelerating effect as a function of the concentration of added gramicidin corroborated this notion: as little as $0.1\,nM$ gramicidin per $10\,\mu M$ of chlorophyll produced a detectable effect. This implied that the functional unit behind this signal contains at least 10^5 chlorophyll molecules, that is, more than 100 electron transport chains. That was the expected number of chains in one thylakoid disk. Taken together, these findings revealed 'the chl-*b* reaction as indicator for an electrical field across the thylakoid membrane' (Junge and Witt 1968) [for the first report, see Witt (1967)]. The newly discovered 'molecular voltmeter' had an astoundingly short rise-time of less than 20 ns (Wolff et al. 1967). The calculated magnitude of the flash-induced voltage jump was about 50 mV after a single flash, and over 200 mV under continuous illumination. The estimate was based on the known density of reaction centres and the specific capacitance of biomembranes (Junge and Witt 1968). These findings gave realm to the electrical component of the protonmotive force, and suggested that the transmembrane voltage alone might present sufficient energy to drive ATP synthesis in terms of Mitchell's hypothesis. Soon it became apparent that both photosystems contributed about equally to the voltage and also to net proton transfer (Schliephake et al. 1968). The difference spectrum of the electrochromic response was shaped as if originating from electrochromic bandshifts, with contributions from carotenoids and chlorophyll *b* (Emrich et al. 1969b). To extract the electrochromic component at any wavelength from the responses to other events, the sensitivity of the decay to ionophores served as a kinetic tag. By the same criterion we discovered an artificial electrochromic dye, rhodamin-*b* (Emrich et al. 1969a), and happily marketed 'molecular voltmeters' as new tools in biophysics (Junge and Witt 1969). Electrochromism in the narrower sense, i.e., the prompt response owed to the dipole moments and/or dielectric polarizability of a dye, has not made a commercial career as a 'voltage probe' outside of photobiology. Even at very high field strengths, say $10^7\,V\,m^{-1}$ (i.e., 50 mV over 5 nm) the wavelength shift is only a fraction of 1 Å, and the resulting absorption change is then too small ($\Delta OD < 10^{-3}$) for ready detection – except in photobiological systems which allow a non-invasive stimulation by light. A more generally applicable solution to this goal, as it has turned out later, have been membrane-adsorbed dyes that undergo a voltage-sensitive isomerization. Although inferior in time resolution, these yield a much larger signal.

Electrochromism as a voltage probe was soon thereafter established in biomimetic model systems (Schmidt et al. 1969, 1971; Reich et al. 1976), and in purple bacteria by Baz Jackson and Tony Crofts (Jackson and Crofts 1969, 1971), see the joint review (Junge and Jackson 1982) and Crofts' historical perspective of the Q-cycle, (this issue). As to the participation in chloroplasts of chlorophyll-*b versus* carotenoids, the latter were surely involved. It was expected that symmetrical pigments without permanent dipole moment, like β-carotene, produced a quadratic response to the electric field by their dielectric polarizability. The observed pseudo-linear response has therefore been attributed to their pre-polarization in van der Waals contact with chlorophyll (see, Sewe and Reich 1977). This early concept was proven when the structure of a light-harvesting-complex (LHC)-antennae protein became available (Kühlbrandt and Wang 1991).

Anyway, we had one essential element of Mitchell's hypothesis, the transmembrane voltage, at hand, and we were going to exploit it further. Surely Horst Witt (see Witt, this issue), but what about me? As indicated previously, I was kind of sceptical about the rigidity of the German university system.

What made me continue after receiving my PhD in early 1968 were the following.

(i) The ambitious and competitive spirit in Horst Witt's institute and the friends there, to name a few: Bernd Rumberg, Gernot Renger (see, Renger 2003) and Ulrich Siggel (who have stayed in the Max-Volmer-Institute); Hannes Stiehl (plastoquinone, later owner of a 'clean room' manufacturing company); Günter Döring (P680, now at the German Bureau of Standards); Hinnerk Emrich (MD, now Professor and director of Psychiatry and Neurology in Hannover); Roland Reich (Professor of Physical Chemistry in Berlin); and, last but not the least, Wolfgang Haehnel (then working on Photosystem I, now on protein *de novo* design, Professor of Biochemistry in Freiburg);

Figure 2. Outside the Volmer Institute, Berlin, with Govindjee (second, right) and his student Rita Khanna (below, right) visiting in 1976, with B. Rumberg (first, right), H. Witt (fourth, right), and the author (second, left, below).

(ii) An extraordinary meeting on molecular biology in the lovely mountainous Schloß Elmau, Bavaria, in 1968, where Manfred Eigen had gathered the cream of biophysical chemistry (including a beaming Francis Crick) in a merger with young graduates; a mind-widening event, really.

(iii) The first official International Congress of Photosynthesis in Freudenstadt, Germany, 1968. At this meeting I gave a short lecture on our data (Junge et al. 1969) and collided with Britton Chance (see Chance, this issue), who had adverse views on the 'carotenoid shifts' and, at that time, was deeply suspicious of Mitchell's hypothesis. The public clash with an expert of his stature was great for a freshly-hatched but fearless PhD with better data. All the more because Brit, despite our disagreement, seemed to enjoy the fight, so much so that he invited me over to the US straight-on, where we became friends. The subsequent stays in his lab, then a focus of worldwide pilgrimage for biophysicists and biochemists, young and old, were pivotal for feeling at home in the wider field.

It was straightforward to use the molecular voltmeter for studies on ATP synthesis. At the Max-Volmer-Institute (see Figure 2), this field was Bernd Rumberg's, and newly chosen in his attempt to escape direct competition and confrontation with Horst Witt. Within the Institute, Horst acted as an ambitious elder brother rather than a father. Aside from obvious disadvantages of this attitude to some, it was advantageous that Horst accepted, while not necessarily respecting, his offspring's strides for independence, and, not the least, that he himself, and the institute as a whole, have remained on the wild side of science.

Bernd had established a preparation protocol for freshly broken chloroplasts and set up the radioactive assay for ATP synthesis. We collaborated. Whereas the earlier work was carried out with fragmented and resealed membranes that were rather leaky to many ions, in freshly broken chloroplasts the electric leakage was greatly reduced. That the interlaced system of grana and stroma lamellae (see the historical perspectives of Staehelin 2003; Albertsson 2003) was largely intact was later quantified by Gerald Schönknecht and Gert Althoff (Schönknecht et al. 1990). They titrated the functional unit of the electrical discharge in freshly broken chloroplasts by adding minimal amounts of gramicidin, and found a detectable acceleration of the voltage decay by as little as one gramicidin dimer per 5×10^7 chlorophyll molecules [compared to as many as 10^5 in fragmented chloroplasts (Junge and Witt 1968)]. This result implies that the whole thylakoid system of a chloroplast, grana and stroma

lamellae taken together (believed to contain about 10^8 chlorophyll molecules total), constitutes one electrically sealed, contiguous sheet. In material of this quality, the decay-time of the transmembrane voltage caused by ion leakage is longer than 100 ms. The slow voltage generation by cytochrome-b_6f is detectable, but was ignored by us in subsequent work.

ATP synthesis: transmembrane voltage as both driving force and regulator of activity – the early approach

In 1969 Hartmut Schröder, Bernd Rumberg and I studied ATP synthesis under excitation of chloroplasts with groups of between one and three closely-spaced flashes of light. The groups were fired repetitively, with long intervals between them. Hartmut determined the ATP yield per flash in the same sample for which I recorded the electrochromic transients. Very few of our many preparations of spinach chloroplasts were sufficiently ion-tight for this purpose – growth season, leaf age, turgor, and speed of handling were all important factors. The major results were as follows (Junge 1970; Junge et al. 1970):

(1) *Transmembrane voltage as major driving force.* The ATP-yield per flash group was halved by insertion of a leak conductance for K^+ (by valinomycin) that exactly matched the one for the proton (by the ATP synthase);

(2) *Threshold voltage.* Under phosphorylating conditions, the voltage decay was fast only if the voltage exceeded a certain threshold. If the starting voltage (directly after firing the flash) was below threshold right away, there was neither an accelerated decay nor ATP formation. This has been interpreted by either voltage regulation of the enzyme activity, or a strongly non-linear current–voltage relationship of the enzyme's proton translocator (Junge 1970);

(3) *Proton (charge)-to-ATP-stoichiometry of 3.3.* The total extent of the initial voltage jump was calibrated against charges per ATP by the known stoichiometric ratio of photosystems to ATP synthases, and the ATP-yield was directly assayed by ^{32}P-radiography. At its time (1970) a stoichiometry of 3.3 was off-scale because everybody else in the community was convinced by a ratio of two, including Albert Lehninger in his Biochemistry textbook (Lehninger 1970).

It is worth considering how the these early insights have survived the passage of time.

(1) That transmembrane voltage can be the major driving force of ATP synthesis was corroborated in a drastic experiment by Horst Witt and his coworkers who showed that CF_OCF_1-doped proteoliposomes synthesize ATP in response to an *externally* applied electric field pulse (Witt et al. 1976). That protons are taken in by the enzyme, and appear at the other side in synchrony with the voltage decay, has been shown by combining the electrochromic voltmeter with kinetically competent pH-indicating dyes for both sides of the membrane (Junge 1987).

(2) The existence of voltage-regulation of the chloroplast enzyme was extensively studied in Berlin (Rumberg and Becher 1984; Bauermeister et al. 1988; Junesch and Gräber 1991) and elsewhere (Strotmann and Bickel-Sandkötter 1984). The oxidized enzyme is activated by both components of the protonmotive force, ΔpH and $\Delta\psi$, and this is physiologically useful. The down-regulation of the enzyme at insufficient protonmotive force, under conditions where the electron transport chain of the chloroplast goes oxidized, serves to prevent ATP hydrolysis in the dark. Responsible for this regulation is an extra-loop of the γ-subunit of the enzyme carrying two apposed cysteines (Moroney et al. 1982; Hisabori et al. 1998, 2002). A voltage threshold has also been established for the activity of an Na^+-translocating ATP synthase (Laubinger and Dimroth 1987; Kaim and Dimroth 1999; Dimroth et al. 2000).

(3) The proton-to-ATP stoichiometry has remained a matter of debate for more than three decades. It has been determined both under static head (quasi-equilibrium) conditions and under off-equilibrium conditions. Published figures increased over the decades from two to three or four. The last figure is the résumé of several groups (van Walraven et al. 1996), and has been corroborated recently by technical improvements which seemed to avoid possible pitfalls of previous approaches (Turina et al. 2003). Today, however, this figure is difficult to reconcile with the supposed structure of this enzyme. One expects the H^+/ATP-ratio to conform to the symmetry ratio between F_O and F_1. By atomic force microscopy, a ring of 14 *c*-subunits has been found in isolates from chloroplast F_O (Seelert et al. 2000), whereas the number of catalytic sites on F_1 is 3 (Abrahams et al. 1994).

The expected ratio based on the structure is therefore $14/3 = 4.7$, which is incompatible with 4 as determined by thermodynamic and kinetic experiments. This discrepancy has not yet been solved. The common belief BS ('before structure') was that the stoichiometry must be an integer. But it is obvious that non-integral stoichiometric ratios are feasible if an elastic power transmission smoothes the non-matching symmetries between the ionic (F_O) and the chemical motor/generator (F_1) (Junge et al. 2001; Cherepanov and Junge 2001; Pänke et al. 2001).

The results of 1970 were ahead of their time: they proved the electrical aspect of Mitchell's theory and indicated the voltage regulation of the enzyme under oxidizing conditions. Because the experiments were carried out on chloroplasts, and based on a spectroscopic probe that was not generally applicable, they appeared as a kind of off-side to the biochemical majority in Bioenergetics. This majority worked with mitochondria and bacteria, and its late 'conversion' eventually gave Peter Mitchell the Nobel prize in 1978. One episode may illustrate the neglect we felt. Peter Mitchell knew the Berlin group very well, and in 1976 he had received his first honorary degree from the Technische Universität Berlin. We sustained a regular correspondence that I loved for its science, humanity and style. In 1990 we were invited to join the celebration of Glynn's 25th anniversary, a marvellous event in his laboratory and home, in Cornwall (see Figure 3). A map was posted in the library whereon red woollen threads linked Glynn House near Bodmin with any place in the world which had contributed to the success of the chemiosmotic theory. The map was comprehensive with one exception, Berlin. I mention this episode as illustration of the interlacing of scientific 'truth' and social acceptance. The multi-authored paper wherein Peter Mitchell was jammed, alphabetically, between his former adversaries, who insisted on their alternative contributions, also illustrates this point (Boyer et al. 1977). G. Gilbert and M. Mulkay's only thinly camouflaged sociological coverage of the chemiosmotic *versus* squiggle discourse (Gilbert and Mulkay 1984), and, fundamentally, Karl Popper's Logik der Forschung (Popper 1935) are worth reading in this context. It is favorable that the lifetime of personal or social bias in science is usually very short.

Thereafter, I left ATP synthesis for a while, one reason being that Bernd Rumberg continued, the other being that Horst Witt promptly hired gifted post-docs just for this topic, i.e., Peter Gräber (now Professor

Figure 3. Peter Mitchell and Peter Rich at the Glynn Lab anniversary, in 1990.

of Physical Chemistry at Freiburg) and Eberhard Schlodder (still at the Max-Volmer). What followed was a migration through different topics within and outside of photosynthesis. In retrospect, there was some logic in the way we later returned to studies of ATP synthesis.

Roaming around: places, topics and methods

In 1979 I moved with my group from the Technical University of Berlin to the Chair of Biophysics at the University of Osnabrück. This move was kind of necessary on the one hand, but risky on the other. 'Going to German Siberia' was Nathan Nelson's comment. The move liberated us from the tight competition in Horst Witt's institute, we were endowed with an unusually large start-up investment, and we changed gradually into another life-style; from the feverish, culturally booming, but sequestered West Berlin, to this lovely old town in northwestern Germany with hills, fields and fens around. The university, founded only in 1974, was still in a fragmentary state. To begin with, we had to convert into labs a suite in a multi-purpose cardboard-and-steel building, a replica of which decorates Moscow's international airport. The great crew of graduate students and post-docs that had moved over from Berlin mastered these tasks in short time and brought widely different research topics from Berlin to Osnabrück: Benjamin Kaupp studied visual transduction in vertebrate rods; Ulrich Kunze, cytochrome-*c*-oxidase; Helmut Schaffernicht, Photosystem I; Richard Wagner, ATP synthase; and Verena Förster, water oxidation. Hawi Trissl joined us with the light gradient technique, Yu Qun Hong (from Shanghai,

Figure 4. Berliners in Osnabrück 1980. Left: almost serious (*from rear to front, from left to right*): Helmut Schaffernicht, Paul Schnetkamp, Richard Wagner, Hawi Trissl, Yu-Qun Hong, Ulrich Kunze, unidentified, unidentified, Ernesta Junge, Margret Offermann, Dorle Trissl, Benjamin Kaupp, Verena Förster, and the author. Right: a little wilder.

China, now in Brookhaven, New York) worked on proton uptake and release, and Paul Schnetkamp (from Nijmegen, The Netherlands) on vision (see Figure 4). In the beginning we did not believe in the longevity of this university, but pleasant new buildings for Biology (we were part of this unit) and Physics, they were finished in 1983, turned the fate. Soon thereafter, together with my collegues Karlheinz Altendorf (Microbiology), Joseph Lengeler (Genetics), and Wolfgang Lueken (Zoophysiology) and others, we founded a collaborative research unit on cellular membrane transport (Sonderforschungsbereich 171). This large, institutional grant, was funded by the Deutsche Forschungsgemeinschaft with three years' revision, it lasted over 15 years, until 1999, and I served as its chairperson.

Of the different topics and systems that I had started in Berlin, Philadelphia, and later in Osnabrück, some were unrelated to, others methodically paving the way or directly aiming at the mechanism of ATP synthesis.

Let me briefly mention two lines of research of the first category: Benjamin Kaupp (now Professor of Biophysics in Köln and Jülich) studied *visual transduction* in vertebrate rods. We disproved the Ca^{2+}-transmitter hypothesis (see e.g., Kaupp and Junge 1977; Kaupp et al. 1980; Schnetkamp et al. 1981; Kaupp and Junge 1982) and the tools derived in these studies helped Benjamin to establish *c*-GMP instead of Ca^{2+} as the second messenger. Studies on *photosynthetic water oxidation* and Photosystem II are still going on and have involved great graduate students, post-docs and collaborators. We

focussed on the electrostatic balance and the interplay of electron and proton transfer in the catalytic centre (see, e.g., Förster and Junge 1985; Lübbers et al. 1993; Haumann and Junge 1994a; Haumann et al. 1994, 1997a, b, 1999; Bögershausen and Junge 1995; Hundelt et al. 1997; Ahlbrink et al. 1998; Clausen et al. 2001, 2004; and reviews in Lavergne and Junge 1993; Haumann and Junge 1999; Junge et al. 2002).

Three lines of earlier work that have technically paved our way to investigate the molecular mechanism of ATP synthesis are: (a) polarized spectrophotometry; (b) the integration of biochemistry and molecular biology in the lab; and (c) improved techniques to monitor proton transfer.

(a) Polarized spectrophotometry. After my futile attempts to implement the 'molecular voltmeter' into Britton Chance's mitochondria in Philadelphia (signals were obscured by too large mixing artefacts) I embarked on *polarized photobleaching and recovery* to study the rotational mobility of cytochrome-*c*-oxidase in the cristae membrane of mitochondria (Junge 1972; Junge and DeVault 1975; Kunze and Junge 1977) and later employed this technique to unravel the spectroscopic band structure of P700 in Photosystem I of green plants (Junge and Eckhof 1974; Junge et al. 1977; Schaffernicht and Junge 1981, 1982). In comparison with fluorescence depolarization, polarized photobleaching and recovery is not limited to a time range of nanoseconds, but, with a triplet probe such as eosin, it extends into milliseconds

[for studies on the F_1-ATPase (see Wagner and Junge 1980, 1982; Wagner et al. 1982)], and to even longer times with a chemically reactive probe such as the transiently oxidized P700 in PSI (Schaffernicht and Junge 1982, 1981). Making use of a permanently photobleached state of the probe eosin we later extended this technique to record rotational motion within the F-ATPase in the range of seconds (Sabbert et al. 1996, 1997). In principle, polarized photobleaching and recovery yields information on chromophore symmetry, resonant energy transfer and rotational motion in the time domain from nanoseconds to seconds.

(b) Integration of biochemistry and molecular biology. *Biochemistry* was brought to us by Nathan Nelson (then Professor of Biochemistry in Haifa, now in Tel Aviv) (see Figure 5 and Nelson and Ben-Shem 2002, this series) and the late Noun Shavit (Professor of Biochemistry in Beersheba). Roland Schmid learned to extract and reconstitute F_1 in thylakoid membranes (Schmid et al. 1976). Nathan taught Helmut Schaffernicht how to purify and immobilize Photosystem I for polarized spectroscopy on the band structure of P700 (Junge et al. 1977). Dear memories of that time in Berlin are: Noun's lab-filling charm, as still remembered from his melodious calls for 'Elsa,' our technician Ilse Columbus; Nathan's improvisations in a chemically inadequately equipped lab, including his special low-pressure liquid chromatography (LPLC, two beakers, silicone tubes, one column, and gravity); and our transiently wifeless living together in my home at the bank of the river Spree. Later, Nathan and Hannah Nelson trained Holger Lill (now Professor of Structural Biology in Amsterdam) in *molecular biology*, and Holger seeded these skills into our Osnabrück lab after returning back from Nathan's 'lab of unlimited resources' at the Roche Institute, Nutley, New Jersey.

(c) Improved techniques to monitor proton transfer. *Proton transfer* has been a topic in my lab right from the beginning. pH-transients can be detected with very high time resolution in both compartments which are separated by the thylakoid membrane. It is pivotal that the total buffering power of the very thin lumen (typical thickness 100 nm) and of the macroscopic suspending medium are about equal in magnitude, although the cumulative volume-ratio between out and in is about 1000 in a typical experiment. In the absence of ad-
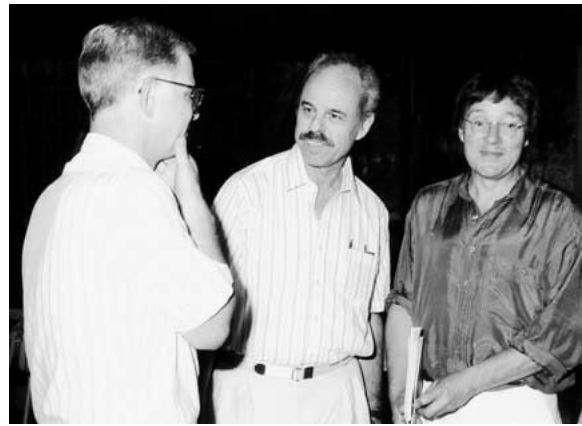

Figure 5. Nathan Nelson (center), Dick McCarty (left) and the author in 1996.

ded buffer, buffering is mainly attributable to the proteins and lipids at both surfaces of the membrane, where the densities of buffering groups are of about equal magnitude. The drastically different size of the respective inner and outer volume is negligible because water is such a poor buffer (see Junge and McLaughlin 1987). The photometric detection with high time resolution of pH-transients in the *suspending volume* of thylakoids is straightforward by using *hydrophilic* pH-indicating dyes (Schliephake et al. 1968; Ausländer and Junge 1974; Junge and Ausländer 1974). Even if penetrating into the lumen, the sheer volume ratio of internal/external, 1/1000, makes the response from the few inside-located dye molecules irrelevant. Winfried Ausländer found a way to record flash light induced pH-transients also in the nanometer-wide lumen of thylakoids (Ausländer and Junge 1975). When neutral red, an amphiphilic and membrane adsorbed pH-indicator, is added to thylakoids, it produces two, oppositely-directed absorption transients in response to excitation of photosynthesis by a short flash of light: one attributable to the pH-transient in the suspending medium (alkalinization); and the other one in the lumen (acidification). The response of neutral red to the pH-transient in the lumen is extracted by adding a non-permeating buffer, e.g., bovine serum albumin (BSA), to the suspending medium (Ausländer and Junge 1975). Only later did it become apparent that non-permeant buffers smaller than BSA, e.g., phosphate, are required for high-speed quenching of pH transients in the narrow space between *tightly appressed* grana thylakoids,

into which BSA does not readily enter (Lavergne and Rappaport 1990; Jahns et al. 1991). When correctly applied, use of neutral red allows a very high time resolution of proton release at the lumen side of the thylakoid membrane. In studies on water oxidation, Michael Haumann observed proton transfer to neutral red in $10\,\mu s$ (Haumann and Junge 1994a). The high velocity of the proton transfer is owed to the bimolecular collision of the surface-mobile proton acceptor, neutral red, with the fixed intrinsic proton donor(s). This mechanism is apparent from the pseudo-linear dependence of the relaxation rate on the concentration of this dye. In studies on water oxidation, the mechanism allowed us to discriminate by kinetic criteria the chemical release of protons, which is limited by an intrinsic time constant, from electrostatically triggered and immediate proton release at the protein periphery [membrane Bohr effects as reviewed in Junge et al. (2002)]. We determined the internal buffering capacity of thylakoids for protons (at pH 7, about 1/20 mol/mol chlorophyll) (Junge et al. 1979), and the magnitude of the pH-transient per short flash of light (at pH 7, about 0.06 pH-units) (Hong and Junge 1983). The response of neutral red was sensitive to the surface potential at the lumenal side of the membrane (Hong and Junge 1983). In other words, in kinetic experiments with thylakoids under flash excitation, neutral red behaves as a surface adsorbed and laterally mobile proton carrier and indicator for the lumenal side of the membrane. On the other hand, its use as an indicator for slower pH transients in mitochondria and chloroplasts is complicated by its redistribution between the luminal and the external sides of the membrane and by dimerization (see, Luvisetto et al. 1991). In the time domain of milliseconds, however, the photometric probes have given a comprehensive view of the transmembrane and the lateral protonic relaxation under the influence of fixed and mobile buffers (Junge and Polle 1986; Polle and Junge 1986a, b, 1989; Junge and McLaughlin 1987). Hemi Gutman (Tel Aviv), Stuart McLaughlin (Stony Brook) and Jérôme Lavergne (Paris) were critical partners during this work and friends.

Electrochromism and neutral red helped us to better understand the ionic side of ATP synthase, as discussed later (for reviews on the work on chloroplasts and purple bacteria till the early 1980s, see Junge 1977, 1982; Junge and Jackson 1982).

Localized *versus* delocalized coupling between proton pumps and ATP synthase

After the chemiosmotic hypothesis had become almost generally accepted in 1978, the properties of the enzyme still did not come into focus since there was so little structural information available. As a 'left-over' the debate over localized-*versus*-delocalized coupling between proton pumps and proton translocating ATP synthase absorbed quite a bit of energy in this field. It dated back to the early sixties. R.J.P. Williams (1961) was probably the first to emphasize a special role of the proton by stating that ADP and P_i in a solution at extremely low pH, would react to yield ATP, by kind of enzyme-free mechanism. Later, when Mitchell had formulated his less dramatic version of how protons might be used for the synthesis of ATP, Williams argued that Mitchell's mechanism cannot hold for the following reason: the outwardly directed proton pumping in a photosynthetic or respiring bacterial cell could *never*, of course, acidify the Pacific Ocean, which, by dilution, presents an almost infinite sink for protons (Williams 1978, 1988). These arguments were valid and they stimulated a wealth of literature on 'localized energy coupling' and 'microchemiosmosis' (see, e.g., Kell 1979; Westerhoff et al. 1984; van der Bend et al. 1985). In the chloroplast field, it was mainly Dick Dilley (this issue) who published experiments that, in his view, proved that ATP synthesis in chloroplasts is driven by some localized pH-difference, and not by the bulk-to-bulk pH-difference between the lumen and the suspending medium (see, e.g., Ort and Dilley 1976; Ort et al. 1976; Dilley et al. 1982; Laszlo et al. 1984; Nagle and Dilley 1986; Dilley 1991).

We held fast against Dick's arguments by monitoring the release of protons into the lumen, and their uptake from it, by the ATP synthase, at some $10\,\mu s$ time resolution. Using neutral red as indicator we found that this dye reports on: (i) the rapid ejection of protons into the lumen, e.g., by water oxidation (Ausländer and Junge 1975; Haumann and Junge 1994a); (ii) ready equilibration with intrinsic and externally added buffers (Junge et al. 1979; Hong and Junge 1983); followed by (iii) proton uptake by the ATP synthase (Junge 1987), or by its proton translocator, F_O (if F_1 is removed) (Schönknecht et al. 1986). It proved that both the pumps and the ATP synthase operate on the lumen, operationally defined as the neutral red accessible space, and on the suspending medium. Strictly speaking, our experiments proved

that a delocalized chemiosmotic mechanism operates in thylakoids *in the presence of neutral red.*

However, it is obvious that neutral red, which acts as a mobile proton carrier along the membrane surface and between surface and 'bulk,' accelerates the equilibration of protons. Perhaps neural red just *created* Mitchellian behavior? Even we, as enthusiasts for Mitchell's concept, observed phenomena that might be interpreted by local proton trapping (Theg and Junge 1983; Polle and Junge 1986c; Jahns and Junge 1990a, b, 1993), and by the existence of energy barriers for protons at the membrane–water interface (Haumann and Junge 1994b; Gopta et al. 1999). These questions have prompted Armen Mulkidjanian and Dmitry Cherepanov, post-docs from Moscow, to resume this topic. They arrive at the conclusion that there is an energy barrier for the passage of ions over the membrane–water interface, because the dielectric permittivity of surface water is lower than that of bulk water (Cherepanov et al. 2003, 2004). Under continuous operation of proton pumps and the ATP synthase and with a calculated barrier height of 60 mV the interfacial pH at the p-side (whereto protons are delivered by the primary pumps) can rise up to 6 units. If this surface acidity existed at the outer side of an alkaliphilic bacterium it might explain, for the first time, how these organisms may synthesize ATP by a basically chemiosmosis mechanism (Cherepanov et al. 2003, 2004). The positive surface of alkaliphilic bacteria faces a bulk at pH 10 whereas the cytoplasmic pH is clamped around neutrality. Because these organisms do not generate a membrane potential greater than −200 mV, their *bulk-to-bulk* protonmotive force in the steady state is insufficient for a chemiosmotic mechanism of ATP synthesis (Guffanti and Krulwich 1992). Our new results have emphasized the *surface-to-surface* protonmotive force for chemiosmotic ATP synthesis in these organisms. However, this feature is probably *not* of general importance. In thylakoids, for instance, the positive side of membrane faces the nanoscopic lumen, which is not an infinite proton sink such as the 'Pacific Ocean.' Because of the smaller dimensions, the proton equilibration between the surface and the thylakoid lumen is rapid, and the formation under steady turnover of a sizeable pH-gradient between the surface and the lumen is not predicted.

ATP synthase: the structure comes into sight

The proton translocating ATP synthase, F-ATPase, is a bipartite enzyme: it has a membrane-intrinsic, proton translocating portion, F_O; and a peripheral portion, F_1, which processes the nucleotides. It is now established that both F_O and F_1 are rotary motor/generators. They are mounted on a common central shaft, and held together by an eccentric stator (see Figure 7). Depending on the predominant driving force, which may be a protonmotive force across the coupling membrane or a high phosphate potential in the solution facing F_1, one portion drives the other as a motor, and the other operates as a generator, producing ATP – or, when running backwards, protonmotive force. It is now generally accepted that the construction principles of $F_O F_1$ are the same in the three kingdoms of life (Archaea, Bacteria, and Eukarya – see Woese, this issue). Today's comprehensive view of this enzyme has emerged from various research objects. Studies on chloroplasts and photosynthetic bacteria have contributed precise kinetic and thermodynamic data, mainly on the ionmotive aspects, studies on other objects have contributed crystal structures (mitochondria, yeast); mutational analysis (*Escherichia coli*); a novel videographic rotation assay (thermophilic *Bacillus* PS3); and information on Na^+-transport (*Propionigenium modestum*).

In the early 1980s we resumed our earlier investigations (Schmid et al. 1976) on the chloroplast proton conductor, CF_O. It was supposedly composed of four types of subunits, I–IV (Nelson et al. 1980). In the current nomenclature, which has been shaped after that for *E. coli*–F_O these subunits are named *a*, *b*, *b'*, and *c*, respectively. Their relative proportions were unknown in the 1980s; there was no structure for F_O in sight. One early stronghold concerned subunit *c*, present in several copies; then, supposedly, six, by titration with a covalently binding inhibitor (Sigrist-Nelson et al. 1978); today, 14, by atomic force microscopy (Seelert et al. 2000). CF_1 was believed to be composed of five types of subunits, α–δ, in stoichiometric ratio of 3:3:1:1:1 (Suess and Schmidt 1982). These subunits could be extracted as a whole to yield the soluble five-subunit CF_1 which, when added back to exposed CF_O, plugged its high proton conductance (Schmid et al. 1976). Subunits αβ formed the core of F_1 and interacted with nucleotides. All subunits of F_O (a, b_2 and c_n) were needed for proton conduction by *E. coli* F_O(EF_O) (Schneider and Altendorf 1985). The function of subunits δ and ε was under contention. They were interesting because the coupling between F_O and F_1, and of proton flow to ATP synthesis, was the most enigmatic part of the function of this bipartite enzyme.

Subunit δ at the interface between F_O and F_1

Siggi Engelbrecht and Holger Lill concentrated on the role of subunit δ 'at the interface between proton flow and ATP synthesis' (see review in Engelbrecht and Junge 1990). When we started in the early 1980s, the association of subunit δ with CF_1 had already been established, but there were also reports on the interaction of subunit δ with CF_O (Andreo et al. 1982; Roos and Berzborn 1983). Our approach to this discrepancy exemplifies the difficulties and errant paths of research before the advent of a molecular structure. In collaboration with Alexandro Viale in Ruben Vallejo's lab in Rosario, Argentina, Yu-Qun Hong observed that the extraction of the four-subunit CF_1 from thylakoids did not produce the proton leakage through CF_O, that was known to occur after the extraction of five-subunit CF_1. Instead, there was a cooperative transient proton trapping, which was attributed to the central acid residue on the multi-copy subunit c (Junge et al. 1984). In a series of articles, Siggi Engelbrecht and Holger Lill established that the purified subunit δ, when added back to CF_1-extracted thylakoids, blocks proton leakage by CF_O (Engelbrecht et al. 1986, 1989b, 1990; Engelbrecht and Junge 1987, 1988, 1990; Lill et al. 1988). The same role of δ has been found in chimeric constructs, with *E. coli* δ bound to *Spinacia* CF_O, and *Spinacia* δ bound to *E. coli* EF_O (Engelbrecht et al. 1989a). This cross-activity of the δ-subunits was surprising given the low sequence identity, 23%, between these proteins with a length of 177 (*E. coli*) and 187 (*Spinacia*) amino acids. The clearly documented ability of δ to plug-up proton conduction by CF_O led us to the speculate about a role of δ as a *conformational energy transducer* between F_O and F_1 (Engelbrecht and Junge 1990), rather than as a proton conductor between F_O and F_1. The structural basis for either function, however, remained enigmatic. Because the first crystal structure of mitochondrial F_1 (MF_1) (Abrahams et al. 1994) showed no mitochondrial equivalent of δ (named OSCP), its position was revealed only later, by other techniques. Crosslinking of δ with αβ (Lill et al. 1996), fluorescence resonant energy transfer (Engelbrecht et al. 1998), and electron microscopy (Wilkens et al. 2000; Rubinstein and Walker 2002) have all located δ at the very top of F_1, far away from F_O. It sits at the end of the stator of rotary F_OF_1, binding with very high affinity [a sub-nanomolar dissociation constant to αβ and to the tip of subunit b (Häsler et al. 1999; Weber et al. 2002, 2003a, b). Therefore δ belongs to the F_1 portion of the enzyme, connecting F_O and F_1 as an essential element of the stator. The earlier, rather speculative concept of δ binding directly to, and acting as a plug of, CF_O, was too simple. It remains conceivable, though, that δ, although primarily binding to the extended subunit b, may contact the c-ring by virtue of the floppy structure of b. Later, we found that δ was not the only F_1-subunit fooling us, since added β and γδε diminished proton flow through F_O, too.

The magnitude of proton conduction by F_O

Figures reported in the literature for the proton conductance of F_O have been scattered over *four orders of magnitude*, from 0.1 fS to 1 pS (1S = 1 A/V). Insufficient time resolution (e.g., of glass electrodes) and uncertainty over the proportions of conducting and non-conducting F_O have caused under- and overestimates, respectively.

The figures in the earlier literature are in the range of 0.1 fS, which is equivalent to a rate of 62 protons s^{-1} at a driving force of 100 mV (Negrin et al. 1980; Friedl and Schairer 1981; Schneider and Altendorf 1982; Sone et al. 1981). This rate is 10 times too low if F_O is able to cope with the turnover of the coupled holoenzyme, F_OF_1. Our first studies on chloroplast F_O were carried out with high time resolution, using electrochromism and pH-indicators. They produced a 100-fold higher figure, 9 fS (or 5600 protons s^{-1} at 100 mV), which is compatible with the maximum turnover rate of the coupled enzyme. This estimate is based on the assumption that all exposed F_O molecules (after removal of F_1) contribute to the relaxation of the transmembrane voltage (Schönknecht et al. 1986). Later, we obtained circumstantial evidence for a major proportion of inactivated F_O, which raised the conductance up to a figure of 1 pS (Lill et al. 1986; Althoff et al. 1989). Such high values are hardly compatible with the diffusive proton supply to F_O, as then noted, and it has remained questionable whether the underlying assumption is correct. At the same time, a conductance of similarly high magnitude was reported in our institute for CF_OCF_1 in a lipid bilayer formed by the 'dip-stick technique,' which is related to patch clamp (Wagner et al. 1989). Gated single channel currents were observed. They peaked at 0.55 pA (at 180 mV), implying a conductance of 0.4 pS, and channels were gated with a sharp activation above 100 mV. The authors attributed this voltage-gated conductance to the proton (Wagner et al. 1989). It remains an open question, whether this

attribution is correct, in particular because the proteoliposomes, which contained the purified c-subunit alone, reveal unspecific cation channels (Schönknecht et al. 1989).

The lack of information on the number of active F_O per membrane area was obviously the major obstacle to a reliable estimate of its proton conductance. Studies on isolated chromatophores from the photosynthetic bacterium *Rhodobacter capsulatus* (Feniouk et al. 2001, 2002) have recently paved the way to overcome the ambiguity over the proportions of active and inactive F_O. The clue was to prepare vesicles so small as to contain, on average, less than one copy of F_O. Then, any *rapid* relaxation of the transmembrane voltage is attributable to the subset of vesicles containing a single F_O molecule, and the conductance of a single F_O can be calculated.

Boris Feniouk prepared chromatophore vesicles with 28 nm mean diameter containing average of 0.3 copies of F_O. Again, a voltage step was generated by a flash of light and its relaxation monitored by electrochromic absorption transients of intrinsic carotenoids, as well as the proton flow by pH-indicating dyes (Feniouk et al. 2003). The rapid phase of the relaxation is attributed to vesicles containing a single copy of F_O. This implies a maximum conductance of 10 fS (at pH 8), which is equivalent to the translocation of 6400 protons at 100 mV. This value was determined, for the first time, without ambiguity concerning active and inactive copies of F_O. The relaxation time is independent of the voltage, in other words, the conductance is Ohmic. F_O conducts protons independently of the nature of the driving force, whether of electrical (transmembrane voltage) or entropic (pH-difference) origin. Both this property, and the Ohmic behavior under electric driving force alone, proves the absence of voltage gating of F_O. The electrical gating of the holoenzyme in chloroplasts (Junge 1970; Junge et al. 1970; Gräber et al. 1977; Witt et al. 1977; Schlodder and Witt 1980, 1981; Schlodder et al. 1982; Junge 1987) and in *P. modestum* (Kaim and Dimroth 1998, 1999; Dimroth et al. 2000) is probably attributable to the interaction of F_1 with F_O, but not to F_O proper. The specificity of F_O for protons over other cations was $> 10^7$, both in the purple bacterium (Feniouk et al. 2003) and in thylakoids from green plants, as previously studied (Althoff et al. 1989). The specificity is lessened in organisms that operate on gradients of Na^+, instead of H^+ (Dimroth 1997). We interpret the properties of F_O in terms of the current rotary model for proton conduction, described below. This model is

based on two proton conducting half channels linking the respective bulk phases by means of the rotating ring of 10–14 copies of subunit c, with its acid residue (Glu or Arg) sitting in the middle of the membrane. The observed Ohmic conduction implies that the two relay groups with widely spread pK-values are each located close to one of the respective membrane–water interfaces. This conclusion is a testable prediction for structural experiments aimed at high resolution of the c-ring and its partner in the membrane, the a-subunit.

A model for torque generation by a rotary proton translocator

Around 1990, the ATPase community was almost certain of an *alternating* mechanism of ATP hydrolysis that involved the cooperation of at least two reaction sites on F_1. This concept owed to the pioneering work of Paul Boyer, who coined the term 'binding change mechanism,' and of Harvey Penefsky, Alan Senior and Richard Cross, to name but few (Boyer et al. 1973; Boyer 1979, 1993; Boyer and Kohlbrenner 1981; Cross et al. 1982; Grubmeyer et al. 1982; Penefsky 1985a, b; Rao and Senior 1987). A 'rotary mechanism' involving three catalytic sites, mechanistically still rather diffuse, was also considered as an option by some.

At the 7th European Bioenergetics Conference in Helsinki in 1991, John Walker and Peter Petersen engaged in a battle over their emerging, and still unpublished, structural models for F_1, which both hinted at a rotary mechanism. Being interested mainly in the proton side of ATP synthesis, I was searching for a viable model to explain torque generation by proton flow. Stimulated by Howard Berg's (Meister et al. 1987, 1989) work on the protonic drive of the bacterial flagellae, with a series of eight 'linear' motor elements operating on the large perimeter of a central disk, I came up with a model for a rotary drive in the much smaller F_O. This model is illustrated in Figure 6.

According to this model, the ion transporting and rotary function of $F_O F_1$ ATP synthase is based on two structural elements, then probable and now corroborated: a ring of several copies of the small subunits c, each carrying one carboxylic residue (Asp or Glu) in the middle of the membrane; and facing this ring, a single copy of the large subunit a. Torque is generated by three properties of this construct: (i) *Electrostatic constraints* which enforce the protonated state of the acid residues on the ring when facing the lipid core,
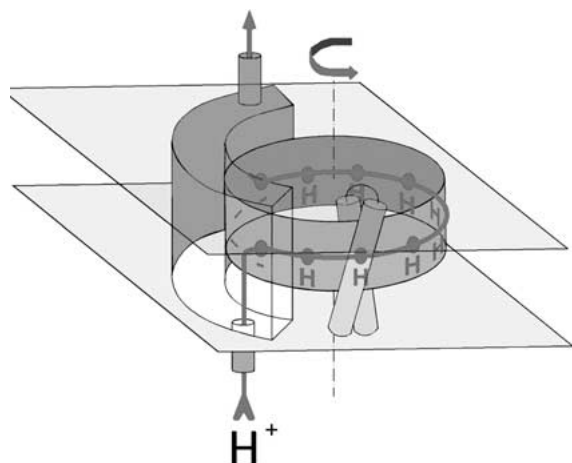
Figure 6. The proton motor of F_O (Junge et al. 1997). See the animation in www.biologie.uni-osnabrueck.de/biophysik/junge.

and the deprotonated state when facing subunit *a*; (ii) The creation of *handedness* by the non-colinear placement of two proton access-channels to the carboxylic residues; (iii) *Brownian rotational fluctuations* of the ring relative to the large subunit *a* (for a review, see Junge et al. 1997). In 1991, I did not publish this model right away, because the late Klaus Lakomiak and I had embarked on an ambitious experiment to prove it. The idea was to block the full rotation of the ring by a covalent reaction with one molecule of N,N'-dicyclohexylcarbodiimide (DCCD), and to trigger limited proton conduction in one direction by light-induced voltage (in chloroplasts). We expected that the ring came to a halt as the attached DCCD molecule hit subunit *a*. We planned then to generate protonmotive force in the opposite direction by photolyzing 'caged proton' in the suspending medium, hoping to detect limited flow of protons in the opposite direction until the ring hit its DCCD nose again, but now at the other end of subunit *a*. Although nicely conceived and technically mastered (both the generation of alternating protonmotive force and the detection worked well) this experiment failed to produce the expected result. We were terribly frustrated. When, in 1993, at an EMBO conference in Freiburg, Germany, John Walker presented his still unpublished structure of F_1, somebody reapproached him by stating that there was then no viable mechanism for torque-generation by F_O, and I projected a drawing of the unpublished model and explained its features. John and others immediately went for it. John asked for permission to use it in his lectures. I agreed, on the condition that he tagged my name to it. This he consistently did,

apparently even in a lecture at the South Western Medical Center in Dallas, Texas (September 1994). From there the model made its way, without the tag, into a paper by Steve Vik (Southern Methodist University in Dallas) and his coworker (Vik and Antonio 1994), and became known in the US, the big market, as the 'Vik-model.' George Oster and his coworkers (Elston et al. 1998) treated the model by statistical mechanics and later adapted it to the then believed other structure (one access channel, only) in Na^+-translocating PF_O (Dimroth et al. 1999). I emphasized that both versions, as, in essence, the concept for the flagellar motor proposed by Meister et al. (1989), shared the three properties that were outlined in Junge et al. (1997). The three common properties are: Brownian relative motion between two proteins; electrostatic constraints; and two non-colinear access-routes to acid groups on the rotor (Junge 1999).

ATP synthase: the structure, eventually, and proving rotation

When the first detailed crystal structure of F_1 from beef heart mitochondria finally appeared in 1994 (Abrahams et al. 1994), a rotary mechanism of catalysis was immediately apparent. Today's concept of the structure of F_OF_1 is given in Figure 7 and three methods to prove rotational motion in F_1 are illustrated in Figure 8. The early structural model of F_1 showed the three catalytic sites each in a different state: with an ATP-analogue; with ADP; and empty. The cranked central shaft, subunit γ, in the middle of the $(\alpha\beta)_3$ pressed against a lever on one copy of subunit β, keeping its nucleotide binding site open, and thereby empty. Having been given advanced access to some structural details, Dirk Sabbert, Siggi Engelbrecht and I were trying to prove inter-subunit rotation by polarized spectrophotometry. It was advantageous that chloroplast CF_1 carried a cysteine as the penultimate residue at the C-terminal end of subunit γ, the central rotary shaft in John Walker's structure. By a maleimide function the triplet probe eosin was covalently attached to this residue. The enzyme body was immobilized on ion exchange globules, as in our previous studies with Photosystem I (Junge et al. 1977; Schaffernicht and Junge 1981 1982). While we were at full speed in these experiments, Richard Cross (see Figure 8) and his coworkers published first evidence for the functional rotation of subunit γ relative to $(\alpha\beta)_3$ (Duncan et al. 1995). Using cleavable crosslinks

between γ and *a* in hybrid enzymes composed of radioactively labeled and unlabelled subunits, their data proved some mobility in minutes, but not on the time scale of the catalytic turnover, and not giving the angular range and directions. In the meantime, we observed rotation with a half-time of 100 ms. When the immobilized but active preparation of F_1 with eosin linked to the end of γ was excited with a linearly polarized laser flash, a small fraction (0.6%) of the dye was irreversibly bleached, which gave rise to a stepped absorption transient. Low energy was chosen for the linearly polarized laser in order to create a preferentially oriented ensemble by photoselection. We found that the polarization anisotropy decayed in 100 ms from a figure of 0.1 to almost 0.02. This decay happened only if the enzyme was active, but not when it was blocked by AMP-PNP. This decay implied the functional rotation of subunit γ relative to $(αβ)_3$ in the time domain of catalytic turnover: the conservative estimate for the angular domain was 200°. The publication appeared in March 1996 (Sabbert et al. 1996).

Howard Berg contacted me soon thereafter and argued that the then expected continuous and unidirectional rotation should give rise to an *oscillation* of the polarization anisotropy rather than to its *relaxation*, as we observed. I argued that the assumption of *continuous* rotary motion in proteins was unrealistic, and that an ensemble of stepped rotors, although initially phase-synchronized by the exciting laser flash (photoselection), becomes rapidly dephased by the stochastic nature of the stepped chemical reactions. Dirk Sabbert and I sent him a theoretical manuscript, which he communicated to *Proceedings of the National Academy of Sciences, USA* (Sabbert and Junge 1997). Therein we had simulated rotary stepping by a Markovian chain reaction, and found that rotary two- and three-steppers produced a relaxation of the polarization anisotropy without appreciable oscillation, whereas a six-stepper gave a damped but detectable oscillation. The oscillation was, of course, more pronounced in a rotary motor with, e.g., 50 steps. The obvious conclusion was that F_1 carries out ATP-hydrolysis by a three-stepped process. We did not know at this time that Howard Berg's group was also working on this matter, aiming at the stepping in the flagellar motor. Shortly after our paper appeared, they published a similar analysis which they had obtained by a different algorithm (Samuel and Berg 1997).

The full paper on the rotational relaxation of F_1 in March 1997 (Sabbert et al. 1997) contained the following additional features: (i) Brownian rotational diffusion of F_OF_1 in the thylakoid membrane with a relaxation time of 200 μs, making the assignment of subunits to 'rotor' and 'stator' an arbitrary choice; (ii) vibrational fluctuation of the probe around its bond axis with subunit γ in nanoseconds within a narrow angular domain; (iii) and in 30 μs in a wider angular domain (probably 360°) as facilitated by domain flexibility at the N-terminal ends of $(αβ)_3$; (iv) the above-mentioned functional, and probably three-stepped, rotation, in 100 ms in the presence of ATP; (v) a limited rotation in the absence of nucleotides.

The last feature was a complication. It could be rationalized, however, in the context of previous work on 'proton slip' in the holo-enzyme, F_OF_1. If the concentration of ADP and ATP is kept below 100 nM, then the chloroplast enzyme conducts protons in a decoupled mode (Groth and Junge 1993). This was interpreted as a release of the 'clutch' on the F_1-portion (Fritsche and Junge 1996). This interpretation was in line with the observation of a more open structure of the $(αβ)_3$-hexagon in the absence of nucleotides than in their presence (Shirakihara et al. 1997).

We set out to investigate stepping by a single-molecule approach, polarized confocal microscopy, again with a probe on subunit γ and with the enzyme body immobilized. Three orientations were detected that were sequentially occupied in a stepped fashion (Häsler et al. 1998) but the data were of poor quality because of the limited photostability of the dye.

The spectroscopic data proving intersubunit-rotation in F_1 had been available since 1996, and were presented, and discussed intensively, in several conferences. Enthusiastically greeted by some, they were not readily accepted by certain top players in photophosphorylation – Heinrich Strotmann and Dick McCarty (see Figure 5) (McCarty and Evron 2000) to name just two. The unavoidable theoretical background of our approach might have been one obstacle; a general dislike of 'chemistry out – mechanics in,' another.

A major problem with our data was the difficult and tedious way in which they were obtained. This

Figure 7. Model of ATP synthase, based on the work by Paul Boyer (left), John Walker (right) and of many other contributors.

Figure 8. Three methods aiming at proving the rotation of subunit γ in F_1. Left: by cleavable cross-linkers (Duncan et al. 1995), middle: by polarized photobleaching and recovery (Sabbert et al. 1996, 1997) and by video-micrography (Noji et al. 1997). Top, left: Richard Cross; top, right: Masasuke Yoshida.
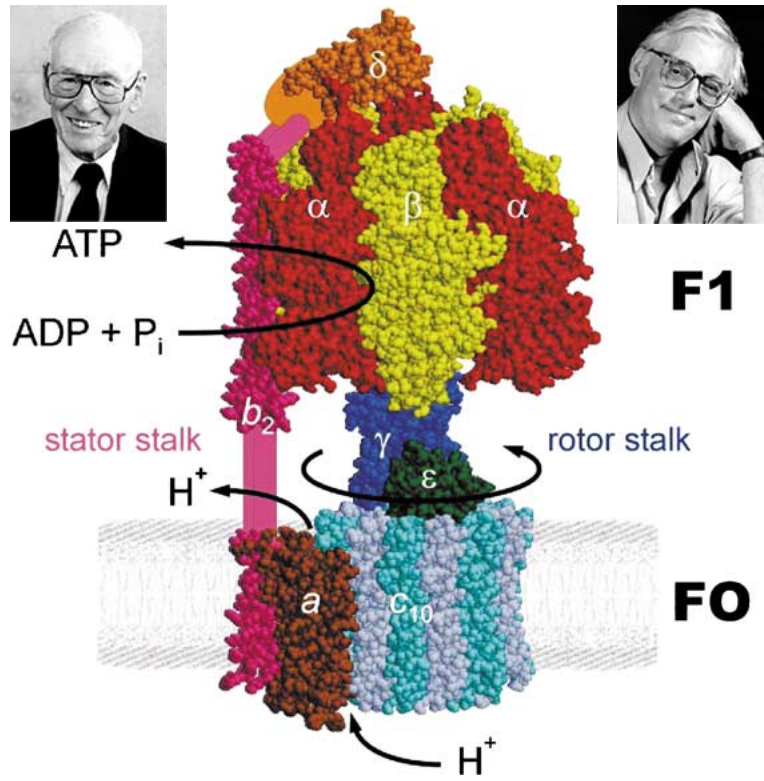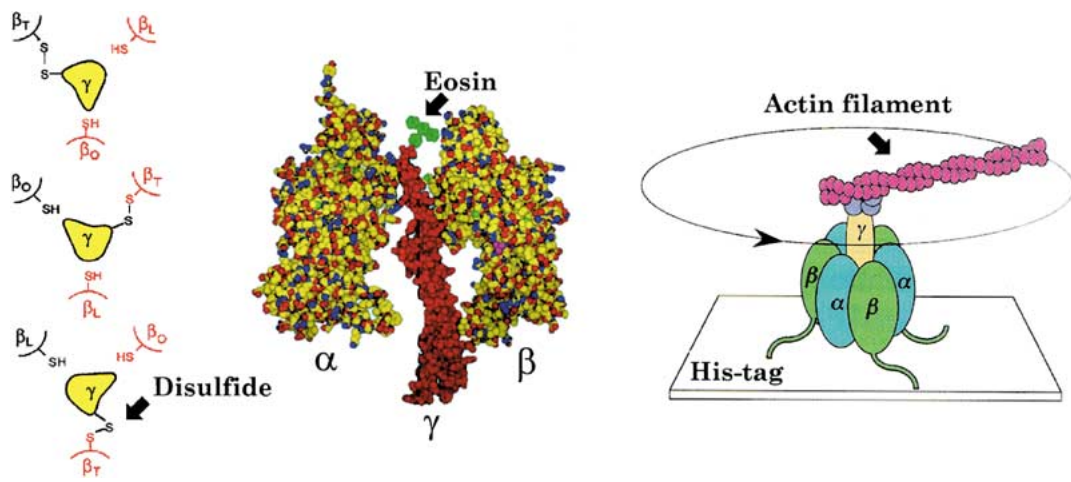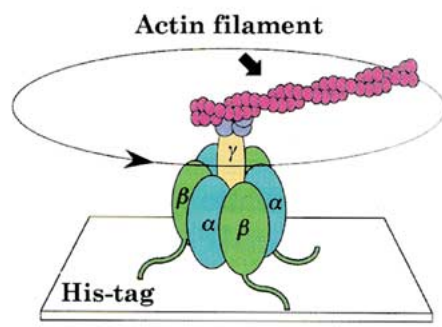
Figure 7

Figure 8

was a subject of major concern in our group. In order to record functional rotation in, say, 100 ms, one had to rely on the permanent bleaching of the probe eosin. While the quantum yield of the triplet, the starting state for bleaching, was high (70%), the yield of bleaching was low and the signals were small. Aiming to resolve small differences between polarized absorption transients, several thousand signals had to be averaged, each resulting from more than 100 samples, all with careful control of the deliberately non-saturating laser output energy. In short, the gruesome feeling of being subject to Cantor's dilemma (Djerassi 1991) forced me back into the lab with Benjamin Junge, my eldest son, lending a hand. In the end, we reproduced the data of Dirk Sabbert's original *tour-de-force*, without matching fully his signal-to-noise ratio.

It came as a great relief to the community that a Japanese team recorded the hydrolysis-driven rotation of subunit γ directly, by videomicrography, using a μm-long actin filament as a visible reporter (Noji et al. 1997). The Nobel prize acknowledging the pioneering contributions of Paul Boyer and John Walker (see Figure 7) followed in the same year. The laboratories of Masasuke Yoshida (see Figure 8) and Katsuhiko Kinosita soon released a series of excellent papers proving the stepped nature of the rotation (Yasuda et al. 1998, 2001, 2003; Adachi et al. 2000). Their experiments were carried out on F_1 from the thermophilic *Bacillus* PS3. Siggi Engelbrecht visited them in Japan, collaborated on the *E. coli* F_1 (Noji et al. 1999), and imported their published technique to Osnabrück for studies on an elastic power transmission between the two motor/generators of the holoenzyme.

ATP synthase: the nanomachine

The ATP synthase is a rather robustly functioning enzyme. Although its various models in pro- and eukaryotic organisms are the product of separate paths of evolution over billions of years, chimeric constructs have been engineered and prove to be functional. Moreover, the enzyme tolerates rather severe artificial modifications [reviewed in Junge et al. (2001) and, for two drastic recent examples, see Cipriano et al. (2002); Prescott et al. (2003)]. And the enzyme copes with the symmetry-mismatch between its two motors: CF_O, supposedly with 14 *c*-subunits (Seelert et al. 2000) drives CF_1, with three αβ-pairs.

To account for these features we have speculated about an elastic power transmission between the two motors (Junge et al. 1997), and treated the consequences of such a transmission theoretically (Cherepanov et al. 1999), as has Oliver Pänke (Pänke and Rumberg 1999) who joined us from Bernd Rumberg's lab to carry this idea further. We extended the video-micrographic assay to the holoenzyme by attaching an actin filament to the *c*-ring of F_O by means of engineered Strep-tags® (Pänke et al. 2000). The goal was to investigate the mechanical properties of the enzyme as a 'nanomachine.' We aimed at the torque, as generated by ATP hydrolysis in F_1, and delivered to its natural recipient, namely the rotor of the ion motor, F_O. Knowing that F_1 progresses in three steps, we expected a smooth, angular progression at F_O if there was an elastic power transmission, and a stepped one if it was rigid. A typical actin filament, 3 μm long, is visco-elastically too strongly damped to sense any stepping when moving in water, because its relaxation time is some 100 ms (Cherepanov and Junge 2001). When driven by the rotary enzyme, however, the filament becomes deformed, and by its curvature it can be used as a spring balance to calibrate the torque. Dima Cherepanov worked out the viscoelastic theory (Cherepanov and Junge 2001) and Oliver Pänke led the experiments (Pänke et al. 2001) to determine the average torque and the angular torque profile as provided at the output. We found an average torque of 50 pN nm. This was greater than values (20–40 pN nm) inferred previously from the rate of rotation, and based on the debatable assumption that the filament feels the viscous drag of *bulk water* (Yasuda et al. 1998). A filament of length 3 μm and radius 5 nm, moving at a height of 20 nm, over the solid support is not only subject to friction by direct encounter with the support, but, even when moving in the fluid, it feels a greater viscosity because of flow-coupling with the adjacent surface. In contrast to previous claims of '100%-efficiency' (Yasuda et al. 1998; Kinosita et al. 2000; Elston et al. 1998; Wang and Oster 1998), which related the lower torque to unrealistically low free energies of ATP hydrolysis in their experiments, our greater figure of 50 pN nm ($50 \, pN \, nm \times (2\pi/3) \times N_A = 63 \, kJ \, mol^{-1}$) matched the phosphate potential in these experiments, 64 kJ mol^{-1}. The estimated '100%-efficiency' of F_1F_O is kind of trivial, because the filament slows the enzyme a 1000-fold, almost to stalling point where, essentially, it operates close to thermodynamic equilibrium (Junge et al. 2001). The efficiency of the enzyme is, of course,

lower if it free-wheels without external load, being subject to kinetic control.

The angular profile of the torque *at the output* was most interesting. Although F_1 is a three-stepper when running freely, the torque profile under load, and measured at F_O, is rather flat, with a weak periodicity of three per round (Pänke et al. 2001). Theoretical considerations reveal that the smoothing can be understood in terms of an elastic power transmission. If the two motors work against each other, as under physiological conditions, the enzyme turnover becomes faster as the internal torsional rigidity of the power transmission becomes smaller. In summary, the elastic transmission is responsible for the kinetic competence of the enzyme under load, and its robustness to structural modifications (Cherepanov and Junge 2001; Junge et al. 2001; Pänke et al. 2001).

Up to this point, the enzyme seems to be described sufficiently for undergraduate textbooks. What has remained open is the detailed chemistry in the nucleotide binding pockets (see Senior et al. 2002), the mechanical throughput from the swinging lever on the β-subunits into this pocket, and the details of the proton motor; in short, the nanoscopic physics of this twin-motor-enzyme. Recent progress in its description (in nanoseconds) by molecular dynamics (MD) is encouraging. It has remained a major challenge for the future to bridge the time gaps between MD-simulations over nanoseconds, protein domain motion in microseconds, and enzyme turnover in milliseconds. The mechanism of ATP synthase will remain a major topic of biophysics for the decade to come. There is no other motor protein which compares with ATP synthase by complexity of function yet simple construction, and by fair accessibility to genetical, biochemical, kinetical and structural analysis.

Acknowledgments

Figure 9. A 1998 photograph of Govindjee (center) in one of his favored activities, bringing people together, here Yosepha Shahak, Tel Aviv, Israel (right) and the author.

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