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Energy Transduction in the Functional Membrane of Photosynthesis
Results by Pulse Spectroscopic Methods

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1. *Methods*

The functional membrane of photosynthesis performs molecular events which are also realized in membranes of photoreceptors, mitochondria, nerves and muscles. These events are light quanta phenomena, electron transfer, electrical field generation, ion translocation, ATP synthesis and hydrolysis respectively. Therefore photosynthesis provides a good example for studying principles of molecular dynamics and energetics in biomembranes in general.

The molecular events in photosynthesis are in action between the beginning of light absorption and the end of the overall process which is terminated by the production of NADPH and ATP. With NADPH and ATP absorbed CO_2 can be reduced into sugar and "everything else".[1]

Valuable information on the molecular events in photosynthesis has been obtained by *optical indication* of the events and by excitation and registration of the indicated events with the *repetitive pulse spectroscopic method.*[2]

In this way three improvements have been realized:

(a) The extremely high sensitivity of the repetitive techniques permits an analysis at conditions of *one single turnover* of the molecular machinery. In this way the interrelationships between the different events became most transparent.

(b) The time resolution of the repetitive technique exceeds the hitherto range by six orders of magnitude and works down to 10 *nanoseconds*. Thereby the analysis is open to all phases of the events between excited singlet states and the formation of the endproducts.

(c) The special optical indications made it possible to extend the analysis—formerly most restricted to redox-reactions—to *six other events.*

After a short introduction to the types of events which have been measured the further presentation is focussed to the electrically energized membrane and to the mechanism of phosphorylation. Subsequently the results are discussed in respect to the current hypothesis of bioenergetics. The report is restricted to results by pulse spectroscopic techniques.

Details on the methods are published in ref. 2. Details on the results are outlined in ref. 3. Because this report is an abstract of what is presented in ref. 3, the reader may be referred in respect of the detailed literature to ref. 3.

2. *Events*

In toto eight events have been analysed in the above explained way.

1. *Metastable states* of carotenoids
2. *Light reactions* of chlorophylls
3. *Redox reactions* during the transfer of electrons.

These three events are indicated—as *in vitro*—by intrinsic absorption changes.

4. 1966—an analysis of the mechanism of ATP generation was opened with the observation of specific absorption changes which are coupled with a *high energy state* of phosphorylation[4]:

1967—these spectral changes have been identified as indicating an *electrical field* across a membrane.[5] A first report on this result has been published in ref. 6. The mechanism of the field indication occurs by electrochromism (shift of absorptbaion nds in a field), see refs. 7 and 7a.

This interpretation has been proved by different independent lines of evidences (see ref. 3):

(i) by the observation that all bulk pigments within the membrane response optically to the event;
(ii) by the shape of the spectral changes which are typical for being induced by a field;
(iii) by the sensitivity of these changes to artificial ion translocators;
(iv) by agreement of this spectrum with that induced by fields on artificial pigment layers;
(v) by artificially embedded dyes as probes for the field.

An electrical field has recently been established also across the inner membrane of photobacteria.[7b]

The field indicating absorption changes ΔA can be used as a molecular voltmeter which can be calibrated in electrical units[8]:

$$\Delta\phi \approx 50\ \text{mV}.\Delta A/{}^{1}\Delta A$$

$\Delta A/{}^{1}\Delta A$ = absorption changes in relation to that produced in a saturated single turnover flash.

5. The field drives ions across the membrane. These *ion fluxes i* change the extent of the field in time. Therefore the time course of the field indicating absorption changes can be used as a molecular ammeter:

$$i \approx 1\ \mu\text{F} \,.\, 50\ \text{mV} \,.\, \text{d}\Delta A/\text{d}t \,.\, 1/{}^{1}\Delta A$$

6. *Proton transfer* ΔH^+ across membranes has been measured in the *outer* phase by artificial indicators. During the transfer of H^+ into the inner phase the indicator is deprotonated. This can be measured in the case of the indicator umbelliferon (UBF) by a change from a non-fluorescing into a fluorescing state of UBF.[9]

7. *Proton accumulation* H^+_{in} in the inner phase has been measured by an intrinsic indicator for H^+_{in} changes. As such an indicator the response of Chlorophyll-a_I-700 has been used.[10]

8. *Phosphorylation* has been measured by the indicator UBF, too. During the ATP generation H^+ is consumed and can be measured by the irreversible fluorescence changes of UBF.[11]

Some results and relationships which have been evaluated between the cited eight events are the following.

3. *Electrically Energized Membrane*

(a) Within one electron transport chain two *light reaction* centres have been spectroscopically observed which drive the transport of electrons from H_2O to $NADP^+$. These centres are an excited Chlorophyll-a_I-700[12] and Chlorophyll-a_{II}-680.[13] These centres have been recognized spectroscopically as coupled in series.[14, 15, 16] This has been proposed in ref. 16a.

(b) The analysis of the *electron transfers* Δe gave inter alia evidence that a pool of plastoquinone PQ ($\sim$ 10 PQ) is the link between the two light centres.[17] Some 100 of these arrangements are covering the surface of one thylakoid. The light driven electron transfer within this network from H_2O to $NADP^+$ converts light energy in form of the reducing power of NADPH.

(c) The observed *electrical potential change* $\Delta\phi$ indicates a primary electron transfer perpendicular to a membrane (charge separation with $\boxed{-}$ outside and $\boxed{+}$ inside).[5]

The generation of $\Delta\phi$ takes place in <20 ns simultaneously with the photoact.[18]

This leads to the idea that the electrically charged membrane ($\frac{1}{2} \,.\, C\Delta\phi^2$) is a further state in which light energy is converted.

(d) The electrical potential change $\Delta\phi$ has been measured as to be proportional to the amount of *moved electrons* Δe[19, 20]:

$$\Delta e \sim \Delta\phi$$

In a single turn-over flash it is $^1\Delta\phi \approx 50$ mV and in a steady-state light $\Delta\phi_{ss} \approx 100$ mV.

(e) The amount of *inward translocated protons* ΔH^+ which is not balanced by counter ion movement is proportional to the previously set up potential change (exchange of $\boxed{-}$ against OH^-, and $\boxed{+}$ against H^+)[8, 20]:

$$\Delta\phi \sim \Delta H^+$$

The amount of inward translocated protons ΔH^+ which is balanced by counter ion movement give rise at steady state light conditions and $pH_{out} = 8$ to a maximal change in the inner phase of the membrane from pH_{in} 8 to pH_{in} 5,[10] i.e. to a *chemical potential gradient* of

$$\Delta pH_{ss} \approx 3$$

(f) For the $\Delta\phi$ generation and ΔH^+ translocation two *light generators hν* per electron chain have been identified[8]:

$$h\nu_I \sim \tfrac{1}{2}\Delta\phi \sim \tfrac{1}{2}\Delta H^+ \qquad h\nu_{II} \sim \tfrac{1}{2}\Delta\phi \sim \tfrac{1}{2}\Delta H^+$$

This supports in an independent way the concept of two light centres reported in (a).

(g) The *discharging* of the electrically energized membrane occurs by a field driven ion flux i ($i \sim \Delta\phi$). Because the $\Delta\phi$ decay is accelerated 20-fold with the increase from pH_{in} 8 to pH_{in} 5 in the inner phase of the membrane it is assumed that the discharging occurs predominantly by field driven *proton* effluxes[21]:

$$\Delta\dot{\phi} = \Delta\dot{\phi}_{H^+} = f(H^+_{in})$$

(h) Only one artificial ion translocator molecule as gramicidine GMCD per 10^5 chlorophyll molecules works already as a shunt for the intrinsic decay. About 10^5 chlorophyll molecules cover an area of about $(5000\ Å)^2$. This area has the same order of size as that of one thylakoid. Therefore it is proposed that the *functional unit* of the electrical events is the membrane of 1 thylakoid[5]:

$$\Delta\phi\text{-unit} = 1\ \text{Thylakoid}$$

4. *Phosphorylation*

For the investigation of phosphorylation the system has firstly been restricted to electrical events by excluding the energetical involvement of ΔpH. This is possible because in a single turnover flash the produced potential change is $\Delta\phi \approx 50$ mV but the pH gradient only $\Delta pH \ll 0{\cdot}1$.

(a) Under the described conditions ATP generation takes place, i.e. phosphorylation is possible in the presence of an electrical potential difference only, without an energetical contribution of a pH gradient.

Whether $\Delta\phi$ and the thereby energized membrane is necessary for

ATP generation or whether $\Delta\phi$ is only a parallel event is answered by the following results.

(b) The extent of the potential $\Delta\phi$ can be changed from ${}^{1}\Delta\phi \approx 50$ mV up to $\Delta\phi_{max} \approx 200$ mV by using longer flashes. The *yield of ATP* per flash increases thereby linearly with the potential change[11]:*

$$\Delta\phi \sim \text{ATP}$$

(c) Under phosphorylation conditions the rate of $\Delta\phi$ decay is accelerated 2-5-fold. From this and the following results it is supposed that phosphorylation is coupled to a field driven proton flux chanelled through a special *ATPase pathway* within the membrane.[22]

(d) According to the interpretation in (c) it is expected that the rate of phosphorylation should be increased when the field driven proton flux is increased. In fact the *rate of ATP* generation increases 20-fold when the intrinsic $\Delta\phi$ decay is accelerated 20-fold by a change from pH_{in} 8 to pH_{in} 5,[23] i.e.

$$\dot{\Delta\phi}_{H^+} \sim \dot{\text{ATP}}$$

(e) From the result in (d) it is evident that phosphorylation is predominantly coupled to electrically driven *proton* effluxes across a ATPase pathway.

(f) The number of H^+ which passes across an ATPase coupled pathway can be read out from the difference in the decay of $\Delta\phi$ with and without phosphorylation (see (c)), because according to section 2.5 the absolute value of ion fluxes can be measured. For the *stoichiometry* H^+:ATP it results that the generation of one ATP is coupled to three field driven H^+[24]:

$$3H^+ \sim 1\text{ATP}$$

(g) A consequence of these results is that *deactivation* of phosphorylation should take place as soon as the intrinsic field driven H^+ flux is outrun by e.g. alcaline ions. This has been proved by addition of alcaline translocators as valinomycine (VMC) [24] and gramicidine (GMCD)[23]:

$$\dot{\Delta\phi}_{K^+} \rightarrow \dot{\text{ATP}} = 0$$

(h) The results in (b–g) indicate that obviously the discharging of the electrically energized membrane by protons is coupled with the generation of ATP. This is lastly strongly supported by the result that when only one artificial ion translocator GMCD per one thylakoid acts as a shunt for the intrinsic discharging of the membrane (see section 3 (h)), phosphorylation is deactivated, too. The *function unit* of phosphorylation is therefore based by the field on one thylakoid[25]:

$$\text{ATP-unit} = 1\ \text{Thylakoid}$$

* A nonlinear dependence has been reported in ref. 24. This deviation is discussed in ref. 3.

5. *Conclusions*

The results are in principle consistent with the electrochemical hypothesis of Mitchell.[26] However the initial generation of the electrical potential by an orientated shift of 2 electrons and the stoichiometry of $3H^+$ per 1 ATP are different from his original concept.

The demonstrated coupling between the electrical events and ATP generation can be written in a short hand drive as

$$\Delta e \rightleftarrows \Delta\phi \rightleftarrows \text{ATP}$$

Two other hypotheses on the coupling between electron transfer and ATP-generation—the chemical[27] and conformational[28] hypotheses—can be written briefly as

$$(\Delta e \rightleftarrows [\sim] \rightleftarrows \text{ATP})$$

The squiggle [~] represents an unknown chemical intermediate in which energy is conserved as covalent bond energy or it represents conformational changes which conserve energy in the folding of numerous protein molecules. The dotted arrows symbolize the subsequent physical and chemical steps by which ATP is synthesized.

The reported results support the acceptance of a coupling of electron transfer and phosphorylation by $\Delta\phi$. The coupling by a squiggle is clearly in contradiction to our results.*

One can argue that the demonstrated coupling of $\Delta\phi$ with phosphorylation takes place from a side path in which $\Delta\phi$ is equilibrated with a squiggle in the mainpath:

$$\begin{array}{c}(\Delta e \rightleftarrows [\sim] \rightleftarrows \text{ATP}) \\ \uparrow\downarrow \\ \Delta\phi\end{array}$$

The as yet available data do, however, also not support this modified squiggle phosphorylation:

(i) The squiggle has as yet never been shown to exist.

(ii) Phosphorylation should be possible without $\Delta\phi$ in suspension without closed vesicles. Treatments in this direction were as yet without success.

(iii) Peculiar properties of the squiggle have to be assumed to explain the observed stoichiometry of $3H^+$ per 1 ATP.

(iv) Generation of the squiggle as well as the subsequent equilibration with $\Delta\phi$ must occur in <20 ns because $\Delta\phi$ generation has been measured to take place in this time (see above). The synthesis of the squiggle must be therefore faster than any electron transfer event apart from that during the photo act. This is a very improbable assumption.

* $\Delta\phi$ can of course induce conformational changes which in turn may support the synthesis of ATP. This step is a result of charging and therefore different from the conformational change hypothesis.

In permanent light H^+_{in} is dumped up and it results at steady state conditions a gradient of $\Delta pH_{ss} \approx 3$ (see above). Evidence for an energetical contribution of a pH gradient to ATP generation was first demonstrated by Jagendorf *et al.*[29] Further detailed proofs have been given in refs. 30 to 33. Also at steady-state conditions a stoichiometry of $3H^+$ per 1 ATP has been observed.[31] This value satisfies, together with $\Delta\phi_{ss} \approx 100$ mV and $\Delta pH_{ss} \approx 3$, the energetical requirement for phosphorylation.[3]

Extending the electrochemical concept for ΔpH contribution it would follow analog to the arguments at single turnover conditions:

$$\Delta e \rightleftarrows (\Delta\phi, \Delta pH) \rightleftarrows ATP$$

The presented lines of evidences for a electrochemical coupling of electron transfer and ATP generation will need further elaboration. For instance the linear dependence of ATP formation with $\Delta\phi$ and the stoichiometry of $3H^+$ per ATP has to be motivated. This is in close connection with the open question for the chemical steps (symbolized by dotted arrows) by which ATP is synthesized.

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