J. Membrane Biol. 82, 113–122 (1984)

The Journal of
Membrane Biology

Bluelight-Induced, Flavin-Mediated Transport of Redox Equivalents across Artificial Bilayer Membranes

Werner Schmidt
Privatdozent für Biophysik, Universität Konstanz, Fakultät für Biologie, D-7750 Konstanz 1, West Germany

Summary. This paper continues our studies of physico-chemical properties of vesicle-bound flavins. Based on previous results, an advanced model system was designed in order to study the mechanisms underlying bluelight-induced redox transport across artificial membranes. The lumen of single-shelled vesicles was charged with cytochrome *c*, and amphiphilic flavin (AF1 3, AF1 10) was bound to the membrane. Upon bluelight irradiation redox equivalents are translocated from exogeneous $1e^-$(EDTA)- and $2e^-$(BH_3CN^-) donors across the membrane finally reducing the trapped cytochrome *c* both under aerobic and anaerobic conditions. The mechanisms involved are explored and evidence for the involvement of various redox states of oxygen, dihydroflavin and flavosemiquinone is presented.

Key Words single-shelled vesicle · membrane redox transport, light-induced · bluelight effect · flavosemiquinone · amphiphilic flavin · superoxide

Introduction

The present paper continues our series on vesicle-associated flavins [43, 48, 51], dealing with the bluelight-induced, flavin-mediated transport of redox equivalents across vesicle membranes. The basic goal of our studies is the elucidation of the primary reaction(s) of the ubiquitous and diverse physiological bluelight responses. These include phototropism of grass coleoptiles, chloroplast rearrangement, shift in biological rhythms, or the enhancement of respiration. Most of these phemomena appear to follow a surprisingly uniform primary reaction mode: (i) the photoreceptor pigment is a flavin, (ii) the photoreceptor is bound to membranous portions of the cell, often the plasmamembrane, (iii) the primary reaction is a redox reaction. For details refer to one of the recent reviews on bluelight physiology [44, 52, 53]. By analogy to better understood primary reactions in photobiology (*cf.* review by Berns [2]) we assume that the photoreceptor relaxation is succeeded by a proton-, an electrical- or a redox gradient, or even by the translocation of small molecules across the membrane. These reactions appear to be physiologically highly efficient transduction steps, being generally adopted by nature. So far they have been substantiated for most photoreceptor molecules which have been purified and subsequently reconstituted with artificial membranes. These systems include phytochrome [14, 28], rhodopsin [34, 37, 61], bacteriorhodopsin [17, 40], stentorin [54, 55] and chlorophyll [5, 11]. More artificial pigment/membrane systems have been described as well [4, 7, 8, 18, 19, 20, 23, 24, 26, 35, 59]. However, due to the ubiquitous appearance of flavins, so far the bluelight receptor flavin has escaped clear-cut identification, purification, and as a result, reconstitution.

As a minimum model system for the bluelight receptor three different amphiphilic flavins bearing C_{18}-hydrocarbon chains at positions 3, 7 and 10 have been synthesized (AF1 3, 7, 10) [31] and anchored within artificial, single-shelled vesicles made from natural and synthetic phospholipids [48]. On this basis we hope to mimic the steric and mechanistic restrictions imposed by the apoprotein and/or the membrane in the natural photoreceptor.

A completely artificial isotropic electron transport chain driven by bluelight via a flavin moiety had been explored by us previously [50]. In includes EDTA, flavin, oxygen (optionally) and cyt *c*. In addition, the mechanisms of cyt *c* photoreduction [63, 64] and cyt *c* binding [36] under isotropic conditions are well understood. On these bases an advanced model system was designed in order to study bluelight-mediated transport of redox equivalents (and protons, work in progress) across vesicle bilayers. Single-shelled vesicles were prepared with cyt *c* trapped in their aqueous lumen as potential electron acceptor, and amphiphilic flavins bound to the vesicle membrane as photoreceptor pigment.

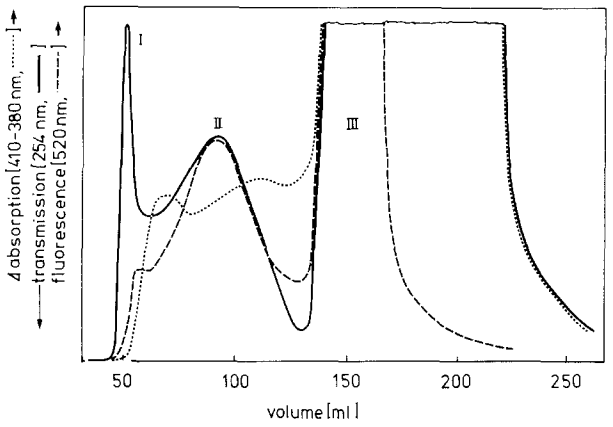

Fig. 1. Elution profiles measured on an individual sonicated egg lecithin/AF1 3/cyt *c* suspension of 5 ml on a Sepharose 4B column. The following entities were monitored: "transmission," i.e. essentially scattering at 254 nm (solid line), flavin content by fluorescence (excitation at 450 nm, dashed line) and cyt *c* content by absorption at the Soret band (dotted line). The three fractions indicated had been previously identified [48] as I: multilamellar layers, II: single-shelled vesicles, and III: micelles and solubilized molecules

EDTA or BH_3CN^- as potential one or two electron donors for the membrane-bound flavin were added to the bulk medium.

This model for the physiological bluelight receptor is completely analogous to the models which were designed to study the reactions of plastoquinone in photosynthetic electron transport of chloroplasts [18, 21], or the ubiquinone-mediated electron transport in mitochondria [18, 19, 20, 23, 38]. From the chemical standpoint the amphiphilic flavins used in our experiments can be regarded as quinones. In addition, flavins themselves are known to be involved in membrane-associated redox reactions: (i) It is commonly accepted that a flavoprotein functions as a transmembrane hydrogen carrier for the first coupling site of the respiratory chain [33]. (ii) In methanogenic bacteria the FAD-specific enzyme D-amino oxidase is membrane-bound [30]. Recent evidence suggests its role in generating transmembrane ion gradients which, in turn, are utilized for ATP-production and methanogenesis (*cf.* [30] for further literature). (iii) A specific flavoprotein/cytochrome *b* complex has been recently isolated from a plasmamembrane fraction purified from coleoptiles of dark-grown *Zea mays L.* [15], which is suggested to be the bluelight photoreceptor for phototropism in these organs. (iv) Nitrate reductase, a complex containing FAD, cyt b_{557} and molybdenum is bound to the plasmalemma of *Neurospora crassa L.* and serves as photoreceptor for a physiological bluelight response [29].

Abbreviations: DML: L-β,γ dimyristoyl-α-lecithin; AF1 3: 7,8,10-trimetyl-3-octadecyl-isoalloxazin; AF1 10: 3,7,8-trimetyl-10-octadecyl-isoalloxazin; EDTA: ethylenediaminetetraacetic acid; SOD: superoxide dismutase; $O_2^-\cdot$: superoxide; cyt *c*: cytochrome *c*; FAD: flavin adenine dinucleotide; FMN: flavinmononucleotide

Materials and Methods

Chemical

A detailed description of the synthesis of amphiphilic flavins has been published previously [31]. FMN was obtained as a gift from Hoffmann La Roche, Basel. The phospholipid L-β,γ dimyristoyl-α-lecithin (DML, N 42803) was purchased from Fluka, Buchs, Neu-Ulm, egg lecithin (P 4139) and superoxide dismutase (S 8254, from bovin blood) from Sigma, München. Ethylenediaminetetraacetic acid (0.1 M Titriplex III, 8431 or 8418), cholesterol (Art 24622) and cyt *c* (Art 24804) were obtained from Merck, Darmstadt; sodiumcyanoborohydride (15.615-9) from EGA-Chemie, Steinheim. The preparation of flavin-loaded single-shelled vesicles has been described in detail previously [48]. For our experiments on membrane transport of redox equivalents with cyt *c* trapped in the vesicle lumen the procedure was modified as follows. Five ml of phosphate buffer (0.01 M, pH 8., 0.1 M NaCl) were saturated with cyt *c*, prior to application to the dry lipid-film preceding sonication. After sonication for a defined period of time in a thermostat-controlled water bath at specific temperatures under argon [48] the suspension was applied to a Sepharose 4B column and progress of the elution was monitored. Figure 1 shows the elution profile (i) essentially based on scattering at 254 nm as measured with an LKB-Uvicord (solid line), (ii) on flavin (AF1 3) fluorescence as measured fluorimetrically (excitation at 450 nm, emission at 520 nm; dashed line), and (iii) on the absorption of cyt *c* (absorption difference between 410 and 380 nm; dotted line). The three profiles differ significantly, indicating different loading properties for cyt *c* and flavin (*vide infra*). The three fractions exhibited by the solid line have been identified previously [48] as (I) multilamellar layers, (II) single-shelled vesicles, and (III) a mixture of lipid micelles and free, solubilized pigment molecules. No cyt *c* and only little AF1 3 coelutes with fraction I (compared to AF1 3, AF1 10 reflects increased binding to fraction I, elution profile not shown). About 10^{-3} times the originally inserted cyt *c* is recovered in fraction II, which was used exclusively for the experiments reported here (collecting the elution volume approximately between 70 and 120 ml yielded material sufficient to perform 20 to 25 individual measurements). Cytochrome *c* is completely solubilized in the vesicle lumen [39]. Vesicles were used immediately after preparation since storage results in progressive formation of fration I, i.e. conglomeration, especially at lower temperatures [43].

Based on a diameter of 250 Å, a vesicle comprises approximately 8000 lipid molecules. Estimating a lipid recovery of maximal 50% of the originally inserted 50 μM [*cf.* 43], fraction II yields about 10^{15} vesicles. With a cyt *c* concentration of 3 μM of the bulk solution and a measured cyt *c* trapping efficiency of vesicles of 10^{-3}, 10^{13} cyt *c* molecules will coelute with fraction II. From this estimate we conclude that at best 1% of the vesicles recovered in fraction II contain one cyt *c* molecule. Because of geometrical reasons, no vesicle will contain more; either it contains one cyt *c* molecule, or none, regardless of its size, i.e. the elution volume. This is the reason for the little pronunciated peak

of fraction II in terms of cyt *c* absorption (Fig. 1, dotted line). Unfortunately there is no simple way to remove the empty vesicles which therefore have to be regarded as "spectroscopical ballast" (scattering).

The precise composition of actual vesicle suspensions (fraction II) in routine preparations cannot be strictly controlled [43, 46, 48], in contrast to the composition of the mixture prior to sonication and chromatography, containing 50 M of lipid and 0.5 M of amphiphilic flavin. As a consequence, the results are presented, compared and normalized on a relative basis (%) rather than in absolute units (which are not very informative if dealing with turbid, highly scattering samples). The effective concentration of cyt *c* and flavin is as low as 10^{-7} and 10^{-6} M, respectively. The observed absorption changes are typically in the range of $\Delta A = 0.001$. This requires specific spectrophotometric apparatuses. Figures represent averages of normalized measurements based on 3 to 5 individual vesicle preparations (Figs. 2, 3, 4, 5, 7). Points in Fig. 6 (temperature dependence) represent the average of 10 measurements.

Spectroscopy

Absorption was measured with a homemade single-beam [47] and homemade dual wavelength spectrophotometer [45] capable of detecting small absorbancies of highly scattering material. Fluorescence was measured with a computerized JY 3 CI spectrofluorimeter (Jobin Yvon) [49], or with a homemade fluorimeter [45]. Photoreduction of cyt *c* or flavin was induced either with a 150 W xenon lamp (ORIEL 6253), yielding blue light of 100 W/m² through a 365 nm interference filter (exciting the S0 → S2 transition of flavin), or by a 100 W halogen lamp, yielding 100 W/m² broad blue light (glass filter, exciting the S0 → S1 transition). Calculations as required for the Hill plots, half-logarithmic diagrams, normalization, etc., were performed with the Hewlett-Packard 9825 microcomputer, in connection with a HP 7225A Graphics Plotter. All measurements were performed at 21 ± 1°C, except for the measurements shown in Fig. 6 (temperature dependencies).

Results and Discussion

Photoreduction and Vesicle-Trapped cyt *c* Kinetics

The *isotropic* electron transport from EDTA via photoexcited flavin to cyt *c* takes place to the same extent, i.e. 100% photoreduction, but different quantum efficiencies both under aerobic and anaerobic conditions. The mechanisms involved have been elucidated previously [10, 50, 60]. On this basis a more advance model for the bluelight-induced, flavin-mediated electron transport chain comprising a membrane moiety has been designed. As shown in Fig. 3, redox equivalents are readily transferred across the vesicle membrane upon bluelight irradiation both under aerobic and anaerobic conditions. Transport of EDTA [48] or cyt *c* [62] are excluded. The efficiency of cyt *c* photoreduction is about 2 to 10% of that under isotropic conditions, depending

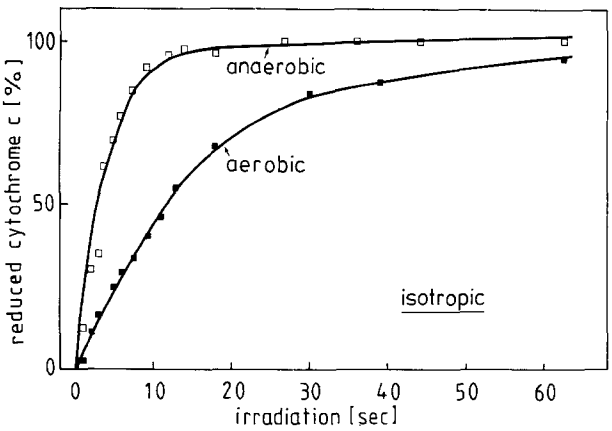

Fig. 2. Normalized isotropic photoreduction kinetics of cyt *c* (8×10^{-7} M) by EDTA (5×10^{-3} M) mediated by FMN (8×10^{-7} M). Both under aerobic and anaerobic conditions the cyt *c* will be completely reduced (as tested by addition of dithionite), even with quite different quantum efficiencies

on the specific flavin/membrane system used, and on the phase of the membrane (*vide infra;* Fig. 6*B*). In control experiments performed without membrane-bound flavins no light-induced electron transport is observed. In contrast to another report [56], ferrocytochrome *c* was not found to be autoxidizable by exogeneous oxygen.

In general, the photoreduction kinetics of the type shown in Fig. 3 do not follow a well-defined order. They rather appear to follow complex underlying electron pathways which have to be categorized by the term "facilitated diffusion" [25]. In one particular system (DML/AF1 10) we observed a strict (pseudo?) first-order reaction under aerobic but not under anaerobic conditions; in another system (lecithin/AF1 3) just the opposite occurs: a (pseudo?) first-order reaction under anaerobic but a more complex one under aerobic conditions (*curves not shown*). This indicates diverse mechanisms for the various systems, strongly dependent on the conditions used.

Inhibitor Experiments

Additional information was obtained from inhibitor experiments. For example, the photoreduction of cyt *c* trapped in DML/AF1 3 vesicles is considerably inhibited by SOD under aerobic conditions (Fig. 3*A*,*B*). This indicates the involvement of superoxide [9, 12, 32] as already established in the isotropic system [50] (*vide supra*). Since the flavin-nuclei are essentially localized in the membrane-water interface [43] and due to its moderate hydrophobicity [51], superoxide anions are presumably generated

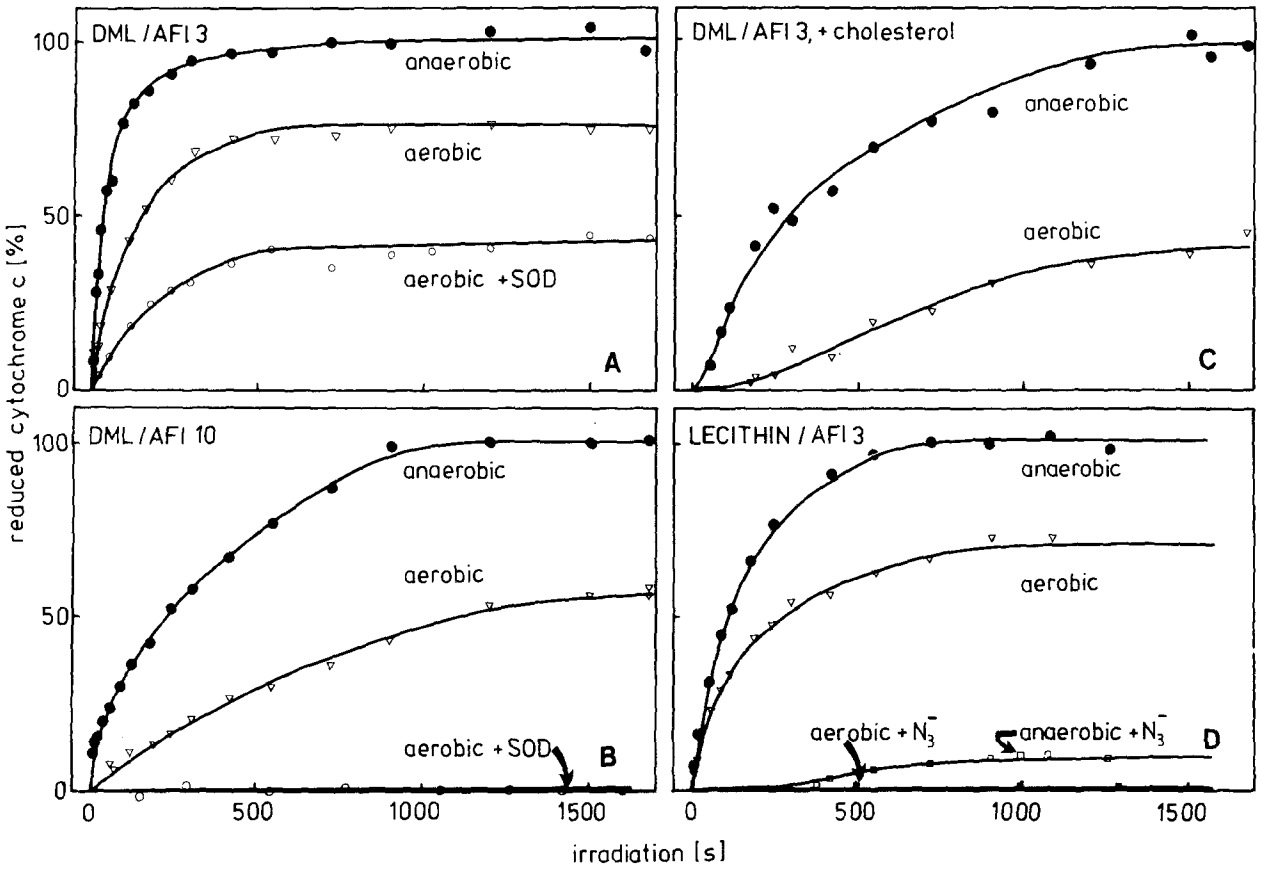

Fig. 3. Normalized photoreduction kinetics of cyt *c* trapped in various single-shelled vesicle/flavin systems by exogenous EDTA. *A.* DML/AFI 3. [EDTA] = 0.066 M; "anaerobic": 90-sec deaeration with Ar (*cf.* Figs. 4, 5*A*, optimum curves). 450 units of SOD significantly decrease the rate of photoreduction in the presence of oxygen. *B.* DML/AFI 10. [EDTA] = 0.033 M; "anaerobic": 90-sec deaeration with Ar (*cf.* Fig. 5*B*, optimum curve). 450 units of SOD abolish the aerobic photoreduction completely. *C.* Similar to (*A*), except that the membrane contains 30% cholesterol. *D.* Lecithin/AFI 3. [EDTA] = 0.167 M; "anaerobic": 90-sec deaeration with Ar. 0.05 M of sodium azide inhibit the membrane transport of redox equivalents completely under aerobic conditions, and by more than 90% in the absence of oxygen

here and easily accessed by the monitoring SOD, which will not intrude into the membrane's hydrophobic interior. Superoxide is sufficiently lipid-soluble and stable, particularly in hydrophobic environment, to cross the vesicle membrane within a few microseconds [9, 41, 42, 57]. Both, the flavin- and the O_2^-·-"selfcontact" are significantly reduced by the membrane [9, 47].

Moreover, azide is known as a highly efficient, even if not very specific quencher of radicals. Fig. 3*D* clearly demonstrates that in this specific instance (lecithin/AFI 3) the transmembrane redox transport is mediated by some radical species, in addition to superoxide. Under anaerobic conditions a minute portion of cyt *c* is reduced by a nonradical, i.e. azide-insensitive species. The inhibitor efficiency, again, depends on the systems and the conditions used (other details of inhibitor action *not shown*).

High concentrations of exogenous dithionite (0.1 M) are capable of reducing the vesicle-trapped cyt *c* completely in all vesicle systems investigated here, including those prepared from egg lecithin. This is in agreement with the general observation of effective reduction of cytochromes of various microorganisms *in vivo* by exogenous dithionite. It is also consistent with the finding by Futami et al. [13] which observed a small but significant dependence of permeability of soy bean lecithin vesicles to dithionite in a concentration-dependent manner, presumably in its neutral form [21].

In this fashion it could be demonstrated that even under anaerobic conditions only approximately 80% of the trapped cyt *c* was photoreduced. Therefore the asymptotic levels reached by the photoreduction kinetics (Fig. 3) reflect some redox equilibrium controlled by opposite tendencies: photoreduction of cyt *c* on one hand, and reoxidation

Table. Aerobic and anaerobic photoredox reactions of systems containing flavin, molecular oxygen (optionally), a suitable electron donor such as EDTA ($1e^-$-donor) or BH_3CN^- ($2e^-$-donor), and cyt c[a]

$$Fl_{ox} + 2H^+ + 2e^- \rightarrow Fl_{red}H_2$$
$$\underline{\underline{Fl_{red}H_2}} + \text{cyt } c\ (3+) \rightarrow FlH\cdot + \text{cyt } c\ (2+) + H^+$$
$$2\ \underline{\underline{FlH\cdot}} \rightarrow Fl_{red}H_2 + Fl_{ox}$$
$$\underline{\underline{Fl_{red}H_2}} + \underline{O_2} \rightarrow FlH\cdot + H^+ + O_2^-\cdot$$
$$\text{cyt } c\ (3+) + \underline{\underline{O_2^-\cdot}} \rightarrow \text{cyt } c\ (2+) + O_2$$
$$2\ O_2^-\cdot + 2\ \underline{\underline{H^+}} \rightarrow H_2O_2 + O_2$$
$$O_2^-\cdot + H_2O_2 \rightarrow OH^- + OH\cdot + O_2$$
$$\text{cyt } c\ (2+) + \tfrac{1}{2}H_2O_2 + H^+ \rightarrow \text{cyt } c\ (3+) + H_2O$$
$$^3Fl^*_{ox} + {}^3O_2 \rightarrow {}^1Fl_{ox} + {}^1O_2^*$$
$$^1O_2^* + \text{cyt } c\ (2+) \rightarrow O_2^-\cdot + \text{cyt } c\ (3+)$$
$$FlH\cdot + \text{cyt } c\ (3+) \rightarrow \text{cyt } c\ (2+) + Fl_{ox} + H^+$$

[a] Under *isotropic* conditions these reactions have been studied previously [9, 32, 50]. The present investigation deals with the membrane-bound (*anisotropic*) conditions. The species underlined are the most likely transmembrane redox carriers.

by hydrogen peroxide [*cf.* 9, 50], singlet oxygen [9], the hydroxyl radical and other compounds (*cf.* Table).

Both aerobic and anaerobic photoreduction of vesicle-trapped cyt *c* is markedly inhibited by cholesterol (Fig. 3*C*). This is in agreement with the generally observed decrease of permeability and fluidity of cholesterol-doped membranes [3, 6].

Dependence on Oxygen and Substrate Concentration

Two aspects of the involvement of molecular oxygen in transmembrane photoreduction of vesicle-trapped cyt *c* need to be discussed. The photoreduction efficiency of cyt *c* shows a clear-cut optimum curve upon progressive deaeration (Fig. 4*A*). This feature is generally found with vesicles made from *synthetic* phospholipids: critical, small amounts of oxygen promote the photochemical production of redox carriers. However, their effect is compensated for by increasing amounts of oxygen, which is a triplet in its ground state and therefore a highly efficient quencher of the flavin triplet (no spin restriction), with the result of a decreased photoreduction rate of cyt *c*. This quenching process by oxygen obviously does not interfere with the rate-limiting step of the electron transport chain in vesicles made from natural phospholipids (Fig. 4*B*).

Similar arguments apply for the dependence of cyt *c* photoreduction in DML/AFI 3 and DML/AFI 10 vesicles on the exogenous concentration of EDTA and BH_3CN^- (Fig. 5). The patterns obtained for one type of vesicles (i.e. same flavin and lipid)

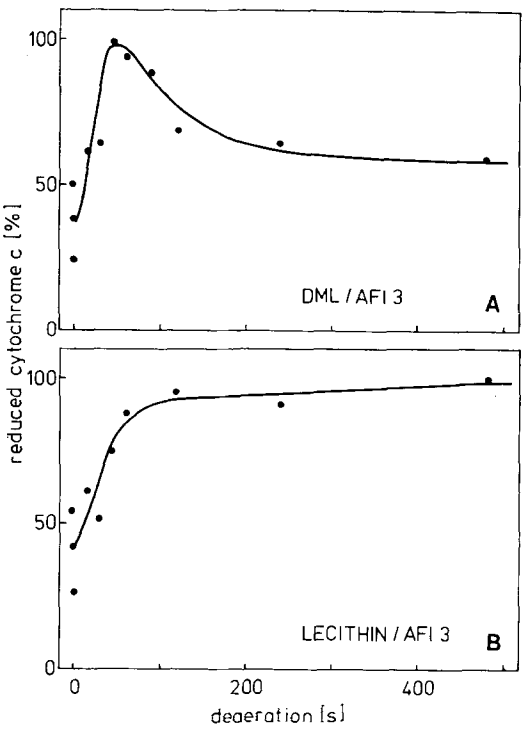

Fig. 4. Dependence of photoreduction efficiency of cyt *c* trapped in the lumen of vesicles on the partial oxygen pressure, as achieved by progressive bubbling of the sample with Ar. *A*. [EDTA] = 0.012 M (*cf.* Fig. 5*A*), 300 sec of irradiation (*cf.* Fig. 3*A*). B. [EDTA] = 0.035 M, 300 sec of irradiation

depend on the substrate used (*cf. A vs. D, B vs. E, C vs. F*), on the lipid (*cf. A vs. C, D vs. F*), or on the flavin used (*A vs. B, D vs. E*). This is taken to reflect different contributions of various mechanisms (Table, Fig. 8). Cytochrome *c* photoreduction in lecithin vesicles, again, does *not* show the feature of an optimum. As general tendency, BH_3CN^- as typical $2e^-$-donor for the excited flavin triplet exhibits a higher electron-donating capability in vesicle systems than the typical $1e^-$-donor EDTA, since maximal photoreduction efficiency is reached at lower concentrations. Cytochrome *c* photoreduction in the DML/AFI 3 system by BH_3CN^- (Fig. 5*D*) has been found to be more efficient under aerobic than under anaerobic conditions, and optimum curves are obtained.

The occurrence of optimum curves (Figs. 4*A*; 5*A*,*B*,*D*) indicates that the rate-limiting step in the electron transport chain is the electron transfer from the exogenous donor to the membrane-bound flavin. However, if a simply "trivial" saturation behavior (Figs. 4*B*; 5*A*, *B*, *C*, *E*, *F*, aerobic) is observed, secondary step(s) appear to be rate limiting, since quenching of the flavin triplet state by oxygen or an electron donor does not affect photoreduction efficiency. This interpretation is consistent with the

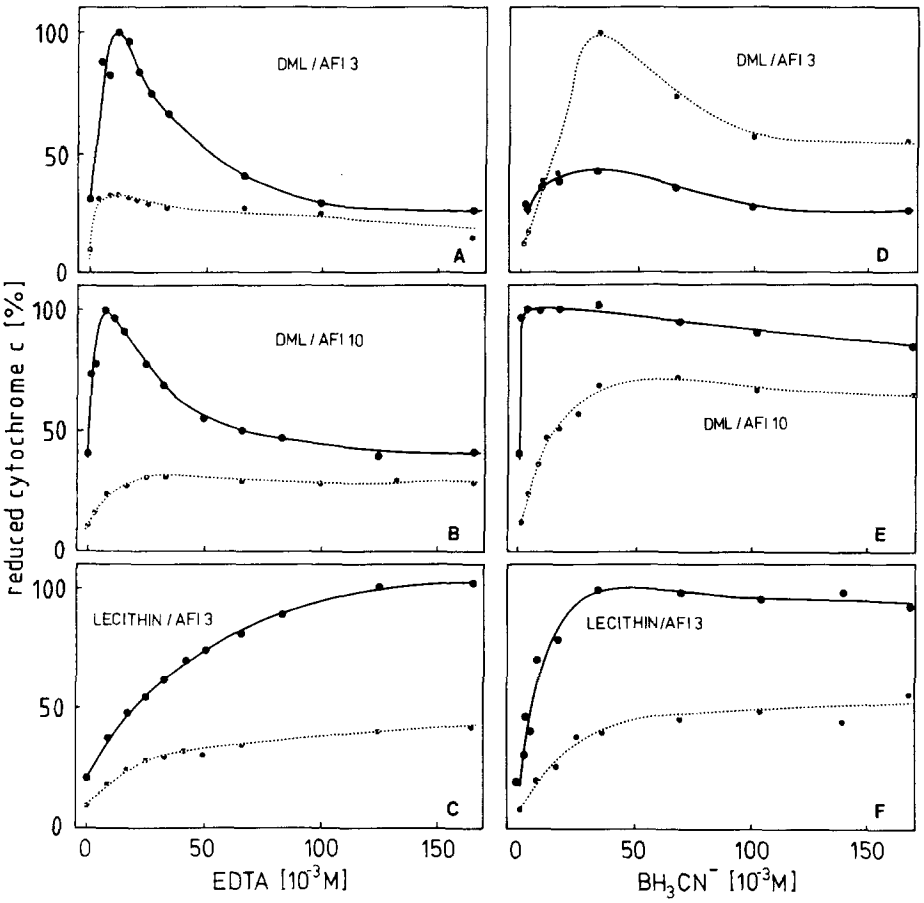

Fig. 5. Dependence of photoreduction efficiency of cyt *c* trapped in single-shelled vesicles as indicated, on the concentration of exogenous EDTA and BH_3CN^-. Dotted lines represent aerobic conditions, solid lines anaerobic conditions after 90 sec of deaeration with Ar (*cf.* Fig. 4, optimum curve). The reduction state of cyt *c* was measured after 300 sec of irradiation

finding mentioned above which suggests distinct rate-limiting steps under aerobic and anaerobic conditions. It is also consistent with the temperature-dependence of the anaerobic photoreduction of cyt *c* trapped in DML/AF1 3 vesicles (Fig. 6*B*, solid line). This reflects the temperature dependence of fluorescence, i.e. in principle the apparent lifetime of the flavin singlet and—through highly effective intersystem crossing of flavin (ϕ = 0.7; [16])—of the flavin triplet. Figure 6*A* shows the analogous comparison of isotropic cyt *c* photoreduction. Only beyond 30°C flavin photoreduction appears to be the rate-limiting step; below 30°C the rate is probably limited by diffusion of the redox partners.

Stoichiometry of Electrons and Light Quanta

For further elucidation of the mechanisms involved in transmembrane electron transport in flavin/vesicle systems, the stoichiometry of light quanta required per cyt *c* molecule reduced was determined (for a similar analysis *see* [65]). For this purpose various photoreduction kinetics of the type shown in Fig. 3 were replotted in Hill diagrams (Fig. 7), all reflecting remarkable linear patterns. In two instances (curves *A, F*) the highest Hill coefficient of n_H = 2.00 was obtained, indicating the requirement of two photons per cyt *c* molecule reduced. The smallest n_H-value of 1.00 was observed for the DML/AF1 3 system under anaerobic conditions (curve *D*). All other kinetics yielded intermediate Hill coefficients, indicating more complex electron pathways. This suggests that under *isotropic* conditions the flavin molecule is photoreduced by a single electron to the radical state [22, 60] which, in turn, disproportionates into the fully oxidized and reduced species (Table). The fully reduced flavin finally reduces a cyt *c* molecule by single electron transfer. On the other hand, in the DML/AF1 3 system under anaerobic conditions the disproportionation of flavin is partly suppressed due to the largely reduced flavin-flavin interaction [46]. Both flavosemiquinone and flavohydroquinone presumably penetrate the vesicle membrane and reduce the vesicle-trapped cyt *c* at the inner membrane/water interface, ending up in the fully oxidized or in the radical form. The redoxequivalent of the latter will be utilized in another cyt *c* reduction, with or with-

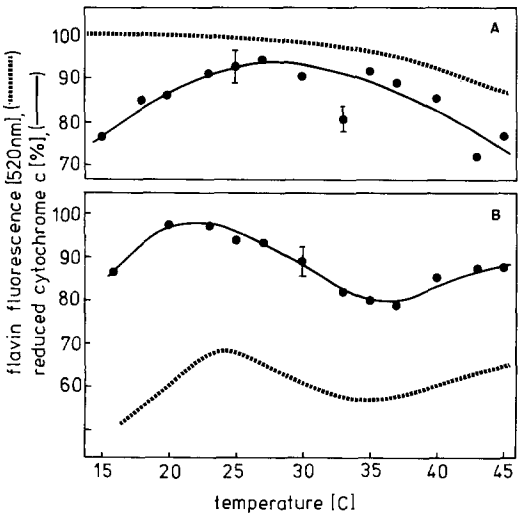

Fig. 6. Dependence of flavin fluorescence (dashed lines, data from [43]) and photoreduction efficiency of cyt *c* by EDTA, as mediated by flavins, on temperature. *A*. Isotropic conditions, [cyt *c*] = 8×10^{-7} M, [EDTA] = 5×10^{-3} M, [FMN] = 8×10^{-7} M. *B*. DML/AFl 3. Effective flavin concentration: 7.5×10^{-7} M, [EDTA] = 0.25 M, 90 sec of deaeration. The redox state of cyt *c* was measured after 300 sec of bluelight irradiation

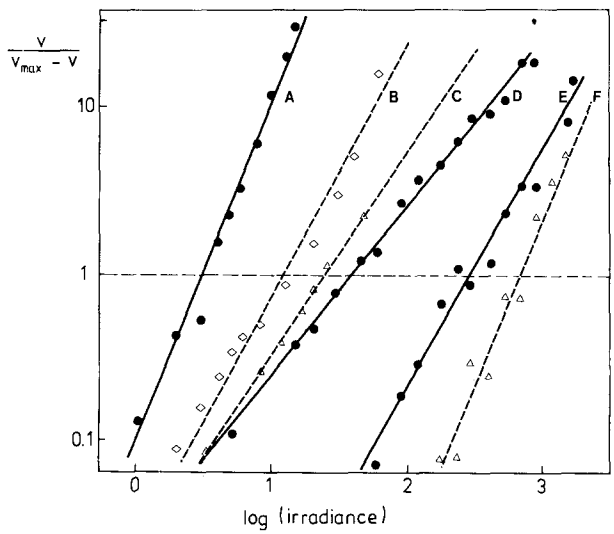

Fig. 7. Hill plots of photoreduction states *v* of cyt *c* as a function of relative number of light quanta needed: *A*: Isotropic; EDTA/FMN/cyt *c*, anaerobic, $n_H = 2.00$ (conditions as in Fig. 2). *B*: Similar to (*A*), but aerobic conditions, $n_H = 1.54$. *C*: cyt *c* trapped in DML/AF1 3 vesicles, aerobic, $n_H = 1.21$ (conditions as in Fig. 3*A*). *D*: Similar to (*C*), but anaerobic conditions, $n_H = 1.00$. *E*: Similar to (*D*), except that 30% cholesterol was incorporated in the membrane, $n_H = 1.42$. *F*: Similar to (*E*), but anaerobic conditions, $n_H = 2.00$

out a preceding disproportionation step involved. As a net result one photon is needed per cyt *c* molecule reduced.

Hinkle [23] was the first who described a completely artificial model system to study membrane transport of redox equivalents from ascrobate to ferricyanide, via the catalytic action of quinoid compounds (dark reactions!). This model has been advanced by Hauska and associates [13, 18–20]. According to these authors quinones are sufficiently lipid-soluble to account for a shuttle mechanism in which the substrate is oxidized on one side and the electron acceptor reduced on the other side of the membrane. Since flavin is a quinoid system and the fully reduced species about 200 times more soluble in the membrane than the oxidized form [*cf.* 1] a similar mechanism is predicted for the present model. Another electron transport system in membranes with an amphiphilic zink porphyrin a photosensitizer has been investigated by Katagi et al. [27]; however no possible mechanism is offered.

Conclusion and Outlook

Figure 8 summarizes the species and mechanisms probably involved in the translocation of redox equivalents across membranes, as mediated by flavins as photosensitizers and EDTA as electron donor, the particular reactions being listed in the Table. The underlined species are suggested as main transmembranous redox carriers. Their individual contribution is strongly dependent on the specific substrate/flavin/membrane system used, and on the particular conditions such as temperature or partial pressure of oxygen.

Upon light excitation the flavin nuclei located in the outer membrane-water interface pick up one electron from exogenous EDTA (analogous interpretation with BH_3CN^- as $2e^-$-donor). The resulting flavosemiquinone either disproportionates into the fully reduced and oxidized forms of flavin, or immediately penetrates the membrane carrying one redox equivalent to reduce a vesicle-trapped cyt *c* molecule. Dihydroflavin, as produced by the disproportionation process is also capable of crossing the membrane transferring two redox equivalents. This is supported by corresponding Hill diagrams, by results obtained with $1e^-$- and $2e^-$-donors and azide as radical quencher. Under aerobic conditions the interaction of dihydroflavin with molecular oxygen will generate the superoxide anion and flavosemiquinone. Superoxide, in turn, penetrates the membrane to reduce cyt *c* within the vesicle lumen. This is supported by the inhibitory effect of SOD. However, vesicle-trapped cyt *c* will not be reduced completely but rather reaches a redox equilibrium which is defined by the various reduction mechanisms described and the concentration of oxidizing reagents as listed in the Table.

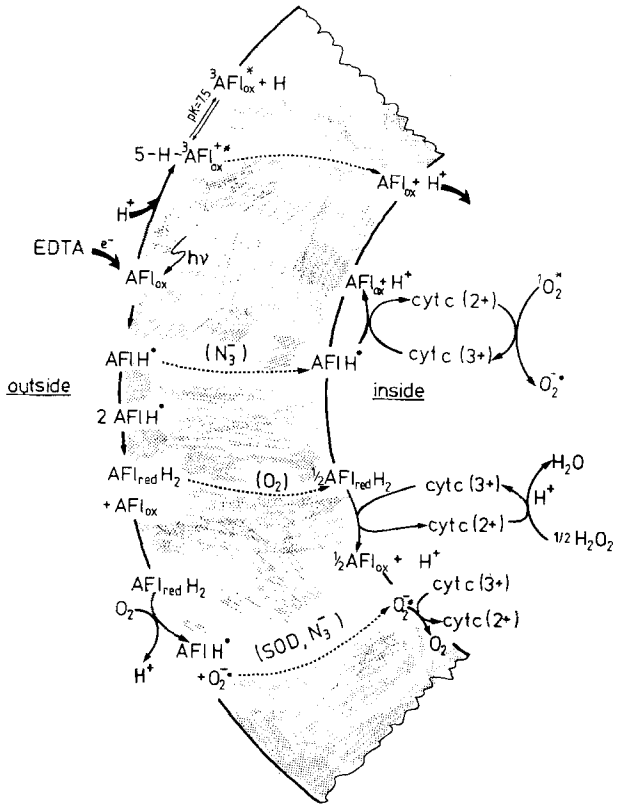

Fig. 8. Sketch of the mechanisms and species probably involved in bluelight-induced, flavin-mediated transport of redox equivalents across membranes of artificial lipid vesicles, as monitored by photoreduction of cyt *c*. The dashed lines indicate the transmembrane redox carriers suggested, with possible inhibitors in brackets. Based upon the strong increase of the N5-pK of flavin from 0 in the ground state to approximately neutral pH in the first excited triplet state under membrane-bound conditions, *cf.* [51], a possible proton transport mechanism is suggested in the upper part

Preliminary but highly significant results obtained with these systems demonstrate that bluelight is capable of inducing reversible pH- and potential gradients across the vesicle membrane. Taking the relatively high activation energies of about 40 kJ/M for these membrane-associated redox processes into account [48], the results presented exclude a purely electronic transmembranous pathway as suggested for membrane-bound carotenoids in photosynthetic membranes [2, 58, 59].

The immediate extrapolation of the interpretation of the results obtained with these model systems to physiological membranes as mentioned in the Introduction appears to be premature. However, some clues presented might be helpful to elucidate the physiological situation.

The author is indebted to Prof. Dr. H. Beinert for reading the manuscript and for many helpful suggestions. Miss G. Wenzl is thanked for excellent technical assistance. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 138, Pojekt A1: Membrane-fixierte Flavine).

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Received 5 March 1984; revised 7 June 1984