Biophys Struct Mech (1983) 10: 61–69

Biophysics of Structure and Mechanism

Clusterformation of 3-Methyl-Dihydroflavin with Ethylenediaminetetraacetic Acid

W. Schmidt and G. Wenzl

Universität Konstanz, Fakultät für Biologie,
Postfach 5560, D-7750 Konstanz 1, Federal Republic of Germany

Abstract. 3-methyl dihydroflavin (3-me-$Fl_{red}H_2$) and ethylenediaminetetraacetic acid (EDTA) aggregate spontaneously in aqueous solution to form clusters in a cooperative manner (*Hill* coefficient $n_H = 2.0$), exhibiting a typical critical micelle concentration (cmc). These clusters can be detected either by *Rayleigh*-scattering, or by a newly formed broad absorption band extending beyond 1,700 nm. Based on measurements of the temperature dependency, the free energy of cluster formation was determined to be as low as $\Delta G = -48$ KJ · mol^{-1}, suggesting the presence of 5–10 hydrogen bonds per cluster. The aggregation requires pH's below 6.5, which is assumed to reflect the N1-pK of 6.5 of free flavins: the anionic form of dihydroflavin prevents clustering. In addition, cluster formation is observed only, if a hydrophobic (methyl-)group is bound to the N3 of the flavin nucleus. This feature has not been observed previously and – as a model reaction – might bear some significance for the coenzyme-apoprotein interaction in flavoprotein catalysis in general.

Key words: Dihydroflavin – EDTA – Cluster formation – Rayleigh-scattering – Activation energy

Introduction

Oxidized flavin, i.e., flavoquinone, is the best known redox state of flavins, irrespective of its limited biological relevance compared to other flavin redox states. However, almost all known biological functions of flavin involve the fully reduced species, i.e., 1,5 dihydroflavin (flavohydroquinone), shuttling between Fl_{red} and $Fl^{\bullet}$ or between Fl_{red} and Fl_{ox} (Hemmerich 1976; Bruice 1976).

The roughly 100 flavoproteins known today are mainly concerned with three important areas of oxidative metabolism: (i) dehydrogenation, (ii) O_2-activation and (iii) single electron transfer (Massey and Hemmerich 1980). Some of them contain FMN (flavinmononucleotide), but most FAD (flavinadenindinucleo-

tide) as a highly anisotropic, tightly bound prosthetic group ($k_d < 10^{-8}$ M). Steric restrictions, as imposed by the apoprotein, are able to control the specific interactions with the flavin redox partners, giving rise to the various enzyme activities (e.g., Blankenhorn 1978; Watenpaugh et al. 1973).

Consequently, the study of the specific flavoenzyme (redox-) mechanisms demands a more detailed knowledge of flavin/protein interaction. Under this aspect, the "cluster effect" described below provides a valuable piece of information.

Materials and Methods

Chemicals

Lumiflavins and lumiflavin-3-acetic acid were kind gifts from Dr. P. Hemmerich, Konstanz.

Flavin mononucleotide (FMN) and 3-methyl-lumiflavin were obtained from Hoffmann La Roche. Flavin adenine dinucleotide (FAD, 24544 and D_2O (8514169) were obtained from Merck, Darmstadt. 3-methyl-lumiflavin was recrystallized from acetic acid/water (9 : 1), dried over KOH at 100° C, 1.3 Pa. The purity of all flavins was tested by TLC (2-butanone/chloroform/methanol = 3/4/6), and by determination of the extinction coefficient in water (ε_{450} = 12,200 $M^{-1} \cdot cm^{-1}$). Nitrilotriacetic acid (NTA, NI 068) was obtained from Schuchardt, München, ethylenediaminetetraacetic acid (EDTA) from Merck, Darmstadt, either solubilized (0.1 M Titriplex III, 8431) or in solid form (Titriplex III, 4818). Sodium oxalate (2579058) was purchased as well from Merck. pH's were adjusted as follows (0.01 M): pH 2/3, sulfate; pH 4/5, acetate; pH 6/7/8, phosphate; pH 9/10/11, borate; pH 12, phosphate. These suspension media are photochemically inert. Except as indicated otherwise, the experiments were performed at 20 ± 0.5° C.

Spectroscopy

Absorption spectra extending up to 1,700 nm (upper limit of the monochromator) were measured with a Cary 14; all other spectra (at room- and liquid nitrogen temperature) with a homemade, computerized single beam spectrophotometer (available wavelength range from 320 to 1,000 nm), and stored on magnetic tape (Schmidt 1982b). *Rayleigh*-scattering was measured with a JY3CI fluorimeter (Jobin Yvon), on-line with a Hewlett Packard 9825 A desk-top computer (Schmidt 1981): the excitation wavelength was set to 350 nm, emission was monitored from 330 to 370 nm, and the peak height was taken as scattering amplitude (cf. Fig. 3). The flavin was progressively photoreduced in the presence of EDTA (cf. Schmidt and Hemmerich 1981), NTA or oxalate, and its redox state assayed by the 450 nm absorbance (Fig. 1). The experiments were performed under anaerobic conditions, which were achieved by extensive deaeration of the sample with argon. If mechanical reproducibility was essential

as for the measurement shown in Fig. 1, the sample was kept in the thermostated cuvette holder, and irradiated successively by means of a light fiber (KL 1512, Schott and Gen, Mainz; caution was taken not to touch the cuvette; simply removing the cuvette and placing it back into the cuvette holder causes small offsets in optical density). This, in turn, was connected to a Prado Leitz projector (250 W, 24 V) with a water filter for heat absorption and a broad band blue glass filter in between, yielding a light intensity of 180 $W \cdot m^{-2}$.

Results

While studying the photoredoxreaction of vesicle-bound flavins (Schmidt and Hemmerich 1981), we observed a peculiar effect not reported before: At a relatively low concentration of 3-me-LFl (3.8×10^{-5} M) and EDTA (10^{-3} M) the usual absorption spectra of flavoquinone and flavohydroquinone are observed, with virtually no absorption above 520 nm (Fig. 1A). However, at [3-me-LFl] = 10^{-4} M, [EDTA] = 5×10^{-2} M and pH = 2. we observed the development of a broad, weak absorption band upon successive photoreduction, which was reversible upon oxygen admission (Fig. 1B). With the spectroscopic equipment available to us (Cary 14) we found still half the absorption of this band at 1,700 nm (ε = 1,200) left, compared to the absorption at 540 nm (ε = 2,460) (Fig. 2). A clearcut isosbestic point at 491 nm suggests that this newly developed extinction is essentially due to absorption rather than scattering. This is further supported both by the employed "end-on" photomultiplier arrangement (collecting 1,000 times more scattered light than with common double beam instruments; Schmidt and Hemmerich 1981; Schmidt 1982 b), and the liquid nitrogen temperature spectrum (Fig. 1C). At this temperature (the sample is not glassy but highly turbid) the extinction is largely due to scattering. However, after subtraction of the baseline (generated by the same volume of frozen buffer) the isosbestic point remains (even if hyposochromically shifted, as expected), as well as the absorption band.

Nevertheless, the development of this broad-banded absorption upon successive photoreduction is accompanied by increasing scattering capability, which again is reversible upon reoxidation (Fig. 3). This is taken to reflect aggregation of single molecules to larger particles ("clusters"). However, it proved impossible to determine the particle size simply by scattering data, since the well-known formula requires knowledge of the particle concentration (which must be determined by another procedure).

In the presence of a fixed concentration of EDTA (2×10^{-2} M, pH = 5), increasing the concentration of 3-me-LFl gives rise to increasing scattering capability (Fig. 4; due to the large effect, scattering is plotted as logarithm of the ratio of the scattering signals of the reduced/oxidized systems; the scattering of the oxidized form is low and largely independent of the flavin or EDTA concentration, and can therefore be used as the basis for normalization). A relatively strong isotope efect is observed in D_2O (Fig. 4, marked point, ΔG is 3% smaller than in H_2O).

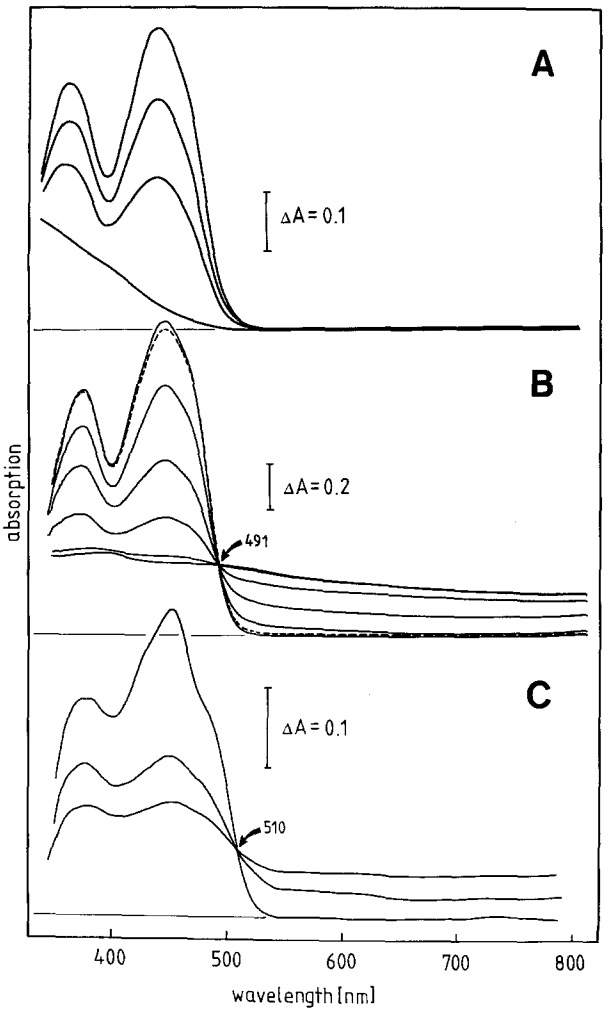

Fig. 1A–C. Absorption spectra of 3-me-lumiflavin in the presence of EDTA at various levels of anaerobic photoreduction. **A** [3-me-LFl] = 38×10^{-6} M; [EDTA] = 10^{-3} M; 20° C, pH = 8; lowest curve: fully reduced species. **B** [3-me-LFl] = 10^{-4} M; [EDTA] = 5×10^{-2} M; 20° C, pH = 2; lowest curve at 450 nm: fully reduced form. Dashed line: spectrum after reoxidation by admittance of air. **C** Same condition as in (**B**), except T = 196° C. After partial anaerobic photoreduction the sample was frozen to liquid nitrogen temperature thereby becoming highly turpid (due to handling, part of the flavin was reoxidized prior to reaching −196° C, lower curve at 450 nm)

A similar dependency of the scattering signal was determined as a function of the EDTA-concentration, keeping the 3-me LFl concentration fixed at 7×10^{-5} M, pH = 5 (Fig. 5). Even at vanishing EDTA-concentrations, in the reduced form of flavin there remains some excess scattering over the oxidized form. Plotting these data in a *Hill*-plot reveals a *Hill* coefficient as high as $n_H = 2.0$, reflecting significant cooperativity. This is in good agreement (i) with the replot of the scattering data of Fig. 3 (Fig. 6 B), and (ii) with the absorption data at 540

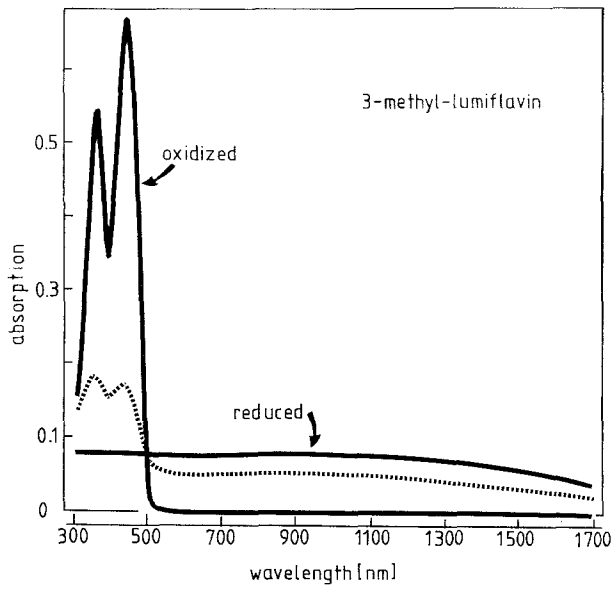

Fig. 2. Absorption spectrum of (3.8×10^{-5} M) 3-me-lumiflavin in the presence of EDTA (1.5×10^{-2} M) at various levels of anaerobic photoreduction; pH 5, 20° C, as measured with a Cary 14 spectrophotometer up to 1,700 nm (highest wavelength available)

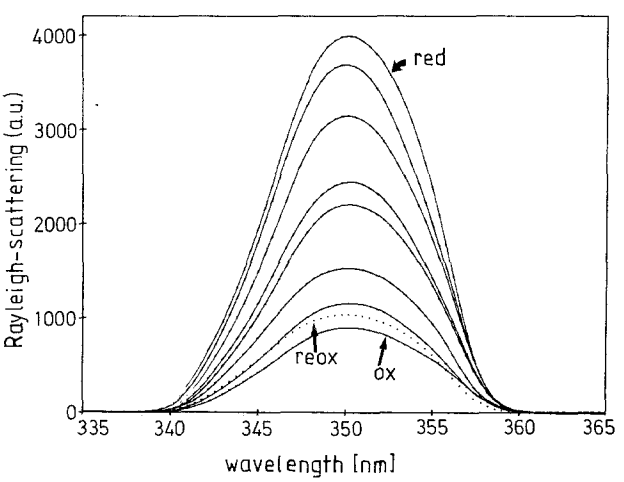

Fig. 3. *Rayleigh* scattering of a sample containing 5×10^{-5} M 3-me-LFl and 1.5×10^{-2} M EDTA, pH = 5, at various degrees of (anaerobic) photoreduction (for details see materials and methods). After reoxidation of the sample by admission of air the scattering attains nearly the original level (dashed line)

nm as in Fig. 1 (Fig. 6 A), as a function of the redox state of the flavin: In case of a non-cooperative cluster formation linear dependencies would result, as indicated by the dashed lines in Fig. 6. However, the actual data clearly reflect what can be termed "critical micelle concentration" (cmc), in a reversible

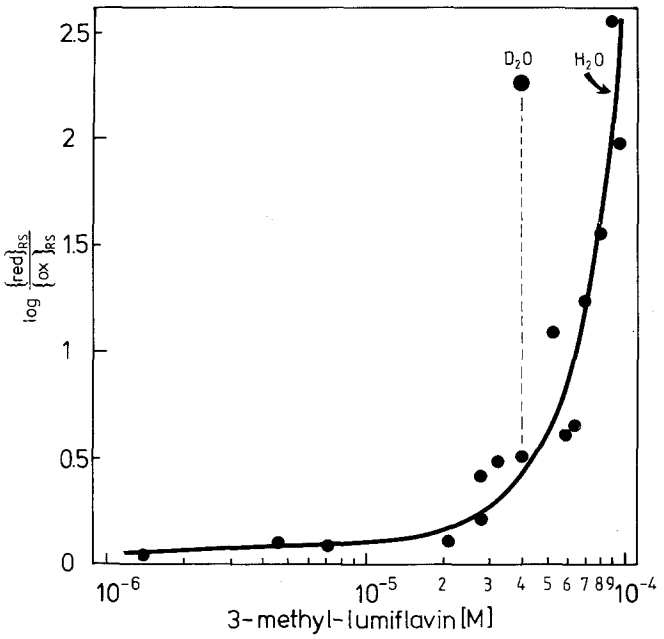

Fig. 4. Dependence of scattering (at 350 nm) of the reduced 3-me-LFl/EDTA system on (3-me-LFl) in H_2O; pH = 5, (EDTA) = 2×10^{-2} M. A relatively strong isotope effect is observed in D_2O (fat point). Due to the large effect, scattering is presented as logarithm of the ratio of the scattering amplitudes of reduced/oxidized forms. At highest *Rayleigh* scattering (factor 315 of red/ox) the sample still looks completely clear to the naked eye

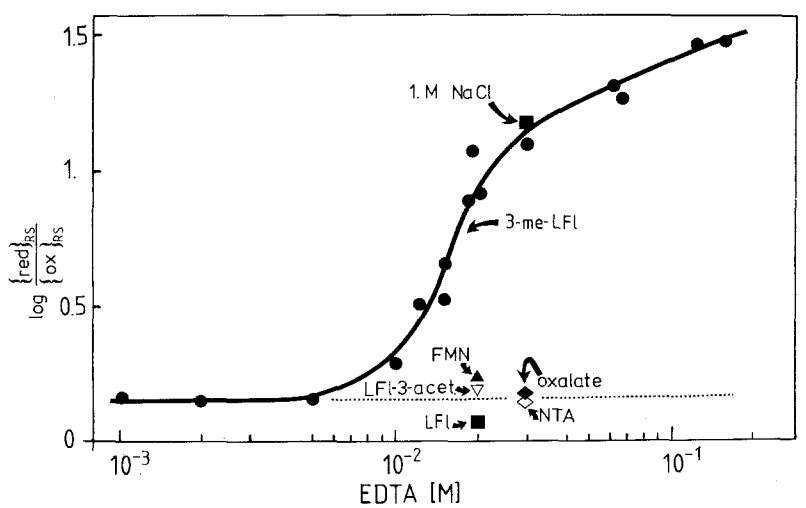

Fig. 5. Dependence of scattering (at 350 nm) of the reduced 3-me-LFl/EDTA system on EDTA (●), NTA (◇) and oxalate (◆), pH = 5. Lumiflavin-3-acetic acid, FMN and (3 H-) lumiflavin results in no clustering effect [(Fl) = 7×10^{-5} M]. 1 M NaCl (■) has no effect on the scattering amplitude of the 3-me-LFl/EDTA-System. A *Hill* plot of these data exhibits a *Hill* coefficient of $n_H = 2.$, representing significant cooperativity. Even in the absence of EDTA (or any other constituent electron donor) there remains excess scattering of the reduced over the oxidized system by a factor of 1.4, reflecting some aggregation of dihydroflavin by itself (cf. Massey and Palmer 1962)

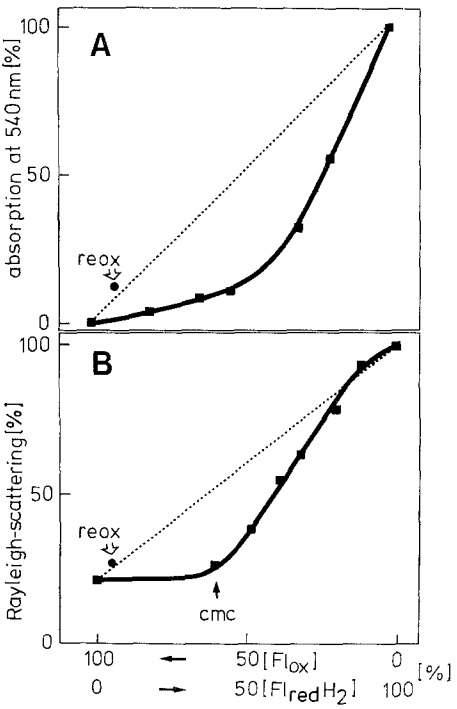

Fig. 6. A Absorbancy, **B** *Rayleigh* scattering (%) of the 3-me-LFl/EDTA complex as a function of the flavin redox state (%), (■), adjusted by successive photoreduction, pH = 5, 8×10^{-5} M flavin, 1.4×10^{-2} M EDTA. After reoxidation by air the marked points (●) are obtained. cmc: "critical micelle concentration". The dashed lines indicate the linear dependency expected for a non-cooperative aggregation mechanism

manner. Flavins bearing a hydrogen atom (LFl, FMN, FAD; 5×10^{-5} M) or a hydrophilic group at positon N3 (LFl-3-acetic acid) do not exhibit significant excess scattering in the reduced form. Similarly, NTA or oxalate (both relatively good electron donors for flavin triplets as is EDTA) have no effect. On the other hand, the cluster formation of 3-me-LFl and EDTA appears to be non-ionic, since it is completely independent of the ionic strength (1.M NaCl has no influence, Fig. 5).

The extent of scattering signal decreases sharply at pH's higher than 6.5 (7×10^{-5} M 3-me-LFl, 2×10^{-2} M EDTA; data not shown). Most likely this reflects the involvement of the N1-pK of 6.5 of dihydroflavin in the aggregation process (cf. Schmidt 1980).

Finally, the measured free energy of cluster formation of $\Delta G = -48.$ KJ · mol^{-1} is equivalent to only 5–10 hydrogen bonds per cluster (*Arrhenius* plot not shown).

Undoubtedly, light in the presence of EDTA as electron donor – under the exclusion of oxygen – is the cleanest procedure to reduce flavoquinone. In order to test for the possibility whether *photo*reduction is a prerequisite for the clustering process to occur, 3-me-LFl was reduced anaerobically with stoichiometric amounts of dithionite prior to the addition of EDTA in the "dark" (red safe light). Obviously, the aggregation of EDTA and 3-me-LFl took place, as well (results are not shown).

Discussion

It has been known for some decades that dihydroflavin is considerably less soluble in aqueous solution than flavoquinone (cf. Beinert 1960). This very characteristic of the "leuco form" has been utilized for the selective precipitation of riboflavin in the industrial production (Hickey 1946).

Dihydroflavin is by no means colorless (Beinert 1960), as usually implied. Concentrated solutions exhibit absorbance up to 560 nm, and an isosbestic point near 520 nm with flavoquinone (Massey and Palmer 1962) which gives them a reddish, color. However, this is only observed below pH ~ 6, i.e., with the neutral form of the molecule, not with the anion (quite similar to the clustering phenomenon described in this paper). Massey and Palmer (1962) and Gibson et al. (1962), explained this coloration of $FMNH_2$ in H_2O with the formation of sandwich polymers. However, since Dudley et al. (1964) observed a similar "endabsorption" in chloroform, this explanation appeared to be at least incomplete (Dudley et al. 1964).

However, we hesitate to adopt these concepts concerning the clustering phenomenon described here. The absorption of the 3-me-lumiflavin/EDTA clusters extends far beyond the hitherto known endabsorption of dihydroflavin (> 560 nm); moreover, EDTA as a binary component is indispensable for clustering. Nevertheless, particle size and long wavelength absorption appear to be concomitant (Fig. 6), i.e., the absorption is due to *inter*molecular interaction of flavin and EDTA, rather than a property intrinsic to flavin. In addition, the structure of EDTA appears to be essential, since NTA (having a very similar structure) shows no effect. The methyl group at N3 of the flavin appears to be essential, as well: Seeming inconsistent with these findings, flavins with a methyl group at N3 are generally *more* soluble in water (Hemmerich, personal communication).

Classical charge transfer (as known to occur between semiquinone and dihydroflavin or between dihydroflavin and flavoquinone as described earlier; cf. Gibson et al. 1962) also appears not to be a plausible explanation: The effect described here is maximal at the *fully* reduced state of flavin (not in the halfreduced state), and the presence of EDTA is prerequisite. What is the electron donor? What the acceptor? Why does only dihydroflavin exhibit such a strong clustering effect rather than the more electron-deficient oxidized form? Finally, the cluster contains more than two individual molecules, as required for a charge transfer complex.

At present we have no unequivocal explanation of this problem, but a clearcut effect remains, and surely reflects a structural (and mechanistic?) feature of a biologically important coenzyme: flavin.

There is overwhelming evidence that the ubiquitous bluelight photoreceptor responsible for many physiological light responses is a membrane-bound flavin (cf. Schmidt 1982a). Recently Hertel et al. (1980) demonstrated that riboflavin and a still unknown flavin termed "FX" is specifically bound to membranes derived from plasmalemma and endoplasmatic reticulum, especially in their reduced forms. On this basis they hypothesize that this redoxdependent interaction might be essential for the primary photoprocess. This feature and the

clustering effect described here might well reflect different aspects of the same phenomenon.

Acknowledgements. The authors wish to acknowledge the interest in this work and the helpful discussions of the late Professor Peter Hemmerich and to thank Professor Vincent Massey for critically reading the manuscript and valuable suggestions. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 138, Project A1).

Note added in proof
Most recently S. Ghisla in this laboratory obtained the final proof by *in vitro* synthesis that FX is identical with 5′-malonic acid riboflavin ester (Ghisla, personal communication)

References

Beinert H (1960) Flavin coenzymes: In: Boyer PD, Lardy H, Myrback K (eds) The enzymes, vol 2. Academic Press, New York London, pp 339–416

Blankenhorn G (1978) Riboflavin binding egg white flavoprotein: the role of tryptophan and tyrosin. Eur J Biochem 82: 155–160

Bruice RC (1976) Models and flavin catalysis. In: Kaiser ET, Kezdy FJ (eds) Progress in bioanorganic chemistry, vol 4. John Wiley & Sons, New York Sidney Toronto, pp 1–87

Dudley KH, Ehrenberg A, Hemmerich P, Müller F (1964) Spektren und Strukturen der am Flavin-Redox-System beteiligten Partikeln. Studien in der Flavinreihe IX. Helv Chim Acta 47: 1354–1383

Gibson QH, Massey V, Atherton NM (1962) The nature of compounds present in mixtures of oxidized and reduced flavin mononucleotides. Biochem J 85: 369–383

Hemmerich P (1976) The present status of flavin and flavin enzyme chemistry. Prog Chem Org Natural Products 33: 451–527

Hertel R, Jesaitis A, Dohrmann U, Briggs W (1980) In vitro binding of riboflavin to subcellular particles from maize coleoptiles and cucurbita hypocotyls. Planta 147: 312–319

Hickey RJ (1946) Precipitation of riboflavin from aqueous solution by bacteriological reduction. Arch Biochem 11: 259–267

Massey V, Hemmerich P (1980) Active site probes of flavoproteins. Biochem Soc Trans Biochem Rev 8: 247–261

Massey V, Palmer G (1962) Charge transfer complexes of lipoyl dehydrogenase and free flavins. J Biol Chemistry 237: 2347–2358

Schmidt W (1980) Artificial flavin/membrane systems: a possible model for physiological blue light action. In: Senger (ed) The blue light syndrome. Springer, Berlin Heidelberg New York

Schmidt W (1981) Fluorescence properties of isotropically and anisotropically embedded flavins. Photochem Photobiol 34: 7–16

Schmidt W (1982a) The physiology of blue light systems. In: Proc. of the 36th Symposium of the British Photobiol. Soc. held in Norwich, September 81, Cambridge University Press

Schmidt W (1982b) Computerized single beam spectrophotometer: An easy set-up. Anal Biochem 125: 162–167

Schmidt W, Hemmerich P (1981) On the redox reactions and accessibility of amphiphilic flavins in artificial membrane vesicles. J Membr Biol 60: 129–141

Watenpaugh KD, Sieker LC, Jensen LM (1973) The binding of riboflavin-5′-phosphate in a flavoprotein: flavodoxin at 2 Å resolution. Proc Natl Acad Sci USA 70: 3857–3860

Received May 19, 1982/Accepted December 22, 1982