Light-Absorption Studies on Neutral Flavin Radicals

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Quantitative light absorption data of the neutral free flavosemiquinone are reported. It is shown that the visible range of the spectrum of the radical is drastically dependent on the solvent. The spectra of the neutral blue flavosemiquinone are compared with those of the blue flavo-protein radicals and conclusions are drawn concerning the environment of the protein-bound flavosemiquinone.

The stabilization of the neutral blue flavosemiquinone through N(5)-alkylation and/or π-complex formation with aromatic compounds is described.

The light absorption characteristics of a tautomeric neutral, but red-coloured, flavosemiqui-none are described. The results are compared with those obtained from the free anionic and protein-bound red flavosemiquinones. The two types of radicals cannot be distinguished from each other by electron spin resonance spectrometry but may be recognized by means of light absorption spectrophotometry. The possible biological significance of the neutral red flavo-semiquinone is discussed. The optical properties of this radical are compared with those of free and protein-bound anionic flavosemiquinones.

A flavin solution is a thermodynamically freely reversible redox-system with amphoteric character. The radical concentration of an aqueous half-reduced flavin solution depends on the radical dispropor-tionation [1] and intermolecular association [2] equilibria which are governed by the acidities of the flavin species involved and by the polarity of the solvent employed. Thus, the actual flavin radical concentration at physiological pH values is 2—5% of the total flavin present [1]. An almost quantitative yield of flavin radical is obtained only at low pH values where all flavin species in the three redox states are cationic, the radical being the cation of lowest acidity [3].

Flavin radicals have been discovered by Michaelis et al. [4]. In 1956, Beinert [5] assigned the weak absorption maximum at about 565 nm of half-reduced aqueous flavin solution to the neutral radical species. However the above mentioned com-plexity of the half-reduced aqueous flavin system prevented the quantitative determination of the light absorption characteristics of the neutral and anionic flavosemiquinone. During the past few years we have worked out conditions to overcome these difficulties in employing either aprotic solvents to prevent intermolecular complexation and/or suitable alkylation of the flavin molecule to prevent disproportionation.

Thus we have reported on the submolecular structure of the anionic [1,6], neutral [7], cationic [8]

and metal-chelated [9] flavin free radicals as studied by electron spin resonance spectroscopy. Quantita-tive data on the light absorption characteristics of the anionic radical and its metal-chelated form have also been published [1,10]. However, no quantitative data concerning the light absorption of the neutral flavin radical have been published yet, except for some preliminary results [3].

Two classes of radicals have been observed with flavoproteins, namely a neutral and an anionic form [11]. This assignment has been confirmed by model studies [1,3]. The former radical exhibits a blue-purple colour in solution whereas the latter appears red.

In this paper we report quantitative data on the light absorption spectra of the neutral flavosemi-quinone and a tautomer thereof in order to establish a quantitative correlation between the free neutral flavin radical in environments of greatly varying polarity as compared to flavoprotein active sites.

MATERIALS AND METHODS

All solvents used were of reagent grade. Aqueous solutions were prepared using doubly distilled H_2O. N(5)-Alkylated 1,5-dihydroflavins were obtained by reductive alkylation of the specific flavoquinone as described previously [7,12] with the exception of N(5)-benzyl-1,5-dihydroflavin which was synthesized by the photocatalyzed phenylacetate method [13].

Light absorption spectra were recorded with a Cary 14 recording spectrophotometer. Anaerobic cells of 0.1, 1.0 or 4.0-cm light path were used.

The flavosemiquinone solutions were prepared either by autoxidation of the $N(5)$-alkyl-1,5-dihydroflavin solution as described earlier [3], or by dissolving the crystalline neutral radical (see below) in the desired solvent under anaerobic conditions. The neutral radical tautomer (Fig. 5) was prepared by catalytic hydrogenation (Pd/SiO$_2$) of the flavoquinonium salt in dimetylformamide or chloroform in the presence of a two-fold excess of triethylamine and mixing the reduced solution thus obtained anaerobically with one equivalent of oxidized starting solution [9]. The flavoquinonium salts were synthesized according to procedures published elsewhere [14].

The blue radical of D-amino acid oxidase was prepared in the following way: a solution of D-amino acid oxidase in 0.1 M pyrophosphate buffer pH 8.5, in the presence of an excess of EDTA was made anaerobic and illuminated as described previously [11] yielding the red flavoprotein radical. To the solution thus obtained a solution of 3-fluorobenzoate in the same buffer was added anaerobically. The final concentration of fluorobenzoate was 0.2 M. Upon addition of the fluorobenzoate the red radical solution immediately turned purple. The light absorption spectrum was then recorded. In contrast to the uncomplexed red radical the purple radical thus generated disproportionated slowly, due to the lower dissociation constant for benzoate binding to the oxidized enzyme than to the radical species.

The spectrum of the radical of *Azotobacter* flavoprotein was determined in the present work by irradiation for 6 days at 12 °C with a bank of 15-W incandescent lights, in the presence of 0.1 M phosphate and 0.1 M EDTA and 5 μM lumiflavin-3-acetate pH 7.0.

The electron spin resonance spectra were obtained with a Varian E3 spectrometer. Flat cells have been employed.

The experiments were conducted at ambient temperatures, if not otherwise stated.

Preparation of Crystalline Neutral
N(5)-Alkyl-monohydrolumiflavin

10 mg freshly prepared 3,5-dimethyl-1,5-dihydrolumiflavin [12], was suspended in 2 ml H$_2$O and two drops of conc. ammonia added yielding a clear solution which was filtered in the presence of S$_2$O$_4^{2-}$ to keep the flavin solution reduced. Then air was bubbled through the solution for 30 min giving a blue solution which was allowed to stand undisturbed in an ice bath for 4 h; the dark-blue crystals were then collected on a glass-sintered filter, washed with water and dried in vacuum at 50 °C for 3 h.

This procedure yielded 50°/$_0$ of $N(3,5)$dimethylmonohydrolumiflavin in bluish-black prisms.

Calculated for C$_{15}$H$_{17}$N$_4$O$_2$ · 0.75 H$_2$O (298.83): C 60.28, H 6.24, N 18.74°/$_0$. Found: C 60.51, H 5.90, N 18.49°/$_0$.

200 mg $N(3)$-methyl-$N(5$-)ethyl-1,5-dihydroflavin [12] was dissolved in 5 ml 1 N ammonia containing a trace of dithionite. This solution was treated with charcoal, filtered and the clear filtrate was collected in a 50-ml Erlenmeyer flask and gently shaken under exposure to air until a faint blue colour of the neutral radical indicated the beginning of autoxidation. Thereafter, the mouth of the flask was covered with a paraffin film allowing slow diffusion of oxygen into the solution which was kept at room temperature for 8 h.

The well-shaped blackish-blue crystals were then filtered, washed with water and dried at 0.01 torr and room temperature for 12 h yielding 120 mg (60°/$_0$) of the pure radical $N(3)$-methyl-$N(5)$-ethylmonohydro-lumiflavin dihydrate.

Calculated for C$_{16}$H$_{19}$N$_4$O$_2$ · 2 H$_2$O (335.35): C 57.30, H 6.91, N 16.71, H$_2$O 10.74°/$_0$. Found: C 57.55, H 6.87, N 16.70, H$_2$O 10.40°/$_0$.

RESULTS AND DISCUSSION

The light absorption spectra of an anaerobic solution of the crystalline $N(3)$-methyl-$N(5)$-ethyl-$N(5)$-monohydrolumiflavin in polar and non-polar solvents are shown in Fig. 1. The colour of the radical solution appears to be blue in aqueous medium and green in organic media (Fig. 1). The spectrum is not affected by variation of the pH in the range of 4.5—9.1. The shape of the spectrum of the ethanol solution is changed dramatically as compared to that of the aqueous solution. Thus, the first absorption band of the spectrum consists now of two distinct maxima, where the maximum at 630 nm is slightly more intense than that at 600 nm. On the other hand, the intensity of the absorption band at about 500 nm decreases sharply as compared to that of the aqueous solution and is about half as intense as the long wavelength band. By going to even less polar solvents a further bathochromic shift of the long wavelength band is observed but leaving the main features of the spectrum almost unchanged. Again the long wavelength maxima increase in intensity and the peak at 500 nm decreases. A similar solvent effect as observed for the long wavelength absorption bands occurs with the 357 nm band of the neutral radical (Fig. 1).

Replacement of the alkyl group at N(5) by other groups, *i.e.* methyl by benzyl, does not affect the pattern of the above described spectra. However, small shifts (2—5 mn) of the *maxima are observed* with the variation of the N(5)alkyl group.
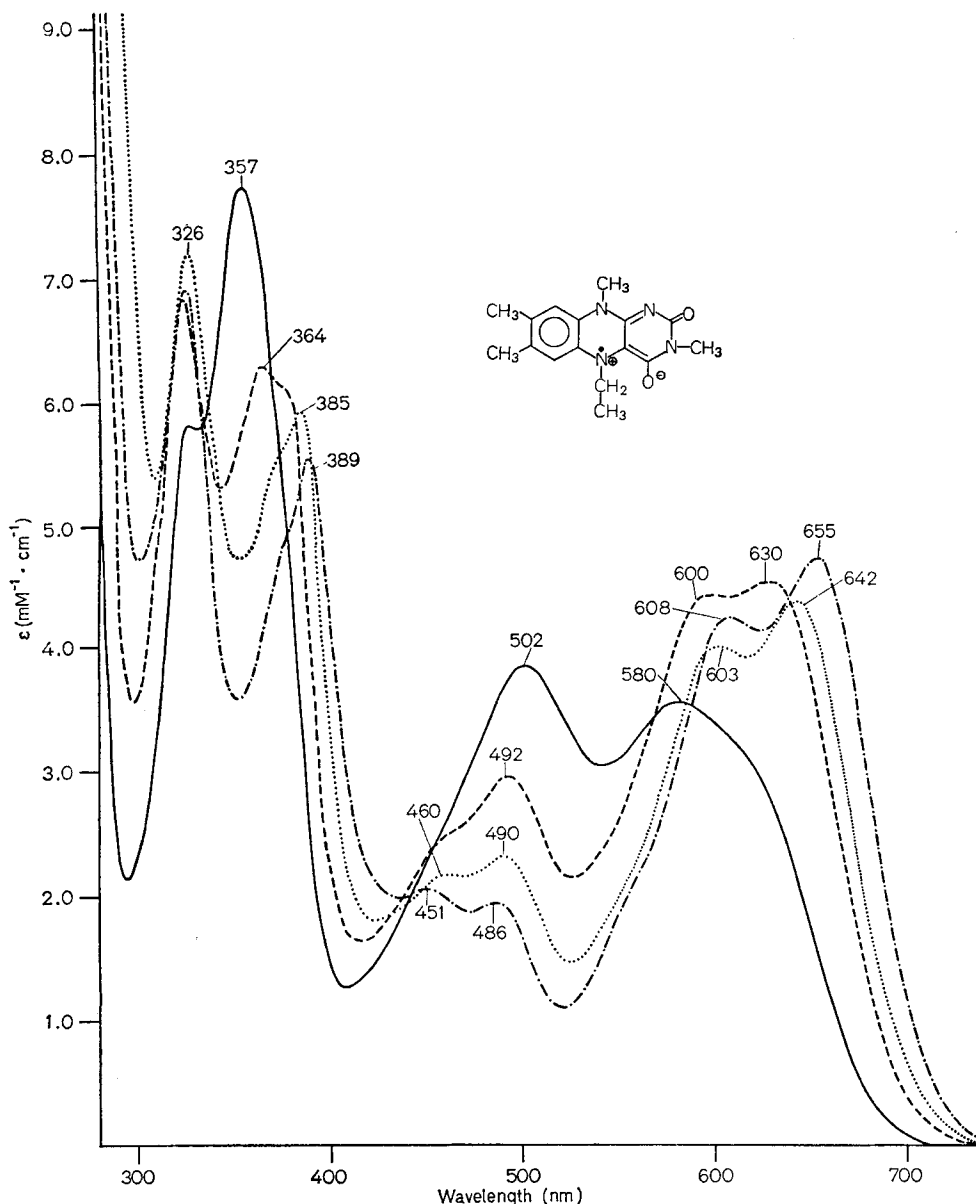
Fig.1. *Light-absorption spectra of* N(5)*-ethyl-*N(5)*-monohydrolumiflavin in various solvents.* A 1 mM stock solution of the crystalline radical was prepared anaerobically in acetonitrile. This solution was appropriately diluted under anaerobic conditions with 0.1 M acetate buffer pH 5.1 (———); ethanol (— — —); chloroform (······) and benzene (—·—·—). The organic solutions contained 5% triethylamine

Furthermore, a similar solvent effect as described above is also observed with N(5)-unsubstituted flavosemiquinones except that in the latter case the light absorption maxima are shifted hypsochromically about 20 nm [3] and the formation of the radical is never quantitative because of the unfavourable disproportionation equilibrium.

In the past few years, a great number of protein-bound flavosemiquinones have been obtained in quantitative yield [11], the radical being stabilized by the protein against disproportionation.

In Fig. 2 the light absorption spectra of the neutral radical forms of several flavoproteins are compared. Despite individual differences in absorption coefficients, it is remarkable how similar in form the spectra are, showing maxima in the region of 350 nm, 480—500 nm and 570—590 nm, with well-resolved shoulders at 340, 460 and 620 nm.
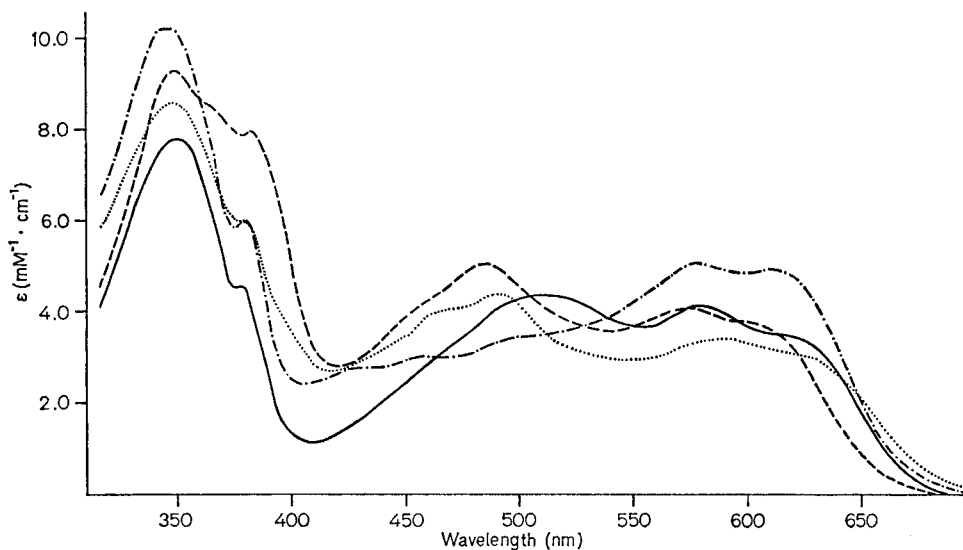
Fig.2. *Optical spectra of neutral-flavoprotein radicals, obtained by the EDTA-light-irradiation technique [11]*. The spectra of the flavodoxin and glucose oxidase radicals are taken from [22] and [24]. The spectra of the radicals of D-amino acid oxidase and *Azotobacter* flavoprotein were obtained as described in Materials and Methods. In all cases the spectra are uncorrected for any reduction beyond the semiquinone level; they represent the maximal development of long-wavelength absorption detected experimentally. –·–·–, *Azotobacter* flavoprotein; ––––, glucose oxidase; ······, D-amino acid oxidase + 3-fluorobenzoate; ——, flavodoxin

A comparison of the overall shape of these spectra with those of the model compound (Fig.1) reveals that the spectra of the neutral flavoprotein radicals resemble more that of the model compound in aqueous solution rather than in organic solvents.

As demonstrated above, N(5)-alkylation of the neutral free flavosemiquinone leads to the stabilization of the half-reduced flavin system, *i.e.* it prevents the disproportionation reaction ("σ-stabilization") [7]. A different stabilization ("π-stabilization") of the free flavin radical system is achieved by means of "stacked" complex formation between the radical and an aromatic compound. The pH dependence of the radical concentration, determined by electron spin resonance spectroscopy [1], of a half-reduced flavin solution in the presence of an aromatic compound revealed that the pK value of the neutral radical is not affected by this complexation.

Fig.3 demonstrates the influence of π-stabilization on the light absorption spectrum of a half-reduced FMN solution in the presence of 1 M $NaClO_4$. The radical yield is about $2.5^0/_0$ of the total flavin concentration present (Curve I). The spectrum consists, as first shown by Beinert [5], of a band centered at 570 nm, due to the neutral flavosemiquinone FlH and a broad infrared absorption characteristic of the flavoquinhydrone complex $[Fl_{red} H_2 \overset{\pi}{-} Fl_{ox}]$ [2]. Another half-reduced FMN solution (Curve II) of the same concentration and pH, but in the presence of 1 M sodium benzoate instead

of $NaClO_4$, gave a light absorption spectrum which suggests that the radical yield increased by a factor of about two and the amount of $[Fl_{red} H_2 \overset{\pi}{-} Fl_{ox}]$ decreased by the same factor. The absorption at shorter wavelengths is mainly due to the oxidized (Fl_{ox}) and fully reduced ($Fl_{red} H_2$) flavin species ($> 90^0/_0$ of total flavin present). Similar effects were recently demonstrated by means of potentiometry [15].

The shape of the FlR-spectrum as shown in Fig.1 is only slightly changed by benzoate. The complex formation is, however, confirmed by the different electron spin resonance hyperfine pattern (Fig.4). This demonstrates that the solvatochroic effect due to "out of flavin plane" water, is still present if one side of the radical is precluded by benzoate.

The red flavoprotein radicals are considered to be the anionic species [11]. This proposal was proved by chemical studies [1]. By this, however, we have observed an additional species which is red coloured in solution like the anionic radical. When all dissociable protons are replaced by alkyl groups, this species can under the given conditions exist only as a neutral radical. In an earlier study, we have established its radical character by electron spin resonance spectroscopy [7,9]. Accordingly, this radical represents the tautomeric form of the neutral blue-coloured flavosemiquinone. In Fig.5 are compared the anionic free radical, the red flavoprotein
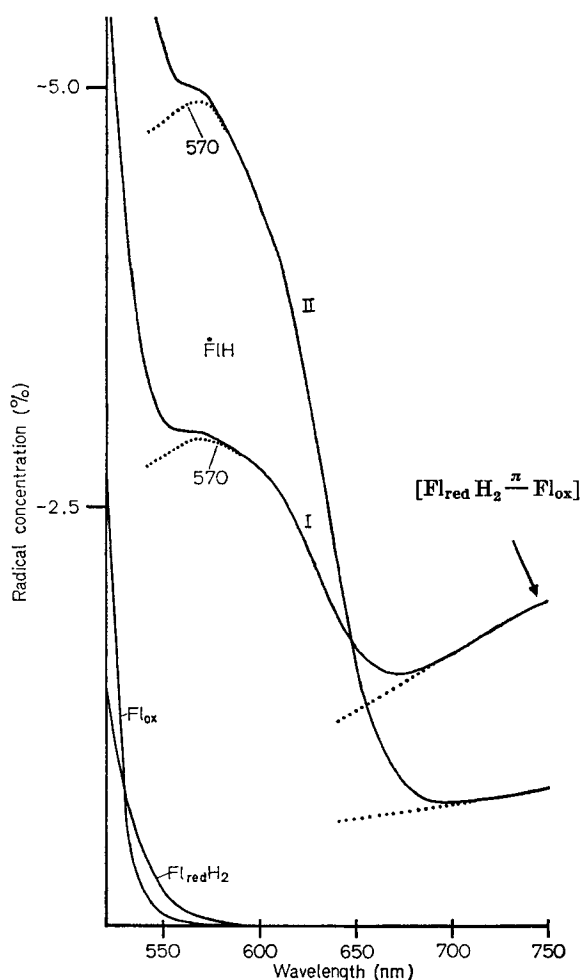
Fig. 3. *Comparison of the light-absorption spectra of a half-reduced 2 mM FMN solution in 0.1 M sodium citrate, pH 6.1.* Curve I: in the presence of 1 M sodium perchlorate. Curve II: in the absence of sodium perchlorate but in the presence of 1 M sodium benzoate. Also the long-wavelength absorption of the oxidized (Fl_{ox}) and the fully reduced ($Fl_{red}H_2$) solutions are given. The desired degree of reduction of the solutions was obtained by anaerobic addition of the appropriate amount of a 100 mM sodium dithionite solution in the same buffer and of the same pH value. The light path was in all cases 4.0 cm. The approximate radical concentration has been calculated by subtraction of half of the absorption of Fl_{ox} and $Fl_{red}H_2$ from that of the experimental curves. The values so obtained are indicated by the dotted lines

radical and the red neutral radical. The light absorption characteristics of the red flavoprotein and free anionic radicals are almost identical. The spectrum of the red neutral tautomer, on the other hand, also shows the characteristic long-wavelength absorption of the anionic radical extending beyond 530 nm, but the first maximum is hypsochromically shifted by 30 nm. The second band is located at 368 nm, *i.e.* in the same region as the corresponding absorption maximum of the anionic species.

In Table 1 we have summarized the spectral data of blue flavoprotein radicals as found in the literature. At first glance it appears that all blue coloured flavoprotein radicals resemble the model radicals in aqueous media. However, a closer examination reveals that the spectra of flavodoxin, glucose oxidase, thioredoxin reductase radicals as well as D-amino acid oxidase radicals in the presence of 3-fluorobenzoate resemble each other and are indeed very similar to the free flavosemiquine spectrum in aqueous solution. The azotobacter flavoprotein radical, on the other hand, resembles more the free flavosemiquinone in organic solutions (*cf.* Fig. 1 and 2), as indicated by the well-resolved double maximum in the 600-nm region and the shape of the spectrum in the 500-nm region. Also the intensity ratios are in close agreement with those obtained from the model compound in organic solvent, *i.e.* ethanol.

These facts indicate that the protein-bound neutral flavocoenzyme radicals of flavodoxin, thioredoxin reductase, glucose oxidase and D-amino acid oxidase are more exposed to the solvent H_2O than that of the *Azotobacter* flavoprotein. Furthermore, in agreement with our earlier suggestion [7] regarding the formation of a hydrogen bond between N(5)H and the apoprotein, the water molecule is in contact with the isoalloxazine ring either above or below but not within the plane of the flavin. This idea is supported by recent measurement of the proton relaxation rate of H_2O conducted with the blue radical of flavodoxin [16] and *Azotobacter* flavoprotein [17]. The concept of the ready H_2O-accessibility of the neutral radical species of all flavoproteins so far detected is further substantiated by the observations of exchange of the electron spin resonance active proton at N-5 by deuterium [18].

That the neutral flavosemiquinone interacts with the solvent H_2O in a specific way, is indicated by the drastic solvent dependence of the visible spectra. But this effect cannot be due to "in-plane" H-bonding at N(5), since 5-alkyl radicals show the same solvatochroic behavior as do 5-protonated analogues. The energy associated with this solvatochroic effect corresponds to an energy change of about 6 kcal/mol.

According to these data and if one accepts "resolved" spectra of oxidized flavoproteins as being due to hydrophobic environment [19,20], the one-electron reduction of flavoquinone seems to provoke a conformational change of the protein. This should be valid for the flavoprotein dehydrogenases, the flavodoxins, thioredoxin reductase and ferredoxin NADP reductase (and in a lesser degree for *Azotobacter* flavoprotein) which all show a resolved "hydrophobic" spectrum in the oxidized state and a purple "hydrophilic" spectrum in the radical state.
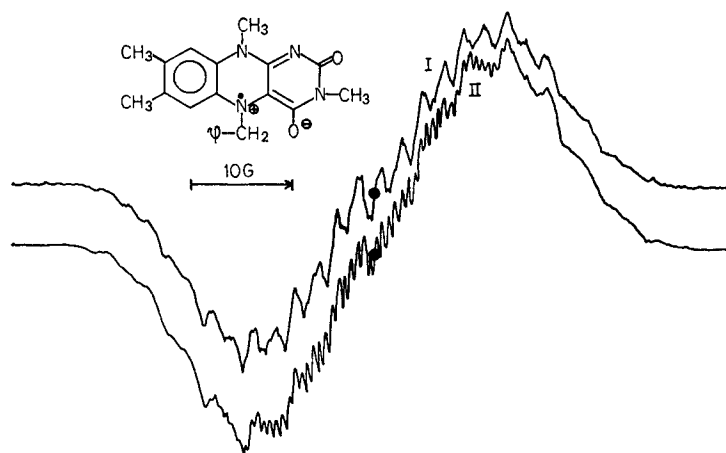
Fig. 4. *Electron-spin-resonance signals obtained from 3-methyl-5-benzyl-monohydrolumiflavin, prepared by air oxidation of the 1,5-monohydro-analog [13] in 50%* methanolic phosphate buffer pH 7 (I), in the absence and (II) in the presence of 1 M ammonium benzoate under the same conditions of measurement.* The additional superhyperfine splitting due to benzoate association is of the order of 1 gauss
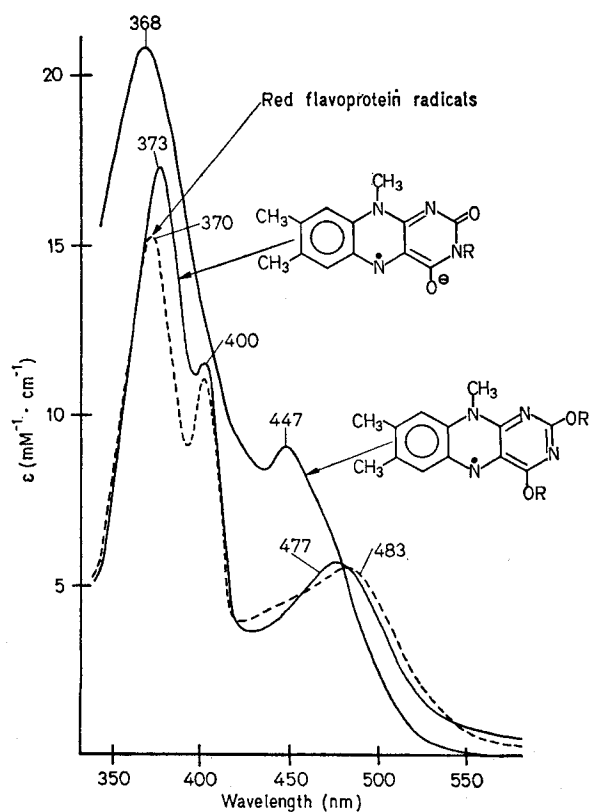

Fig. 5. *Comparison of the light-absorption spectra of the red protein-bound and free flavosemiquinones.* The spectrum of the red flavoprotein radical was taken from [11] and that of the anionic free flavosemiquinone from [1]. The spectrum of the neutral red flavosemiquinone was obtained as described in Materials and Methods

Finally, our data demonstrate that the light absorption spectrum of the neutral tautomeric red radical is rather similar to that of the anionic radical. Also the electron spin resonance spectra are practically identical [7,9]. These facts make it difficult, apart from the quantitative differences in their absorption coefficients at 450—480 nm, to distinguish the two types of radicals from each other. These red coloured neutral flavosemiquinones are in the free state stabilized only by appropriate alkylation of the flavin nucleus. A similar stabilization might be brought about by flavin binding to apoproteins which extend a stabilizing H-bridge towards N(1) instead of N(5). A possible example for the quantitative transformation of a blue neutral protein-bound flavosemiquinone into its red tautomer might be the observation of Ludwig et al. [21], that red-coloured radical crystals are obtained upon crystallization of the radical of clostridial flavodoxin, whereas in solution the radical is blue-purple in colour. The crystals obtained from our chemical model, however, do not seem to differ in colour from the saturated aqueous solution.

The data presented by the present authors on chemically or enzymatically stabilized flavin radicals should be compared with corresponding results on the transient radicals of unmodified free flavin, as studied by pulse radiolysis [28]. The excellent agreement points to the high utility of the latter method.

Note Added in Proof. The magnetic properties of monocrystalline blue neutral flavosemiquinone are presently under investigation by K. Maier and H. J. Keller, Institut für Anorganische Chemie, Universität Heidelberg, Germany.

Table 1. *The light-absorption characteristic of free and protein-bound neutral flavosemiquinones*

Compound	pH	λ_{max} (ε) nm (mM⁻¹ cm⁻¹)						References
Flavodoxin[a]	6.0	627[b] (3.8)	580 (4.50)	505 (4.70)		377 (5.5)	350 (7.65)	[22]
Thioredoxin reductase[a]	7.6	635 (4.36)	588 (4.6)	495 (4.95)	467 (4.6)	384 (4.6)	340 (8.5—8.7)	[23]
Glucose oxidase	5.80	610[b] (3.85)	570 (4.15)	485 (5.20)		382 (8.20)	350 (9.50)	[24]
Azotobacter flavoprotein	7.0	610 (4.92), 615 (5.10[c])	578 (5.10), 580 (5.44)[c]	505 (3.5)	460 (3.02)	380 (6.0)	345 (10.2)	this paper [25]
3-Methyl-FMN-Azotobacter flavoprotein	7.0	615 (4.15)	580 (4.25)	487 (3.52)	460 (3.25)	380 (5.80)	357 (7.7)	[26,27]
Deoxy-FMN-Azotobacter flavoprotein	7.0	607 (3.78)	572 (3.98)	474 (4.37)	448 (4.40)	380 (6.22)	350 (9.28)	[26,27]
Iso-FMN-Azotobacter flavoprotein	7.0	655[b] (2.03)	606 (2.6)	520[b] (2.4)	466 (3.7)		354 (7.5)	[26,27]
2-Thio-FMN-Azotobacter flavoprotein	7.0	670 (5.42)	620[b] (3.32)	485 (2.60)	415 (1.98)		365 (5.00)	[26,27]
D-Amino acid oxidase in the presence of 0.2 M 3-fluorobenzoate	8.5	620 (3.15)	590 (3.43)	490 (4.37)	460[b] (4.0)	380[b] (6.0)	348 (8.6)	this paper
N(5)-Ethyl-lumiflavin	5.0	580 (3.60)	502 (3.90)	357 (7.70)	326 (5.80)			this paper
	ethanol	630 (4.50)	600 (4.40)	492 (3.00)	364 (6.30)	325 (6.80)		this paper
	CHCl₃	642 (4.40)	603 (4.00)	490 (2.35)	460 (2.00)	385 (5.90)	326 (7.20)	this paper
	benzene	655 (4.70)	608 (4.25)	486 (1.95)	451 (2.05)	389 (5.60)	326 (6.90)	this paper
Lumiflavin	7.0	570[d]						[3]
Lumiflavin	CHCl₃	622[d]	588[d]					[3]
O(2,4)-Diethyl-flavosemiquinone	CHCl₃	447 (9.00)	368 (21.00)					this paper

[a] The values shown are extrapolated from the published data.

[b] Shoulder.

[c] The higher values shown are from [27] and are calculated values; the other values shown were experimental ones obtained in this work by irradiation under anaerobic conditions with EDTA.

[d] ε not known because of high disproportionation of the system.

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