238. Water Relaxation Measurements on Semiquinones of Various Flavoproteins

by **Antonie J.W.G. Visser, Jan L. de Wit** and **Franz Müller**[1])

Department of Biochemistry, Agricultural University, De Dreijen 11, 6703 BC Wageningen, The Netherlands

and **Herman J.C. Berendsen**

Department of Physical Chemistry, State University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

(29.VI.82)

Summary

The water relaxation rates of several flavoproteins in the semiquinone state have been investigated by the spin echo technique. The results indicate a rather unspecific interaction between water and the protein-bound flavosemiquinones. An average interaction distance of 0.3–0.5 nm has been estimated. From the temperature dependence of the rate constants the free energy of activation for proton exchange is calculated to be about 17 kJ/mol. The rate of proton exchange is around 10^{11} s^{-1} for the flavoproteins studied. The protein-bound flavosemiquinones investigated are accessible to water regardless of their ionic state.

The large difference in relaxation rates of water protons between D- and L-amino-acid oxidases is noticeable. Oxynitrilase exhibits the highest whereas *Azotobacter vinelandii* flavodoxin shows the lowest water relaxation rate of the flavoproteins studied. The results are discussed in relation to the visible-light absorption properties of the flavoproteins.

1. Introduction. – From a comparison of visible absorption spectra of free flavins[2]) in apolar and polar solvents with those of protein-bound flavins in the oxidized state it has been, and still is postulated [1–3] that the microenvironment of the prosthetic group in some flavoproteins is of hydrophilic and in others of hydrophobic nature. In our opinion it is dangerous to draw such conclusions solely from a particular shape of light absorption spectra of flavoproteins in a particular redox state, because these spectra are rather complex and do not allow for an identification of the various effects which might contribute to the perturbation of the electronic spectra of

[1]) Author to whom correspondence should be addressed.

[2]) Flavin = 7, 8-dimethyl-10-substituted isoalloxazine = 7, 8-dimethyl-10-substituted-2, 3, 4, 10-tetrahydrobenzo[g]pteridine-2,4-dione; FMN = riboflavin 5′-monophosphate ('Flavin mononucleotide'); FAD = flavin-adenine dinucleotide.

0018-019X/82/8/2422-09$01.00/0

flavins when bound to apoflavoproteins. This latter statement is supported by recent model studies [4].

One of the most suitable techniques to test the above-mentioned proposal, at least in the case of the flavin semiquinone, is the determination of nuclear relaxation rates of water protons enhanced by paramagnetic species [5] [6]. Many flavoproteins yield the flavosemiquinone almost quantitatively upon suitable chemical reduction [7] [8], and therefore, lend themselves as natural spin labels for water relaxation studies. If one assumes that upon one-electron reduction of oxidized flavoprotein no gross conformational change occurs then the results obtained from the protein-bound flavosemiquinone could, with some caution, be extrapolated to the oxidized state of the flavoenzyme. This is a reasonable assumption[3]) considering the crystallographic [9] [10] and NMR. data [11] [12] on the flavodoxins from *Clostridium MP*, *Desulfovibrio vulgaris* and *Megasphaera elsdenii*. To test the hypothesis we studied various flavoproteins. Such a study might also contribute to a better understanding of the broad biological function of flavoproteins.

A similar study was performed by *Palmer & Mildvan* [13] on the flavodoxin from *M. elsdenii*. Their data and conclusions will be compared with ours.

2. Experimental. – *Materials and methods.* – *Sample preparation.* All samples were prepared in 0.1M sodium phosphate or pyrophosphate buffers except for flavodoxin from *M. elsdenii* where the phosphate buffer was 0.02M. All samples contained 0.1M ethylenediaminotetraacetic acid (EDTA). The flavoprotein concentration varied from 15 to 30 mg/ml. The flavoprotein solution (0.3 ml) was placed in a 5-mm NMR. tube, sealed with a serum cap and made anaerobic by repeated degassing under vacuum and flushing with Ar. This cycle was repeated at least four times, the final phase was a gas flush. The concentration of the samples was determined by light absorption using published extinction coefficients for the various flavoproteins in the oxidized state.

The flavoproteins were isolated from various sources according to published procedures (see *Table 1*). The flavoproteins were reduced to the semiquinone level either by addition of sodium dithionite or by illumination (EDTA as electron donor) [8]. For illumination a *Philips SP 500* high-pressure Hg-lamp was used and the light was filtered through a *Zeiss* blue filter transmitting the strong 436-nm Hg-line. The maximal semiquinone formation was determined by measuring the spin-spin relaxation time as a function of either the illumination time or the concentration of sodium dithionite added. The minimum of the curve thus obtained corresponds to the maximum semiquinone formation. The radical yield was also judged by light absorption spectrometry using published extinction coefficients of the semiquinones.

Instruments and data handling. The spin-lattice (T_1) and spin-spin (T_2) relaxation times were measured with the diamagnetic (oxidized) and paramagnetic (one-electron reduced), oxygen-free flavoprotein solutions on a 14 MHz spin-echo apparatus constructed by *Edzes* [14] at the University of Groningen. For the T_2-measurements a normal *Carr-Purcell-Gill-Meiboom* pulse program was used. The program used to measure T_1 was described by *Edzes* [14] and consisted of a $180\text{-}(t\text{-}90\text{-}\tau\text{-}180\text{-}2\tau\text{-}180_{\pi}\text{-}\tau\text{-}90_{\pi})n$ pulse sequence where the subscript π denotes a 180° phase shift. In this way a rapid determination of relaxation times is obtained while possible errors in the pulse width are corrected for. The signal was detected at 14 MHz, the echoes integrated and sampled in a signal averager (4 to 8 signals per measurement). The digital data were processed by computer resulting in relaxation times with less than 1% standard error.

For temperature-dependent measurements a *Varian* temperature accessory was used. The temperature was measured on the surface of the sample with the aid of a copper-constantan thermocouple and a digital voltmeter. The accuracy of the determination of the temperature was within 2 K. A least-

---

[3]) Crystallographic studies and NMR. spectra showed that no gross conformational change in the enzyme occurs upon one-electron reduction.

squares program was used to fit the values, obtained at different temperatures, to the *Solomon-Bloembergen* equations [15].

Methodology. $1/T_{1p}$ and $1/T_{2p}$ were calculated by subtracting the diamagnetic contribution (oxidized flavoproteins) from the paramagnetic one (semiquinones). $1/T_{1p}$ and $1/T_{2p}$ are given by the *Equation 1* [16]:

$$1/T_{1p} = p \cdot q/(T_{1m} + \tau_m);\ 1/T_{2p} = p \cdot q/(T_{2m} + \tau_m) \quad (1)$$

where p is the concentration of paramagnetic molecules relative to the concentration (111 M) of water protons; q is the number of water protons coordinated to the paramagnetic molecule; T_{1m} and T_{2m} are the spin-lattice and spin-spin relaxation times, respectively, of a proton coordinated to the paramagnetic molecule [15] and τ_m is the residence time of a proton in the coordination sphere [17]. Since $1/T_{1p}$ and $1/T_{2p}$ are different in all our cases, and decrease with increasing temperature *(Fig., vide infra),* T_{1m} and T_{2m} become $\gg \tau_m$, so that *Equation 1* reduces to:

$$1/T_{1p} = p \cdot q/T_{1m};\ 1/T_{2p} = p \cdot q/T_{2m} \quad (2)$$

If p and q are known, $1/T_{1p}$ and $1/T_{2p}$ directly yield $1/T_{1m}$ and $1/T_{2m}$, respectively.

To analyze the temperature-dependent behaviour of $1/T_{1p}$ and $1/T_{2p}$ for the flavoprotein systems, we have to assume that r and A/h, the distance and the hyperfine coupling constant between the paramagnetic molecule and a proton within the coordination sphere, are temperature-independent. The temperature dependence of the relaxation rates [15], is then given by: *i)* the rotational correlation time of the paramagnetic molecule, τ_R, which for spherical molecules can be obtained from the *Stokes-Einstein* relation, *i.e.* $\tau_R = 4\pi\eta R^3/3kT$ [17]; *ii)* the rate of exchange of water protons in and out of the coordination sphere, $1/\tau_m$, which can be represented by the *Eyring* equation $1/\tau_m = kT/h\ \exp(-\Delta G/RT)$, ΔG = free energy of activation [17]; and *iii)* the spin-lattice (τ_{1e}) and spin-spin (τ_{2e}) relaxation times (known from EPR. experiments [18] [19]) of the electron spin of the paramagnetic molecule.

Under the condition that τ_m determines all correlation times in the *Solomon-Bloembergen* equations [15] and assuming $\omega_I\tau_m \ll 1$ ($\omega_I \approx 90 \times 10^6\ s^{-1}$, the angular measuring frequency), the relaxation rates are given by

$$1/T_{1m} = D\left(6\tau + \frac{14\tau}{1+\omega_s^2\tau^2}\right) + E\frac{2\tau}{1+\omega_s^2\tau^2} \quad (3)$$

$$1/T_{2m} = D\left(7\tau + \frac{13\tau}{1+\omega_s^2\tau^2}\right) + E\left(\tau + \frac{\tau}{1+\omega_s^2\tau^2}\right) \quad (4)$$

where $\tau = \tau_m$ and $D = \gamma_I^2 g^2 \beta^2 S(S+1)/15r^6$ and $E = 4\pi^2 S(S+1)A^2/3h^2$.

For $\omega_s\tau \gg 1$, the difference between $1/T_{2m}$ and $1/T_{1m}$ is determined by the hyperfine coupling constant A/h, while $1/T_{1m}$ is determined by dipolar interaction. The shape of the curves (assuming activation behaviour for the temperature dependence of τ_m) determines τ_m, given the electron-spin angular frequency ω_s of $5.8 \times 10^{10}\ s^{-1}$. The parameters were determined from both equations by least-squares deviation from experimental results.

3. Results. – *Table 1* represents the results of the measurements on flavoproteins together with data on the ionic state of the semiquinone, the molecular weights of the proteins and the pH of the solutions studied.

For an appropriate evaluation of the *Solomon-Bloembergen* equations [15] it is essential to estimate the values of the contributing parameters. Since in most flavoproteins the association constant between apoenzyme and prosthetic group is very large (tight binding) it is assumed that $\tau_R > 10^{-8}$ s for the proteins studied. Both τ_{1e} and τ_{2e} are known to be $\sim 10^{-7}$ s [18] for a free flavin radical in solution at RT.

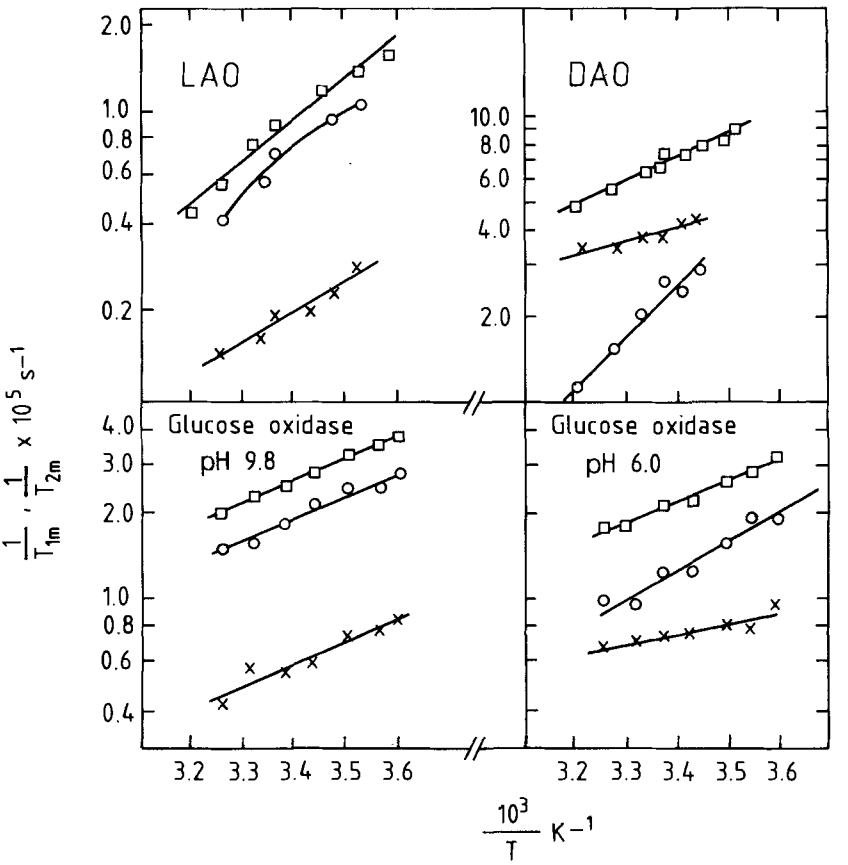


Figure. *Determination of the activation energy of the interaction of water molecule(s) with protein-bound flavosemiquinones* (The inverse paramagnetic spin-spin ($1/T_{2m}$, □) and the inverse paramagnetic spin-lattice ($1/T_{1m}$, ×) relaxation times of various protein-bound flavosemiquinones are plotted semilogarithmically *vs.* the reciprocal absolute temperature. Circles (○) indicate the difference $1/T_{2m} - 7/6\ 1/T_{1m}$. The assumed value of q = 2; LAO = L-amino-acid oxidase; DAO = D-amino-acid oxidase)

These values are used as a first approximation since electron-spin relaxation times are not known for protein-bound flavins. The adopted lower limit of τ_R and estimated value of τ_{1e} suggest that both $\omega_I\tau_R$ and $\omega_I\tau_{1e}$ are larger than unity. If one of the correlation times (τ_R or τ_{1e}) determines T_{1m} and T_{2m}, the temperature coefficients of T_{1m} and T_{2m} must be opposite [15]. In fact they have equal signs *(Figure)*. Therefore the relaxation behaviour of the water protons in the paramagnetic environment is determined by τ_m. At low temperature both $1/T_{1m}$ and $1/T_{2m}$ are proportional to τ_m and show activation behaviour. At higher temperature deviations occur since τ_m becomes shorter ($\omega_s\tau_m$ approaches unity). This is particularly evident by plotting $1/T_{2m} - 7/6\ 1/T_{1m}$, required for an estimate of the hyperfine coupling constant, against the reciprocal temperature. The difference is not constant indicating short-

Table 1. *The inverse paramagnetic spin lattice ($1/T_{1m}$) and inverse paramagnetic spin-spin ($1/T_{2m}$) relaxation times of various protein-bound flavosemiquinones* (Some physical and chemical data of the flavoproteins and their solutions are given (for details see *'Materials and methods'* in *Sect. 2*); temperature 298 K. Assumed value for q = 2)

Flavoprotein	Prosthetic group/ Molecular Weight	Ionic State of Semiquinone	pH	$1/T_{1m}$ ($\times 10^5$ s^{-1})	$1/T_{2m}$	Isolation procedure
L-amino-acid oxidase	2 FAD/130000	Anionic	7.4	0.19	0.92	[20]
D-amino-acid oxidase	2 FAD/ 90000	Anionic	8.3	3.81	6.72	[21]
Glucose oxidase	2 FAD/186000	Neutral	6.0	0.74	2.09	[22]
Glucose oxidase		Anionic	9.8	0.56	2.55	[22]
Oxynitrilase	1 FAD/ 75000	Anionic	7.0	4.71	31.60	[23]
A. vinelandii flavodoxin	1 FMN/ 23000	Neutral	7.0	0.13	0.15	[24]
M. elsdenii flavodoxin	1 FMN/ 15000	Neutral	6.9	0.62	1.12	[25]
M. elsdenii flavodoxin		Neutral	6.9	0.53[a])	1.06[a])	
3-Methyl-5-ethyl-lumiflavin semiquinone		Neutral	7.0	0.15[a])	0.15[a])	

[a]) Published values of *Palmer & Mildvan* [13] multiplied by 111M (proton concentration of H_2O) for a direct comparison with our values.

ening of τ_m (see the *Figure*). The calculated data obtained as described in *Section 2 Methodology* are presented in *Table 2.*

4. Discussion and conclusion. – Comparing our results with those of *Palmer & Mildvan* [13] the following similarities are evident. The spin-lattice and spin-spin molar relaxation times of the 3-methyl-5-ethyllumiflavin semiquinone are of the same order of magnitude (*Table 1*, our values not shown). This is also true for the values obtained from the semiquinone of *M. elsdenii* flavodoxin. The temperature

Table 2. *Physical constants of the interaction of water with protein-bound flavosemiquinones*

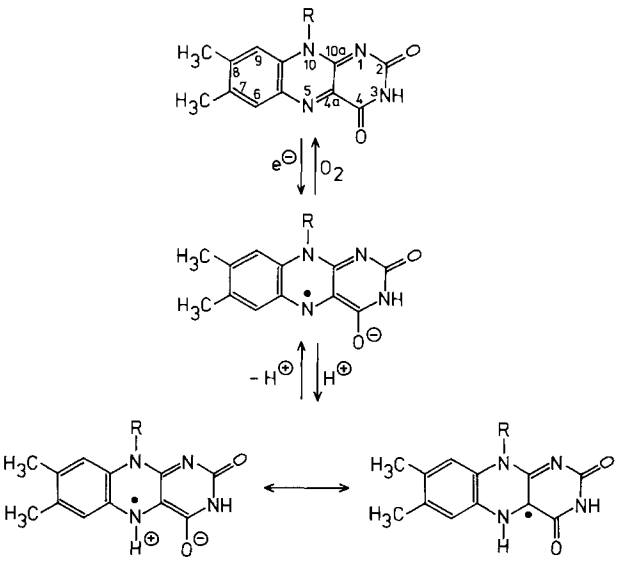
Flavoprotein	$\tau_m \times 10^{11}$ s[a])	E_a[b]) kJ/mol	r[c]) (in nm) q = 4	r[c]) (in nm) q = 2	A/h(10^3Hz)[d]) (for q = 2)
L-amino-acid oxidase	14.0	21.4	0.51	0.46	1.57
D-amino-acid oxidase	30.0	9.3	0.35	0.31	3.27
Glucose oxidase (pH 6.0)	20.0	7.1	0.45	0.40	2.29
Glucose oxidase (pH 9.8)	20.0	15.1	0.46	0.41	3.68
Oxynitrilase	33.0	–	0.35	0.31	31.6
A. vinelandii flavodoxin	2.6	17.8	0.47	0.42	very small
M. elsdenii flavodoxin	~ 1	4.6[e])	–	–	8.0[e])
3-Methyl-5-ethyl-lumiflavin semiquinone	4.4	6.8	3.59	3.19	very small

[a]) Residence time τ_m, calculated from the *Eyring* equation (*cf. Methodology* in *Sect. 2*), T = 298 K.
[b]) Activation energy, calculated from *Arrhenius* plots of $1/T_{1m}$ (*cf.* the *Fgure).*
[c]) Distance between flavosemiquinone and water (q = 4 refers to the involvement of 2 water molecules, q = 2 refers to one water molecule).
[d]) Hyperfine coupling constant.
[e]) Value of *Palmer & Mildvan* [13], A/h corrected for the value of τ_{1e} (10^{-7}s instead of 7×10^{-7}s).

dependence of the relaxation rates are also in full agreement, as are the conclusions that the relaxivities of the water protons in the paramagnetic environment are determined by τ_m. The values of τ_m in *Table 2* further validate this conclusion. These facts imply that also at the lower frequency used in this study (14 MHz instead of 24.3 MHz) the same assumptions can be made as it was described in [13]. In our opinion, however, the data do not really allow to reach any conclusion about the number of H-bonds formed between the paramagnetic species and H_2O (one, two or three) as proposed by *Palmer & Mildvan* [13].

EPR. data [18] [19] [26] and theoretical calculations [27] show that about 70% of the spin density is localized in the center part of the isoalloxazine ring of flavin, specifically on the N(5)- and C(4)-atoms in a nearly co-planar semiquinone conformation. Furthermore, in the neutral flavosemiquinone C(4a) carries a larger spin density than N(5) whereas in the anionic flavosemiquinone the situation is reversed *(Scheme)*. Taking this into consideration, inspection of *Table 2* leads to the following conclusions.

Scheme



1) There are no differences in τ_m or r for glucose oxidase at pH 6 (neutral form) and pH 9.8 (anionic form), but the hyperfine coupling constant for the neutral form is smaller than that for the anionic form. This observation is in agreement with the spin distribution in the neutral and anionic flavosemiquinone.

2) Oxynitrilase shows a hyperfine coupling constant of approximately one order of magnitude larger than those of the other proteins, while the distance is of the same order of magnitude as calculated for the other flavoproteins. This suggests that the electron spin density on the N(5)-atom of the flavin bound to oxynitrilase is

much larger than that in the other proteins. Such changes could be induced by the apoprotein forcing the flavosemiquinone into a less planar conformation. Recent model studies [28] and theoretical calculations [27] on flavosemiquinone cations support this view.

3) Comparing all anionic forms it is noticed that their hyperfine coupling constants (A/h) vary much more than the other parameters. Therefore variations in A/h probably reflect spin-density variations in the center part of the flavin ring system, while the behaviour of the other parameters suggests a rather unspecific interaction between water and the isoalloxazine ring. Subtle changes in the ring conformation of flavosemiquinone induced by specific interactions with active site amino-acid residues might be responsible for the large variations in the hyperfine coupling constants.

The relaxation rates given in *Table 1* can be interpreted in terms of the degree of accessibility of the protein-bound flavosemiquinone by water molecule(s). The results clearly show that all protein-bound flavosemiquinones are accessible to water irrespective of their ionization state. This is supported independently by static experiments where it has been shown that the electron spin resonance active proton at N(5) of neutral flavoprotein radicals is exchangeable against a deuteron [29]. Differences in accessibility are, however, only revealed by kinetic techniques of a high time resolution, *i.e.* relaxation measurements. The degree of accessibility varies rather strongly among the flavoproteins studied. Among the protein-bound neutral flavosemiquinones that in glucose oxidase seems the most easily accessible, followed by that of *M. elsdenii* flavodoxin and *A. vinelandii* flavodoxin. These facts support our notion that visible absorption spectra are not very useful for judging the accessibility of water to flavoprotein active centers since water does even have access to the (so-called) 'hydrophobic', protein-bound flavosemiquinone of *A. vinelandii* flavodoxin. The accessibility of isoalloxazine to water is, therefore, not the only factor determining the overall shape of the visible absorption spectra of protein-bound flavosemiquinones. The shape of the visible spectrum of a particular flavoprotein is more likely the result of several factors such as specific interactions with amino-acid residues like H-bonding and *van der Waals* forces.

It has been proposed that the stabilization of protein-bound neutral flavosemiquinones, as compared to that of the free molecule, is brought about by H-bond formation between H–N(5) and amino-acid residue(s) of the protein [18]. The short proton residence times (see *Table 2*) observed for *M. elsdenii* and *A. vinelandii* flavodoxins indicate rapid exchange of water protons. However the relatively large hyperfine interaction A/h in *M. elsdenii* flavodoxin suggests the presence of H-bonded water. On the other hand the higher accessibility of the neutral flavosemiquinone in glucose oxidase, also considering the fact that the accessibility is about the same for the anionic radical, suggests that in glucose oxidase no H-bond with amino-acid residue(s) is present and that the micro-environment of the active site of this flavoprotein is rather polar. This proposal is also in better agreement than the previous one [18] with the observation that the pK_a value of the protein-bound semiquinone occurs at lower pH values (7.5, [8]) than that of the free flavin radical (pH 8.2, [30]).

For the anionic, protein-bound flavosemiquinones it is difficult to explain the results in a similar detailed manner because no crystallographic data and no solvent-dependent model studies are available. Nevertheless, it is remarkable that the light absorption spectra of all anionic flavoprotein radicals show maxima between 485 nm and 490 nm [8]. The absorption maximum of the corresponding free flavin radical is at 477 nm in DMF [30]. It is noteworthy that differences exist within the class of these flavoprotein radicals *(Table 1)*. The most intriguing observation is, however, the very good accessibility and the rather high calculated coupling constant for oxynitrilase as compared to the other anionic flavoprotein radicals. This observation suggests direct H-bond formation between the anionic flavosemiquinone of oxynitrilase and water. The latter example might well illustrate that in all other flavoproteins studied water molecules located in the outer coordination sphere of the flavin are exchanged rather than water molecules directly interacting with the flavin.

We thank Mrs. *J.C. Toppenberg-Fang* for typing the manuscript, Mr. *B.J. Sachteleben* for the preparation of the drawings, Prof. *Pfeil* for the genereous gift of oxynitrilase and Dr. *A. de Kok* for a sample of L-amino-acid oxidase.

This study has been carried out under the auspices of the *Netherlands Foundation for Chemical Research* (S.O.N) with financial aid from the *Netherlands Organization for the Advancement of Pure Research* (Z.W.O).

REFERENCES

[1] *H.A. Harbury, K.F. La Noue, P.A. Loach & R.M. Amik*, Proc. Natl. Acad. Sci. U.S.A. *45*, 1708 (1959).
[2] *K. Yagi*, in 'Oxidases and Related Redox Systems', T.E. King, H.S. Mason and M. Morrison, eds., University Park Press, Baltimore 1973, p. 217.
[3] *P. Hemmerich*, in 'Transport by Proteins', G. Blauer and H. Sund, eds., Walter de Gruyter & Co., Berlin, New York 1978, p. 123.
[4] *J.K. Eweg, F. Müller, A.J.W.G. Visser, C. Veeger, D. Bebelaar & J.W.D. van Voorst*, Photochem. Photobiol. *20*, 463 (1979).
[5] *M. Cohn & J.S. Leigh*, Nature *193*, 1037 (1962).
[6] *A.S. Mildvan & M. Cohn*, in 'Advances in Enzymology', F.F. Nord, ed., Interscience Publishers, New York 1970, Vol. 33, p. 1.
[7] *F. Müller, M. Brüstlein, P. Hemmerich, V. Massey & W.H. Walker*, Eur. J. Biochem. *25*, 573 (1972).
[8] *G. Palmer & V. Massey*, Biochemistry *5*, 3181 (1966).
[9] *S.G. Mayhew & M.L. Ludwig*, in 'The Enzymes', P.D. Boyer, ed., Academic Press, New York 1975, 3rd Edition, Vol. XII, p. 57.
[10] *K.D. Watenpaugh, L.C. Sieker & L.H. Jensen*, in 'Flavins and Flavoproteins', T.P. Singer, ed., Elsevier Scientific Publishing Co., Amsterdam 1976, p. 405.
[11] *T.L. James, M.L. Ludwig & M. Cohn*, Proc. Natl. Acad. Sci. U.S.A. *70*, 3292 (1973).
[12] *F. Müller, C.G. van Schagen & W.J.H. van Berkel*, in 'Flavins and Flavoproteins', K. Yagi and T. Yamano, eds., Japan Scientific Societies Press, Tokyo 1980, p. 359.
[13] *G. Palmer & A.S. Mildvan*, in 'Structure and Function of Oxidation-Reduction Enzymes', Å. Åkeson and A. Ehrenberg, eds., Pergamon Press, Oxford 1972, p. 385.
[14] *H.T. Edzes*, J. Magn. Reson. *17*, 301 (1975).
[15] *N. Bloembergen, E.M. Purcell & R.V. Pound*, Phys. Rev. *73*, 679 (1948).
[16] *T.J. Swift & R.E. Connick*, J. Chem. Phys. *37*, 307 (1962).
[17] *R.A. Dwek* in 'Nuclear Magnetic Resonance in Biochemistry', Oxford Press, Oxford 1973, p. 42.

[18] *F. Müller, P. Hemmerich, A. Ehrenberg, G. Palmer & V. Massey,* Eur. J. Biochem. *14,* 185 (1970).
[19] *F. Müller, P. Hemmerich & A. Ehrenberg* in 'Flavins and Flavoproteins', H. Kamin, ed., University Park Press, Baltimore 1971, p. 107.
[20] *D. Wellner & A. Meister,* J. Biol. Chem. *235,* 2013 (1960).
[21] *V. Massey, G. Palmer & R. Bennett,* Biochim. Biophys. Acta *48,* 1 (1961).
[22] *B. E. P. Swoboda & V. Massey,* J. Biol. Chem. *240,* 2209 (1965).
[23] *W. Becker, U. Benthin, E. Eschenhof & E. Pfeil,* Biochem. Z. *337,* 156 (1963).
[24] *J. W. Hinkson & W. A. Bulen,* J. Biol. Chem. *242,* 3345 (1967).
[25] *S. G. Mayhew & V. Massey,* J. Biol. Chem. *244,* 794 (1969).
[26] *W. H. Walker, A. Ehrenberg & J.-M. Lhoste,* Biochim. Biophys. Acta *215,* 166 (1970).
[27] *R. J. Platenkamp, M. H. Palmer & A. J. W. G. Visser,* J. Mol. Struct. *67,* 45 (1980).
[28] *F. Müller, H. J. Grande, L. H. Harding, W. R. Dunham, A. J. W. G. Visser, J. H. Reinders, P. Hemmerich & A. Ehrenberg,* Eur. J. Biochem. *116,* 17 (1981).
[29] *G. Palmer, F. Müller & V. Massey,* in 'Flavins and Flavoproteins', H. Kamin, ed., University Park Press, Baltimore 1971, p. 123.
[30] *A. Ehrenberg, F. Müller & P. Hemmerich,* Eur. J. Biochem. *2,* 286 (1967).