134. The Binding of Flavins by Apoflavodoxins from *Peptostreptococcus elsdenii* and *Azotobacter vinelandii* as Studied by Temperature-Jump Technique

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Summary

The binding of various flavins by apoflavodoxins from *P. elsdenii* and *A. vinelandii* has been studied by the temperature-jump technique using fluorescence detection. *P. elsdenii* apoflavodoxin interacts only with flavins possessing 5 carbon atoms in the N(10) side chain and a terminal phosphate group. Employing a wide range of concentrations of deoxy-FMN[2]) and apoflavodoxin only one relaxation process was observed, indicating a one-step binding mechanism. With native flavodoxin no relaxation could be observed.

The kinetic parameters of the interaction of *A. vinelandii* apoflavodoxin with various flavin analogs (Structure **I**) have also been investigated. The interaction between apoflavodoxin and flavin derivatives carrying an ionizable, terminal functional group on the side chain becomes very weak when the number of the side chain carbon atoms is decreased below 4. This observation is interpreted in terms of repulsive forces due to negatively charged amino acid residues located in the flavin side chain binding region of the apoflavodoxin. All complexes studied revealed only one relaxation process. This observation is in contradiction with published results [10]. The published traces are instrumental artifacts.

Introduction. – Flavoproteins can be divided into 2 classes with respect to their interaction with the prosthetic group. In one class the prosthetic group can be reversibly released whereas in the other class the flavin[2]) is covalently linked to the polypeptide chain (for a review see [1]). Methods have been developed for the preparation of stable apoenzymes from flavoproteins of the first class (*e.g.* [2] [3]). Among these flavoproteins there are a number of low-molecular-weight proteins especially suitable for a study of the kinetic parameters of the flavin-apoenzyme interaction. We chose to work with *Peptostreptococcus elsdenii*

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[2]) Flavin = 3,4-dimethyl-10-substituted isoalloxazine = 3,4-dimethyl-10-substituted-2,3,4,10-tetrahydrobenzo[g]-pteridine-2,4-dione; FMN = riboflavin-5′-monophosphate.

and *Azotobacter vinelandii* flavodoxins because their physical and chemical properties have been investigated in detail [4–7]. The interaction between FMN[2]) and apoflavodoxin from *P. elsdenii* has been investigated at low pH values and various ionic strength [8]. Since apoflavodoxins form rather stable complexes with FMN in the neutral pH region the described techniques [8] cannot be used to obtain information about the kinetic parameters of the flavin-apoenzyme interaction in this pH region.

The relaxation method [9] has been used by *Barman & Tollin* [10] to investigate the kinetics of the interaction of FMN with apoflavodoxins from *A. vinelandii* and *P. elsdenii* [10] and the flavodoxin from *Desulfovibrio vulgaris* has also been studied by this technique [11]. The latter enzyme exhibited a single relaxation time indicating a single one-step binding mechanism. Since the former enzymes showed two relaxation times, it was concluded that the phosphate group of FMN triggers a conformational change upon binding to these apoenzymes [10]. The fact that the mechanism of binding of FMN by *P. elsdenii* and *A. vinelandii* apoflavodoxins is different from that of *D. vulgaris* protein is surprising in view of the many similarities between the flavodoxins from *A. vinelandii* and *D. vulgaris* [7]. Several explanations might account for the 2 different kinetic patterns observed, the most obvious being that the dissociation constants of flavodoxins from *P. elsdenii* and *A. vinelandii* are much smaller than those of flavodoxin from *D. vulgaris* and of the complex between apoflavodoxin from *A. vinelandii* and riboflavin. The dissociation constants for native *P. elsdenii* and *A. vinelandii* flavodoxins are, in fact, so small and the flavin fluorescence of the complexes so low that we questioned whether meaningful data could be obtained from fluorescence measurements following a temperature-jump.

In order to test the 2-step binding mechanism proposed by *Barman & Tollin* [10] we have carried out an investigation using the temperature-jump relaxation technique and studied the binding of modified flavins to various apoflavodoxins.

Materials and methods. – Flavodoxin was isolated from *A. vinelandii*, strain OP, and purified according to the method of *Hinkson & Bulen* [12]. The apoenzyme was prepared by the method of *Edmondson & Tollin* [13]. Flavodoxin from *P. elsdenii*, LC 1, was isolated and purified according to the method of *Mayhew & Massey* [4]. The apoenzyme was prepared by dialysis against 2M KBr in 0.1M sodium acetate, pH 3.8 [5]. The concentration of the apoenzyme was determined by titration with pure FMN [14] using a fluorescence technique. An excitation wavelength of 450 nm was used and the fluorescence emission observed at 520 nm. Similarly the dissociation constants for complexes between various flavins and apoflavodoxins were determined. Static fluorescence measurements were carried out at 22°. Concentrations of holoenzymes were determined using the published extinction coefficients of 10,200 $mol^{-1}cm^{-1}$ for flavodoxin from *P. elsdenii* [4] and 10,600 $mol^{-1}cm^{-1}$ for flavodoxin from *A. vinelandii* [13].

The kinetics of binding of flavin analogs were determined at 22° (final temperature) at pH 8.5 in Tris HCl or pyrophosphate buffer (0.1M) containing 0.1M KNO_3 as a conducting electrolyte. As identical results were obtained in both buffer systems, no specific salt effects or effects caused by a pH jump (occurring in Tris-HCl buffers as a result of the temperature-jump) were thus observed under these conditions. Samples were prepared from stock solutions using equimolar concentrations of flavin and apoenzyme.

The temperature-jump relaxation apparatus (*Messanlagen* GmbH, Göttingen, Germany) was modified for fluorescence measurements by placing appropriate mirrors behind 2 of the 4 cell windows. This modification caused a 3-fold enhancement of the sensitivity of the instrument. The

336 nm line from a high pressure Hg-lamp was used as the exciting light source while a non-fluorescent filter, type KV 411 *W. Schott*, Jena, separated the exciting light from the emission light. Prior to a kinetic determination, the temperature-jump cell compartment was equilibrated to 14°. At approximately 4 min intervals the temperature of the solution was increased $8 \pm 0.3°$ by means of a calibrated high-voltage discharge (20 kV) from a 0.05 μF capacitor and the resulting relaxation traces were stored in a *Datalab* transient recorder DL 905. The data were recorded by plotting the output *via* a strip chart recorder. Our instrument maintains the higher temperature for about 2 s, after which cooling starts to occur. The exponential heating time was approximately 11 μs for an 8.3° temperature change.

We report here some factors leading to artifacts using fluorescence detection in temperature-jump experiments when working with flavins and flavoproteins. First, free flavins bearing a hydroxyl group at C(2′) are subject to intramolecular photoreduction (*e.g.* [15]) in the presence of electron donors such as EDTA or Tris, which appears as a decrease of the fluorescence intensity on the 100 ms time scale or longer. Second, the well-known effect of electrolysis caused by the discharge of the capacitor generates some gaseous products which pass the windows as a stream of bubbles causing an apparent increase of the fluorescence intensity. This effect occurs within 5 s after the temperature-jump under normal viscosity conditions (water, RT.) but can be slowed down by adding glycerol. Third, stray light from the exciting light beam can form a large part of the light detected when fluorescence intensities are low and it is therefore important to establish the optimum conditions for the secondary filters. This has been checked independently by placing the temperature-jump cell in a conventional spectrofluorimeter equipped with a photomultiplier possessing the same sensitivity as the one used in the temperature-jump instrument.

The flavin derivatives (Structure **I**) used in this study were prepared according to published procedures: riboflavin-5′-monosulphate (**Ib**) [17]; $N(10)$-ω-hydroxyalkyl flavins containing various numbers of methylene groups (**Ie**, n = 2–5) [18]; the latter compounds were phosphorylated to **Id** by the method of *Flexser & Farkas* [19]. The analogous carboxylic acids were synthesized according to *Föry et al.* [20]. Each of the derivatives gave one spot by thin layer chromatography in at least 2 different solvent mixtures, and were therefore judged to be pure.

Results and discussion. – In applying the temperature-jump relaxation technique to study fast chemical reactions, certain conditions have to be fulfilled. For instance in a bimolecular reaction, the interaction between the reactants should not be too strong or too weak. In addition the system must possess a favourable change in enthalpy, so that a measurable displacement of equilibrium occurs by the instantaneous heating of the system. In the study conducted by *Barman & Tollin* [10] native flavodoxins from *A. vinelandii* and from *P. elsdenii* were employed. However, FMN is tightly bound by the apoenzymes of these proteins [5] [13] suggesting that they are not suitable for this technique. Indeed temperature-jump experiments performed on the native enzymes using fluorescence detection yielded experimental curves [10] difficult to interpret unambiguously because of the poor signal-to-noise ratio of the experimental traces. In order to overcome this problem, we have used modified FMN derivatives which exhibit a weaker interaction with the apoenzymes mentioned so that a more favourable fluorescence change could be observed upon temperature perturbation of the complexes.

a) Studies with flavodoxin from P. elsdenii. The interaction between the apoenzyme and various FMN derivatives modified at positions 2, 3, 6 and 8 of the isoalloxazine[2]) ring has been studied [21]. Furthermore, from the fact that riboflavin does not bind to the apoenzyme it has been concluded [7] that the 5′-phosphate group of the flavin is required for effective binding. Our measurements with FMN derivatives having side chains of varying length and a variety of terminal groups on the side chain (Structure **I**) confirm this view. The only

compound that interacted with the apoenzyme was deoxy-FMN[3]) (**Id**, n=4) and this was already known [21] to interact with the apoenzyme about 3 orders of magnitude less strongly than FMN. With this system we tested the proposal [10] that the 5′-phosphate group triggers a conformational change in the apoenzyme (E) and leads to a 2-step binding mechanism:

$$\text{FMN}+\text{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{FMN}\ldots\text{E}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{FMN-E} \tag{1}$$

Temperature perturbation of the deoxy-FMN-apoenzyme complex yields an increase in fluorescence intensity *(Fig. 1)*. The analysis of the experimental traces with a good signal-to-noise ratio revealed that only one relaxation process was involved in the reaction, indicating the one-step mechanism for binding of the flavin on the apoprotein:

$$\text{dFMN}-\text{E} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{dFMN}+\text{E} \tag{2}$$

This has been verified by using a wide range of concentration of the components of the system. A linear relationship was found between the sum of the concentrations of free deoxy-FMN and apoenzyme and the corresponding relaxation times *(Fig. 2)*. The perturbation of equilibrium (2) follows the equation:

$$1/\tau = k_1([\bar{\text{E}}]+[\overline{\text{dFMN}}])+k_{-1} \tag{3}$$

where k_1 and k_{-1} are the bimolecular and monomolecular rate constants, respectively, and $[\bar{\text{E}}]$ and $[\overline{\text{dFMN}}]$ are the new equilibrium concentrations of apo-

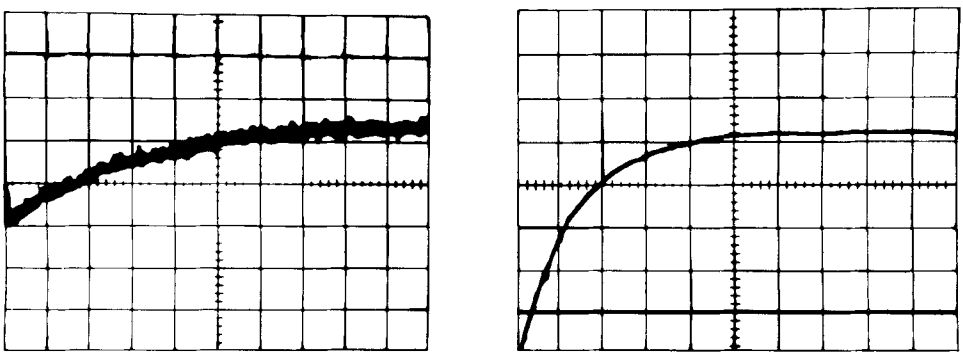


Fig. 1. *Experimental relaxation traces for the equilibrium displacement of* P.elsdenii *apoflavodoxin and deoxy-FMN* in 30 mM phosphate buffer, pH 8.5, in the presence of 0.3 mM EDTA and 0.1 M KNO_3, final temperature 22°. Fluorescence detection has been employed, excitation wavelength was 377 nm. Upward deflection represents an increase in fluorescence intensity.

A) Total protein concentration 2.2 μM, total flavin concentration 2.2 μM. Time constant 1 ms, 10 mV/large vertical division and 100 ms/large horizontal division.

B) Total concentration of both the protein and the flavin was 13.3. μM. Time constant 5 ms, 50 mV/large vertical division, 200 ms/large horizontal division.

[3]) Deoxy-FMN(dFMN)=3,4-dimethyl-*N*(10)-(5-hydroxypentyl)-isoalloxazine-5′-monophosphate.

enzyme and free flavin, respectively. From the intercept on the ordinate of the plot of *Figure 2* $k_{-1}=0.8\ s^{-1}$ and from the slope $k_1=3.6\times 10^5\ mol^{-1}ls^{-1}$ [4]) can be calculated. These values yield a dissociation constant of the system of 2.2 $\mu mol\,l^{-1}$. This latter value is identical with the one obtained by static fluorimetric titration experiments performed under the conditions of the temperature-jump experiments, *i.e.* 22° and buffer containing 0.1 M KNO_3. This and the other values reported below were calculated graphically according to the method of *Bensi & Hildebrand* [23] assuming a 1:1 complex formation. The deoxy-FMN-apoenzyme complex was very weekly fluorescent. The value of 2.2 $\mu mol\,l^{-1}$ differs by a factor of *ca.* 5 from that (0.43 $\mu mol\,l^{-1}$) published by *Mayhew & Ludwig* [21]. Similarly the association rate constant [21] is about one order of magnitude smaller than the value reported in this paper. The reason for this apparent discrepancy probably lies in the difference in conditions (ionic strength and temperature)[5]).

The association-rate constant for deoxy-FMN is of the same order of magnitude as for FMN [5] indicating that the side chain hydroxyl groups are not of great importance for the formation of the complex. However, they contribute considerably to the stabilization of the flavin-apoenzyme complex because the dissociation rate constant is about 2 orders of magnitude greater for deoxy-FMN [5].

Our data confirm that the apoenzyme exhibits a high specificity with respect to flavin binding, *i.e.* a side chain consisting of 5 carbon atoms and a terminal

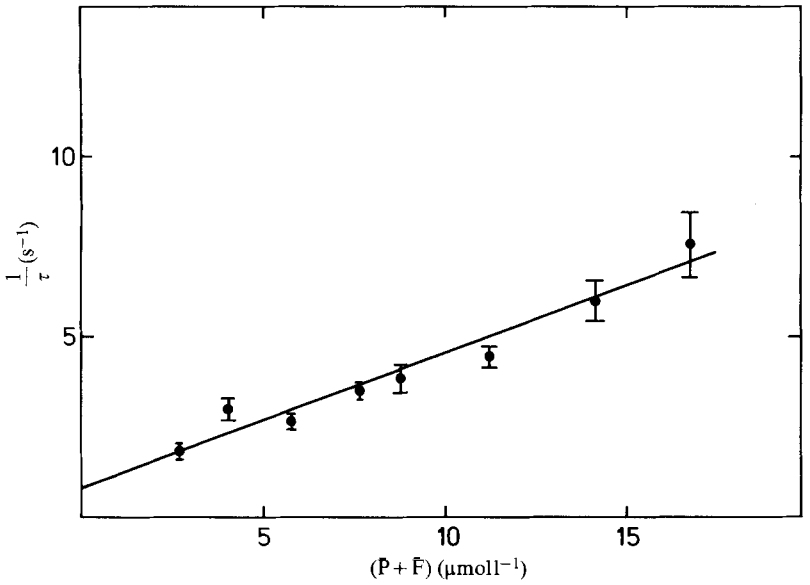


Fig. 2. *Plot of reciprocal of the observed relaxation times* vs. *the sum of the concentration of free deoxy-FMN and apoflavodoxin from* P. elsdenii. The experimental conditions were those of *Fig.* 1.

[4]) The bimolecular association rate constants determined with FMN and various apoflavodoxins all fall within the range 10^5 to $10^6\ mol^{-1}ls^{-1}$ [21].

[5]) This is a reasonable assumption considering published results [8] [10] [26] which show that the rate constants depend strongly on the salt concentration of the solution.

phosphate group are minimal requirements. From these results it also follows that the phosphate group plays a more important role in the interaction between flavin and apoenzyme than the side-chain hydroxyl groups. From X-ray studies on flavodoxin from *D. vulgaris* [23] and *Clostridium MP* [24] it is known that the phosphate group of FMN is bound to the apoenzyme through several hydrogen bonds. It is a very reasonable assumption that the same situation also exists in flavodoxin from *P. elsdenii*. In addition in the latter enzyme the phosphate binding site must be constructed in such a way that a small deviation from the phosphate conformation leads to a complete loss of the binding capacity (*cf.* **Ia** *vs.* **Ib**, and **Ie** (n = 5)).

Our results are in contradiction with the postulate of *Barman & Tollin* [10], *i.e.* one against 2 relaxation times. This discrepancy cannot be explained in terms of possible differences between native flavodoxin and the complex in question since the chemical and physical properties of the 2 complexes appear to be very similar [13]. Therefore, we performed experiments with native flavodoxin under the conditions described in [10] also considering the technical precautions mentioned in "Materials and methods". No relaxation was found in the time scale of 1 s. However, when the experiments were carried out in the time range of about 1 min [*cf.* 10] similar traces were found. Since studies with *A. vinelandii* flavodoxin have led to the proposal of the 2-step mechanism [10] the possible causes for the discrepancy will be discussed below.

b) Studies with flavodoxin from A. vinelandii. Apoflavodoxin from *A. vinelandii* differs from that of *P. elsdenii* apoflavodoxin in that it forms stable complexes with many flavin derivatives, even with lumiflavin [13]. This property allowed a more detailed study of the proposed mechanism [10] than was possible with flavodoxin from *P. elsdenii*. All of the models tested (Structure **I**) irrespective of the terminal functional group on the side chain showed only one relaxation time over a wide range of concentration of the reactants. Thus these experiments are in agreement with results described above for *P. elsdenii* apoflavodoxin and deoxy-FMN. The relaxation process observed is in accordance with eq. (2) indicating that no detectable conformational change occurs during the time course of the experiment. This conclusion is further supported by the fact that plots of $1/\tau^2$ against the concentration of the reactants yielded straight lines in accord with eq. (4) [25]:

$$1/\tau^2 = 2k_1k_{-1}([\bar{F}]+[\bar{P}])+k^2_{-1} \qquad (4)$$

These experiments cannot exclude, however, a conformation change occurring on a time-scale faster or slower than used here. The results are summarized in the *Table*. All compounds carrying a terminal ionizable group (**Ib**, **d**, **e**) show a lower association rate constant than the ones carrying a terminal hydroxyl group (**Ic**). A possible explanation is that dehydration of the terminal ionized group prior to binding is necessary. A similar observation was made by *Edmondson & Tollin* [13] who used stopped flow spectrophotometry to measure the rates of flavin-binding by apoflavodoxin from *A. vinelandii*. It was suggested that the lower association rate constant observed with FMN as compared to that of riboflavin might be due to a phosphate-triggered conformational change. The

Table. *Rate constants and dissociation constants for the binding of flavin derivatives to* A.vinelandii *apoflavodoxin*[a])

$$F + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F-E; \quad K_d = \frac{[F]\,[E]}{[F\text{-}E]}$$

Compound	k_1 ($mol^{-1}s^{-1}$)	k_{-1} (s^{-1})	K_d ($\mu mol\,l^{-1}$)	
			[b])	[c])
Ib	2.6×10^5	0.35	1.3	2.3
Ic n=2	1.3×10^6	4.2	3.2	4.4
n=3	4.2×10^6	4.4	1.1	1.4
n=4	2.0×10^6	4.6	2.3	1.3
n=5	1.5×10^6	4.7	3.1	2.0
Id n=1[d])				>1000
n=2[d])				>1000
n=3	2.8×10^5	0.3	1.1	2.5
n=4[e])	8.6×10^5	0.06	0.07	~0.01
n=5	6.0×10^5	0.2	0.33	0.35
Ie n=1[d])				>1000
n=2[d])				>1000
n=3	3.0×10^5	4.0	13.0	19.0
n=4	4.6×10^5	2.0	4.4	4.7
n=5	3.0×10^5	0.9	3.0	3.3

[a]) All experiments were conducted in 0.1M pyrophosphate buffer, pH 8.5, and 0.1M KNO_3. [b]) Calculated from the given rate constants. [c]) Determined by fluorimetric titration experiments. [d]) Interaction with the enzyme too weak to be measured. [e]) Because of the quite strong interaction with the apoenzyme the given values have to be considered qualitatively.

present study does not support this proposal. Nevertheless, from the data presented in the *Table* it might be concluded that the rate constant of association of flavin with apoflavodoxin is not only influenced by the degree of hydration of the reactants but also depends on the charge of the flavin and the net charge of the apoflavodoxin at a given pH. Thus, the effect of hydration is illustrated by the fact that under identical experimental conditions the association rate constants are in the order of 10^7 $mol^{-1}ls^{-1}$ for lumiflavin [10] (**I**, $R=CH_3$), which is the least polar molecule among the flavin derivatives discussed here, in the order of 10^6 $mol^{-1}ls^{-1}$ for riboflavin [10] and its analogs **Ic**, and in the order of 10^5 $mol^{-1}ls^{-1}$ for FMN [10] and flavin derivatives carrying an ionizable side chain terminal group (**Ib**, **d**, **e**). The influence of the net charge of apoflavodoxin on the association and dissociation reactions, on the other hand, can be derived from published data [8] [26] [27] showing that the rate constants of the reaction of FMN with either *P. elsdenii* or *A. vinelandii* apoflavodoxin are pH and ionic strength dependent.

The results demonstrate another interesting fact. The uncharged derivatives (**Ic**) gave complexes with dissociation constants in the $\mu mol\,l^{-1}$ range. Introduction of an ionizable terminal group into compounds **Ic** yielding **Id** or **Ie** leads to a very weak interaction between the apoflavodoxin and the flavin derivatives **Id**, **Ie** (n=1, 2) *(cf. Table)*, thus making temperature-jump studies impossible. How-

ever, as the number of methylene groups of the side-chain increased (**Id**, **e**, $n>2$), the interaction between the components became again comparable to the other compounds tested. This observation could be interpreted in terms of repulsive forces; *i.e.* the protein side chain binding region probably possesses negatively charged group(s) distanced about 0.3–0.5 nm from the N(10) atom of the isoalloxazine ring system. This interpretation is supported by previous kinetic studies conducted with flavodoxins from *P. elsdenii* [8] and *A. vinelandii* [27] at pH values between 2 and *ca.* 7, indicating that 2 glutamic acid residues are located in the flavin binding region of the proteins. Our results indicate that these glutamic acid residues are strongly involved in the binding of the flavin side chain. Furthermore, the finding of *Meighen & MacKenzie* [28], that the activity of the bioluminescent reaction of bacterial luciferase depended on the number ($n>2$) of methylene groups of the flavin derivatives **Id** and **Ie**, is probably also related to repulsive forces.

With 3 exceptions (**Id**, $n=4$, 5; **Ie**, $n=3$) the dissociation constants of the complexes investigated are similar *(Table)*. The dissociation constants calculated from the given rate constants agree reasonably with those determined by static fluorescence titration experiments *(Table)*. Our data also allow us to describe the kinetic parameters which govern the stability of a given complex. The stability of the complexes between apoflavodoxin and compound **Ic** seems mainly governed by the association rate constants, which vary by a factor of *ca.* 3, whereas the dissociation rate constants (the inverse of which is related to the lifetime of the complex) are about the same for all 4 complexes. The association rate constants of the complexes of apoflavodoxin with the charged compounds **Ib**, **Id**, **Ie** vary by a factor of *ca.* 60, the stability of these complexes is therefore governed by the rate constant of dissociation. From the results obtained with **Id** and **Ie** *(Table)* 2 facts become obvious. First, the most stable complex formed within the homologous series of **Id** is that with $n=4$, a phenomenon probably related to the influence of repulsive forces mentioned above. It must be pointed out that the structure of this compound is very similar to that of the prosthetic group FMN. Second, the stability of the complexes formed with compounds **Ie** increases with increasing number of methylene groups. This indicates that the most effective electrostatic interaction between the constituents of the complex requires a distance of *ca.* 1 nm between the N(10) atom and the terminal ionizable side chain group of the flavin.

As mentioned above, the proposal of *Barman & Tollin* [10] was based mainly on experimental traces obtained with native *A. vinelandii* flavodoxin. When we performed temperature-jump relaxation experiments with native *A. vinelandii* flavodoxin no relaxation was observed in 1 s after the temperature change but we could reproduce the published, experimental traces when we used a 1 min time scale [10]. The observations were therefore similar to those with native *P. elsdenii* flavodoxin (*cf.* above). Since these and the published data [10] were obtained under limiting instrumental conditions (working with very weakly or non-fluorescent molecules) these relaxation curves might possibly arise from artifacts; the following experiments proved that this conclusion was correct. When native flavodoxin was replaced by an identical concentration of egg albumin

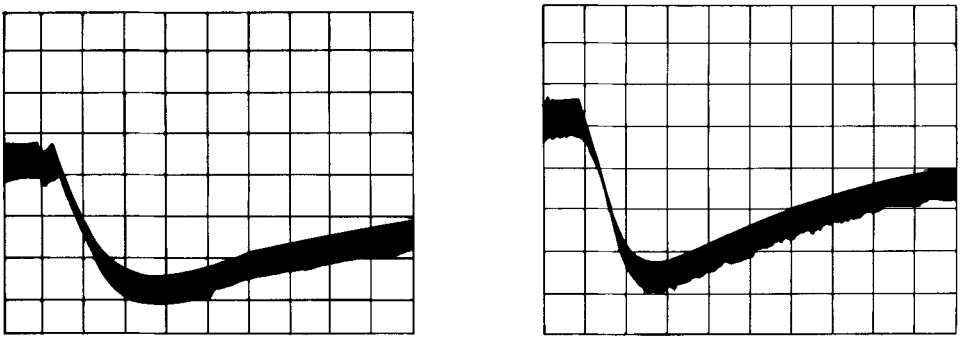
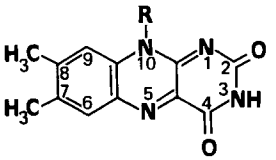


Fig. 3. *Experimental relaxation traces of "blank" solutions after a temperature-jump of 8.3°. Final temperature was 22°.* A *Schott* 485 glas filter was used in the fluorescence detection mode. The time constant was 1 ms. Upwards deflections represent an increase in light intensities. A large horizontal division corresponds to 5 s.

A) (left side) Egg serum albumin solution ($A_{280}=5.0$) in 30 mM phosphate buffer and 0.1 M KNO_3, pH 8.5 (20 mV/large vertical division). Incident wavelength was 366 nm.

B) (right side) 0.1 M KNO_3 solution in 30 mM phosphate buffer, pH 8.5 (100 mV/large vertical division). Incident wavelength was 366 nm; essentially the same trace was obtained when 440 nm as incident wavelength was used.

or by a 0.1 M KNO_3 solution alone the relaxation traces shown in *Figure 3A* and *B*, respectively, were obtained. The shapes of these traces are very similar to those obtained with the native flavodoxins (*cf.* [10], *Fig. 5, 8-10*). They are dependent on the incident wavelength and width of the diaphragm, placed in the incident light beam; *i.e.* a minimum diaphragm width caused an initial downward deflection followed by an upward deflection or *vice versa* reaching the original value within 1 min. The described artifacts are probably caused by stray light from the solutions and/or cell walls. Furthermore, the results obtained from the deoxy-FMN-apoflavodoxin complex *(cf. Table)* suggest that the temperature-jump technique cannot be applied to the native flavodoxins, because of the even stronger interaction with the natural prosthetic group. Even if the proposed 2-step binding mechanism [10] as opposed to our results would be accepted strong arguments against the analytical procedure of calculating the rate constants from the published traces (low signal-to-noise ratio and cooling effects, *cf.* [10]) can be brought forward. Our recalculation of the relaxation times using the published rate constants [10] and the appropriate equation [25] shows values of $\tau_1^{-1}=0.164\ s^{-1}$ and $\tau_2^{-1}=0.05\text{-}0.07\ s^{-1}$ instead of $0.137\ s^{-1}$ and $0.08\ s^{-1}\text{-}0.1\ s^{-1}$, respectively. Furthermore, since both equilibria relax at times not too far apart, the relaxations are coupled to each other and therefore both relaxation times



Ia R = $-CH_2-(CHOH)_3-CH_2OPO_3H_2$
Ib R = $-CH_2-(CHOH)_3-CH_2OSO_3H$
Ic R = $-(CH_2)_n-CH_2OH$, n = 2-5
Id R = $-(CH_2)_n-CH_2OPO_3H_2$, n = 1-5
Ie R = $-(CH_2)_n-COOH$, n = 1-5

become concentration dependent [25] which was not previously considered [10][6]. In addition, as shown recently by *Gafni et al.* [29], the calculated standard deviations of 2 relaxation times, which differ by a factor of 2 or less, can be as high as 40% for the longer relaxation time. From this it can be concluded that the procedure applied by *Barman & Tollin* [10] is invalid.

Conclusion. – From our data all of the flavins studied are bound to apoflavodoxins from *P. elsdenii* and *A. vinelandii* in a simple one-step process. This is in contrast to the conclusion of *Barman & Tollin* [10] who proposed a 2-step binding of FMN but a one-step binding of flavins which lacked the terminal phosphate group on the side chain. Our conclusions are in accord with those of *Dubourdieu et al.* [11] who used stopped flow and temperature-jump techniques to study the binding of flavin and protein in flavodoxin from *D. vulgaris*; these authors also conclude that the mechanism of binding involves a single step. The fact that the mechanisms of flavin binding in the 3 proteins are similar is not too surprising since the proteins have other physico-chemical properties and biological functions in common: X-ray crystallography has shown that the overall 3-dimensional structures of flavodoxins from *D. vulgaris* [23] and *Clostridium MP* [24] are very similar, and that, although different amino acid side chains interact with the flavin in the 2 structures, the flavin binding sites are also not too different.

The kinetics of the interaction between **Ib** and **Id** (n=4) *(cf. Table)* and the apoflavodoxin from *A. vinelandii* were also studied by independent methods. From stopped flow spectrofluorometry it was found that $k_1 = 5.6 \times 10^5$ $mol^{-1}ls^{-1}$ and $K_d = 0.2$ μmol l^{-1} for **Ib** [10] and from conventional fluorescence quenching technique $k_1 = 5.7 \times 10^5$ $mol^{-1}ls^{-1}$ and $K_d \sim 0.01$ μmol l^{-1} were found for **Id** (n=4) [13]. These values are in fair agreement with those given in the *Table* and provide further support for the one-step binding mechanism. It should be noticed however that any conformational changes that follow the initial bimolecular binding process would not be detected by these techniques if they occur much faster or much slower than the time resolution of the techniques.

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[6]) When the curves in [10] that were computed to the experimental traces are combined, a straight line with zero intensity is obtained, as would be expected of course for a reaction composed of 2 very similar relaxation times and in which the corresponding changes in light intensity are equal in amplitude, weighted equally, but of opposite sign.

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