STUDIES ON THE INTERACTION OF RIBOFLAVIN 5'-PHOSPHATE WITH PROTEINS

with special attention to

BACTERIAL BIOLUMINESCENCE

CENTRALE LANDBOUWCATALOGUS
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BIBLIOTHEEK
DER
LANDBOUWHOGESCHOOL
WAGENINGEN

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S T E L L I N G E N

1. Door uitsluitend uit te gaan van N^5- alkyl gesubstitueerde flavines, bestemmen Kemal et al. van te voren de plaats, waarop de hydroperoxide groep aan het flavine molekuul addeert. Het onbesproken laten van andere plaatsen van additie toont een onjuiste houding aan t.o.v. de (biochemische) problemen, waarvoor ze hun modellen aandragen.

   C.Kemal, T.W.Chan en T.C.Bruice, Proc.Natl.Acad. Sci.U.S. (1977) 74,405

2. Bij de beschrijving van de bepalingen voor de levensduur van erytrocieten wordt door Wintrobe een onvoldoende verklaring gegeven voor het verlies van radioactief chroom uit de cellen.

   M.M.Wintrobe, Clinical Hematology (1975) p.198

3. De experimentele gegevens van Morise et al. bevestigen niet hun conclusie, dat de intermoleculaire energie-overdracht van het luciferase naar het groen-fluorescerend eiwit, plaats vindt via een "Förster-mechanisme".

   H.Morise, O.Shimomura, F.H.Johnson en J.Winant Biochemistry (1974) 13,2656

4. Bij chromatografie van biochemisch materiaal wordt vaak onvoldoende gelet op de storingen, die optreden door concentratie van indifferente stoffen.

   W.W.Ward en R.J.Fastiggi Anal.Biochem.(1972) 50,154

5. De bewering van Morin et al. dat de vis Photoblepharon zijn lichtorgaan gebruikt om naast het aantrekken van zijn prooi, ook roofdieren te ontvluchten, is onwaarschijnlijk en wordt door geen van hun waarnemingen gesteund.

   J.G.Morin, A.Harrington, K.Nealson, N.Krieger, T.O.Baldwin en J.W.Hastings Science (1975) 190,74

6. "Negatief base-overschot" is een voorbeeld van een begrip dat de overdracht van kennis negatief beinvloedt.

b.v. R.J.M. Croughs en H.C. Hemker, De fysiologische basis van klinisch laboratorium onderzoek (1976) p.307

7. Waar, bij patiëntenbesprekingen, zoals bijvoorbeeld gepubliceerd in het Nederlands Tijdschrift voor Geneeskunde, een uitgebreider laboratoriumonderzoek vermeld wordt, dienen de gevonden waarden onderling duidelijk in overeenstemming te zijn, of dienen fysiologisch onwaarschijnlijke combinaties verklaard en/of besproken te worden.

8. Voor de beoordeling van manuscripten, ter publicatie in een wetenschappelijk tijdschrift, zijn de namen van de auteurs overbodige informatie.

C. McCutchen, The Sciences (1976) July/August p.25

9. Het cijfersysteem, zoals toegepast in de praktijk van het hedendaags onderwijs, werkt belemmerend op docent en student in het aanwakkeren van andere motivaties dan de onderlinge competitie.

R. Gast
Studies on the interaction of riboflavin 5'-phosphate with proteins, with special attention to bacterial bioluminescence
Wageningen, 19 april 1978.

Aan Iep, Rut en Daaf

Aan mijn M

Aan mijn ouders

Allen,die aan de totstandkoming van dit proefschrift hebben bijgedragen,ben ik zeer dankbaar.

"It is true
when one's reason runs a fever
one believes
as in a dream
to grasp this understanding;
but when one wakes up and the fever is gone
all one is left with are litanies of shallowness".

ERWIN CHARGAFF

A Fever of Reason
the early way

Annual
Review of
Biochemistry
1975

LIST OF ABBREVIATIONS

A_{280}	Absorbance at 280 nm
A. vinelandii	Azotobacter vinelandii
BF	the postulated chromophore of the BFP
BFP	blue fluorescence protein
B. harveyi	Beneckea harveyi
BSA	bovine serum albumin
DEAE-	diethyl amino ethyl-
dFMN	7,8-dimethyl-N(10)-(ε-hydroxypentyl)-isoalloxazine 5'-phosphate
D. vulgaris	Desulfovibrio vulgaris
E	enzyme
EDTA	ethylene diamine tetraacetic acid
F	flavin: 7,8-dimethyl-10-substituted isoalloxazine
FMN	riboflavin 5'-phosphate, oxidised form
$FMNH_2$	two electron reduced form of FMN
FP	flavoprotein
G+C	guanine + cytosine
I	ionic strength
k^o	reaction rate constant extrapolated to zero ionic strength
K_d	dissociation equilibrium constant
K_d^o	dissociation equilibrium constant extrapolated to zero ionic strength
K_M	Michaelis constant
MES	2-(N-morpholino)ethanesulfonic acid
P	protein
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
P. elsdenii	Peptostreptococcus elsdenii
P. fischeri	Photobacterium fischeri
P. phosphoreum	Photobacterium phosphoreum
Q_B	quantum yield of bioluminescence
ϕ_F	quantum yield of fluorescence
SDS	sodium dodecyl sulphate
TRIS	tri(hydroxymethyl) amino methane
$\bar{X}$	equilibrium concentration of reactant X
Z_X	charge of reactant X

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Ik wil geen schrijver meer lezen,wie men
het aanvoelt,dat hij een boek wilde maken,
doch slechts een zodanige,wiens gedachten
onvoorzien tot een boek werden.

Friedrich Nietzsche

I N T R O D U C T I O N

This thesis deals with two subjects,which are related with each other,Firstly,a detailed study is presented on flavin-protein interaction.There are around 50 different biochemical reactions catalysed by flavoproteins.The specificity for a particular reaction is dictated by the protein moiety of the enzyme.However in order to understand how the flavin is able to carry out such a variety of different reactions (1,2),a profound knowledge of the interaction with the protein moiety is required,even on the atomic level.For this reason relatively simple flavoproteins,flavodoxins were chosen for the studies presented in this thesis,in order not to complicate the interaction that is being studied,by such things as a monomer-dimer equilibrium or other prosthetic groups that also bind to the apoprotein.Secondly,the study on flavin protein interaction was applied to the bioluminescence of bacteria.Soon after Strehler (3) published the observation of the emission of light in cell-free extracts of luminous bacteria,it became evident that flavin played some kind of role in the reaction mechanism (4).Later it appeared likely from the experiments of Mitchell and Hastings that the excited state,prior to light emission,is an enzyme-bound complex,involving flavin (5).Then Eley et al. (6) proposed the luciferase-bound flavin cation as the emitting species in bacterial bioluminescence. Experiments performed by Murphy et al. (7),showing that FMN could be removed from a long-lived intermediate in the in vitro reaction were the reason to study flavin-luciferase interactions in more detail.Results of these studies are published in this thesis.During these studies,moreover,the real in vivo bacterial emitter was discovered and some of its properties are described.

FMN (riboflavin 5'-phosphate) is derived from the water-soluble vitamin riboflavin. The existence of the latter, as a yellow-green fluorescent pigment in milk whey was noted as early as 1879 by Blyth, who called it lactochrome. Its structure was established by synthesis in 1935 by Kuhn, Karrer and their coworkers. The isolation of a yellow enzyme from yeast by Warburg and Christian (8) in 1932 was the onset to a number of discoveries that flavins play an important functional role in many biological systems. Through the recognition that riboflavin is a part of the vitamin-B complex, the important link between a vitamin and a corresponding cofactor of an enzyme was made about the same time. The use of flavin as a cofactor in enzymatic reactions is due to its redox behaviour (9). The iso-alloxazine ring system is the active part of the molecule.

The flavin molecule "has the choice" of adding one or two electrons to the substrate in one reaction step. Another reason for the versatile role flavins play in nature, might be the fact that the standard redox potential of a protein-bound flavin can be dramatically shifted as compared with this potential for its free form (e.g. 11,12). Flavoproteins are dehydrogenation catalysts. Upon dehydrogenation of the substrate, the flavin moiety of the enzyme becomes reduced. The specificity towards the substrate identifies the enzyme. In general the reduced flavoprotein in turn, becomes substrate for a reaction with another electron acceptor, thereby regenerating the oxidized form of the prosthetic group. Specificity towards these latter acceptors is much less pronounced and this observation can be used to recognize groups of flavoproteins. The free reduced flavins react very rapidly with a wide variety of acceptors (13), which implies that the mentioned specificities are imposed on the flavin by the binding with the different apo-enzymes.

A knowledge of the nature of this binding might give us insight in the cause of the specificities of enzymes. Furthermore, we also may learn a lot about the requirements in general for such a powerful catalytic action as enzymes exhibit. Therefore the binding between flavins, notably FMN, and proteins have been studied as early as 1936 by Kuhn and coworkers (14). They reported on the interaction of the iso-alloxazine moiety of FMN

with the apo-enzyme of the "old yellow enzyme".These findings were later confirmed by Theorell and Nygaard who also found a strong interaction between the phosphate group of FMN and the apo-enzyme (15).They suggested that the negatively charged phosphate group is bound to a positively charged amino group of the protein (16).Aromatic amino acid residues were suggested as candidate groups from an apoprotein for the interaction with the iso-alloxazine moiety of the flavin.In this way the observed quenching of the fluorescence from the flavin,which occurs in most flavoproteins,could be explained,as was proposed by Weber (17).Indeed a complete blocking of flavin binding was observed upon iodination of one phenolic group of the apo-enzyme (18).However interpretation of this type of studies is not unambiguous as iodination might severely alter the spacial structure of the enzyme,making it thus unfit for binding the flavin.Flavins are released from the enzyme by a variety of methods,one method being more succesfull in a particular case than another.Treating the flavoprotein with a high concentration of a salt -KBr (19) $CaCl_2$ (20) or $(NH_4)_2SO_4$ (21) - at a particular (low) pH,or denaturing the enzyme with trichloro-acetic acid (22) or guanidinium salt (23) usually releases the flavin from the enzyme.Therefore it can be concluded that the binding of the flavin to the apo-enzyme is noncovalent and the forces involved must be of electrostatic and/or hydrophobic character.A different situation exists in succinate dehydrogenase together with around six other enzymes,where the flavin is covalently bound,via the 8-alpha position to an amino acid residue of the polypeptide chain (24,25).

For one class of flavoproteins,the flavodoxins, a lot of progress has been made in the research of the forces involved in flavin-protein binding,through X-ray studies (26,27).This class of flavoproteins, that serve as electron carriers at a low potential, has been reviewed comprehensively recently (28).In the structures so far examined,it appears that the iso-alloxazine ring system is not totally buried in the inside of the enzyme molecule,but partly exposed to the solvent,while the ribityl side chain is buried almost completely within the interior of the folded polypeptide chain.The aim of the investigations

reported in the first part of this thesis was to obtain more insight into the dynamics of the flavin-apo-enzyme binding in these flavodoxins.

The second part of this thesis deals with the role of FMN in the activation and emission process of bacterial bioluminescence. The binding of FMN to bacterial luciferase has been studied for both the oxidized (29) and the reduced form (30,31) of the co-enzyme. Bound to this enzyme $FMNH_2$ has a much higher electron affinity than in the free form. This results in a comparatively slow reoxidation when exposed to air (32). Consequently Lee and Murphy (33) showed that in order to outcompete the rapid oxidation of free $FMNH_2$ -the so called auto-oxidation -, one has to use at least a concentration of over 30 micromolar of the bacterial luciferase, while studying the fate of $FMNH_2$ in this system. A brief introduction to the subject of bacterial bioluminescence will be given at this point.

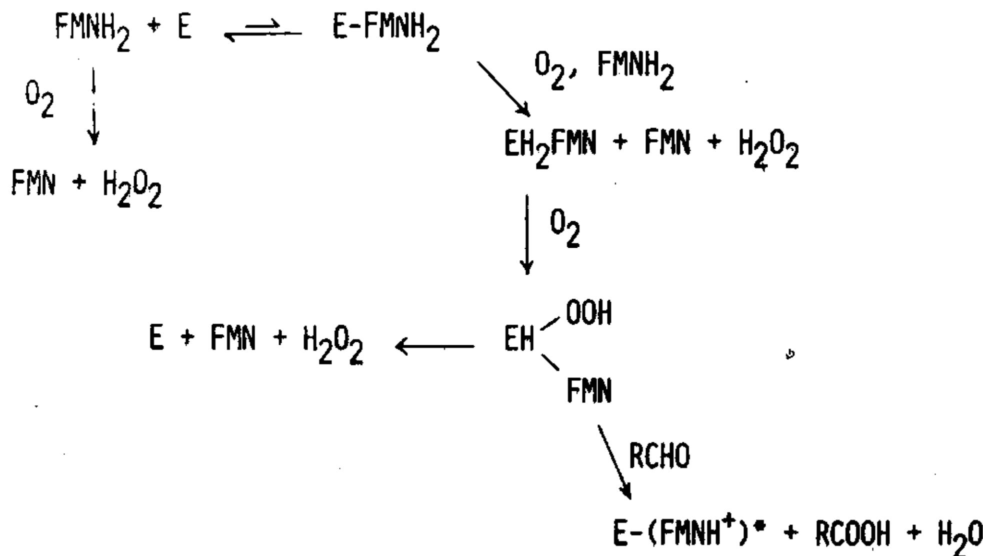
Bioluminescence (34), the emission of light by living matter, has fascinated people from the earliest times. The luminescence of flesh, now known to be caused by saprophytic bacteria, was known to Aristotle who wrote in "DE ANIMA" of things that "give light in the dark". In 1742 Baker for the first time suggested that the "phosphorescence" of dead fish and flesh might be due to "animacules". Before that time many of the fundamental discoveries concerning bacterial bioluminescence were made without the realisation that living organisms were involved. In this way the famous Robert Boyle proved in 1668 that the luminescence is dependent on air, by using his air pump. Now we know that it is the oxygen part of the air that is the active factor. In 1875 Pfüger inoculated a medium after filtering the bacteria, thus proving that the bioluminescence originated from the bacteria. Around the turn of the century then a host of papers were published on luminescent bacteria. Kutscher isolated in 1895 a luminous Vibrio from the river Elbe, a species very much related to the Vibrio cholera. This bacterium is the only luminous fresh water species, but there are only a few studies done on it. All of the other luminous bacteria are of marine origine and today classified into three main groups (35). The most widely studied species are: for group A (with a molar % Guanine +

Cytosine in their DNA of 39.0 - 40.5)Photobacterium fischeri; for group B (with a molar % G+C of 41.2 - 43.8)Photobacterium phosphoreum and for group C (with a molar % G+C of 45.0 - 48.2) Beneckea harveyi.Although for a beetle an in vitro luminescent reaction was already demonstrated as early as 1885 by Dubois (36),it was not untill 1953 that this was done for bacteria by Strehler (3).Since the bioluminescent reaction of bacteria has been studied mostly on extracts, with the result that more is known about the in vitro reaction than about the in vivo one.In vitro bioluminescence is an enzyme-catalysed chemiluminescence.The enzyme catalysing the reaction is called luciferase.Bacterial luciferase catalyses the oxidation of $FMNH_2$ and a long-chain aliphatic aldehyde,with molecular oxygen as final electron acceptor.It is likely that free $FMNH_2$ is not available within the cell and flavoproteins have been isolated that have activity in the light reaction (37,38).Both H_2O_2 and the corresponding long-chain aliphatic acid have been identified as products of the reaction (39 - 41),with the same quantum yield (Q_B) of formation as the quantum yield of utilisation of the aldehyde. Bacterial luciferase is a protein of molecular weight 80 000 and has no prosthetic group bound to it (42).It may be reversibly dissociated into two subunits which differ slightly in molecular weight (43).

The already mentioned association of $FMNH_2$ with the luciferase is the first step in the in vitro reaction sequence.After reaction with molecular oxygen relative stable intermediates are formed.Finally aldehyde can be added and production of light occurs (44).If no aldehyde is added to the reaction mixture,the intermediate eventually decays to produce FMN,H_2O_2 and free luciferase.The proposed reaction mechanism is presented in Scheme 1.

The decay time of the intermediate,both in the absence or in the presence of aldehyde,is dependent upon the type of luciferase.For the type isolated from Beneckea harveyi,the lifetime can be extended to about an hour,as will be described in chapter 4.

As far as the emitting lumiphore is concerned, it became soon clear that FMN itself, although a product of the in vitro reaction, could not be considered a fair candidate, because its fluorescence maximum is too far red-shifted, as to make it possible, by perturbation, to match the bacterial emission spectrum, whose maxima range from 476-505 nm. In chapter 5 a review is given of all the different proposals that have been made for the emitting species. All current speculations as to the nature of the emitter are based on the similarity of the fluorescence spectra with those obtained from the bioluminescence reaction. In this thesis a novel protein is presented that fulfills all of the requirements to qualify it for the in vivo emitter. The protein is found in the extracts of the luminous bacteria.



SCHEME 1

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Niet het stellen van vragen,maar het
geven van antwoorden is vaak onbescheiden.

Oscar Wilde

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STUDIES ON THE BINDING OF FMN BY APOFLAVODOXIN FROM *PEPTOSTREPTOCOCCUS ELSDENII*

pH AND NaCl CONCENTRATION DEPENDENCE

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SUMMARY

1. The pH and ionic strength dependence of the interaction of FMN with apoflavodoxin has been studied by fluorometry in the pH region 2–5, at 22 °C.

2. The rate constant of dissociation and the dissociation constant were experimentally determined; the rate constants of association were calculated at a given pH value. These constants depend on the ionic strength. The plots of these constants against the square root of the ionic strength are straight.

3. Our data have been interpreted in terms of the Brönsted theory, which relates chemical reaction rates to ionic strength. The data indicate that the apoenzyme reaches its maximum net positive charge at pH 2.0–2.6. The calculated net charge in this pH region is between 11 and 12 and is in agreement with the theoretical value of 12 as deduced from the primary structure of the protein. The isoelectric point of the holoenzyme is about 4.

4. The rate constant of association extrapolated to zero ionic strength is $3.2 \cdot 10^5 M^{-1} \cdot s^{-1}$ and is pH-independent.

5. The rate constant of dissociation and the dissociation constant extrapolated to zero ionic strength depend on the pH. The results are explained by assuming that there are two protein ionizations with a pK value of 3.4; these ionizing groups are possibly close to the FMN binding site.

INTRODUCTION

Flavodoxins are small flavoproteins which contain one molecule of FMN as prosthetic group and function as electron carriers of low redox potential in reactions catalyzed by crude extracts of a variety of micro-organisms (e.g. refs. 1–7). Their

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Abbreviation: Mes = 2-(*N*-morpholino) ethanesulfonic acid.

physico-chemical properties have been extensively studied (e.g. refs. 8–11), and recently the crystal structures of flavodoxins from *Clostridium* MP [12] and *Desulfovibrio vulgaris* [13] have been determined at high resolution. The primary structures of several of them are also known [14–17]. The small size of these proteins and the ease with which stable apoenzymes can be prepared from them, make the flavodoxins very suitable for studies on the kinetics of the interaction between protein and flavin. Based on their reactions with different flavins, apoflavodoxins fall into two groups: those in one group (*Clostridium pasteurianum*, *Peptostreptococcus elsdenii* and *Clostridium* MP) are specific for flavins at the level of FMN [18, 19]; proteins in the second group (e.g. *Azotobacter vinelandii* and *D. vulgaris* flavodoxins) show much less specificity, forming complexes also with riboflavin and lumiflavin analogs [20, 21]. In order to further define the specificity of apoflavodoxins in the first group, we have undertaken a detailed kinetic study of the interaction of *P. elsdenii* apoflavodoxin with FMN.

MATERIALS AND METHODS

Flavodoxin from *P. elsdenii* LC 1 was isolated and purified according to the method of Mayhew and Massey [6]. The apoenzyme was prepared by dialysis against 2 M KBr in 0.1 M sodium acetate, pH 3.9 [18]. Concentrations of apoenzyme were determined by titration with pure FMN [22], prepared by affinity chromatography according to Mayhew and Strating [23]. Concentrations of holoenzyme were determined using the published extinction coefficient of 10 200 $M^{-1} \cdot cm^{-1}$ at 445 nm [6].

In experiments conducted at low ionic strength special attention was paid to the pH value of the buffer solutions [10]. The pH value was checked before and after each kinetic run and only those experiments in which the pH value did not vary more than 0.05 of a pH unit were evaluated. For different pH regions the following buffers were used at concentrations not exceeding 3 mM: pH 2, HCl; pH 2.6, glycine/HCl; pH 3.2–3.8, formic acid; pH 3.8–5.1, acetic acid; and Mes for higher pH values. To obtain solutions of a particular ionic strength, two solutions (A and B) of each buffer were prepared. Solution A consisted of the buffer of low ionic strength whereas solution B contained buffer plus 0.5 M NaCl. Solutions A and B were mixed in the appropriate ratio. The pH values of the solutions prepared in this way were then checked and if necessary adjusted. Double glass-distilled water was used throughout.

The kinetics of the dissociation of holoflavodoxin into its constituents were determined by adding 10 μl of a solution of holoenzyme (0.1 mM in 3 mM Mes, pH 6.30) to 1 ml of buffer. The increase of fluorescence emission at 524 nm due to the dissociation of the flavin from the holoenzyme was then followed on an Hitachi Perkin Elmer MPF-2A spectrofluorometer using an excitation wavelength of 450 nm. The dissociation reaction was also followed by measuring the increase in protein fluorescence emission using an excitation wavelength of 295 nm and an emission wavelength of 333 nm. The equilibrium was usually reached within 3 min and could be maintained for at least 30 min. Even when the pH of the equilibrium was 2.6 the reaction was more than 95% reversible upon changing the pH rapidly to 6.3. The equilibrium constant was determined from the experimental increase in fluorescence by comparing it with that of an equimolar amount of free FMN under the same conditions. The experiments were conducted at 22 °C unless otherwise stated.

RESULTS AND DISCUSSION

As shown by fluorometric titration experiments [10], *P. elsdenii* apoflavodoxin forms a rather stable complex with FMN at neutral pH. To obtain information about the kinetics of dissociation of the holoenzyme it is therefore necessary to use experimental conditions where the interactions between the apoenzyme and prosthetic group are much weaker. Previous work [18], which showed that the apoenzyme can be prepared by acid treatment of the holoenzyme in the presence of KBr, indicates that the flavin-protein interactions are weak at low pH. We have therefore studied the dissociation and association processes in the pH range 2–5.

The dissociation of the holoenzyme (E-FMN) into its apoenzyme (E) + FMN has been followed by measuring the increase of the flavin fluorescence emission until the new equilibrium of reaction 1 was established.

$$\text{E-FMN} \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{E} + \text{FMN} \qquad (1)$$

Under equilibrium conditions the dissociation constant of this reaction is

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[\text{E}]\,[\text{FMN}]}{[\text{E-FMN}]} \cdot \frac{\gamma_{\text{E}} \cdot \gamma_{\text{FMN}}}{\gamma_{\text{E-FMN}}} \qquad (2)$$

where γ is the activity coefficient of the various species. Since γ is not known, the experimentally determined dissociation constant is

$$K_d' = \frac{k_{off}'}{k_{on}'} = \frac{[\text{E}]\,[\text{FMN}]}{[\text{E-FMN}]} = \frac{x_e^2}{a - x_e} \qquad (3)$$

where a is the initial concentration of the holoenzyme and represents 100% fluorescence yield if complete dissociation occurs, x the concentration of dissociated holoenzyme (calculated from the observed fluorescence at a given time) and x_e the concentration of dissociated holoenzyme at equilibrium. If the dissociation of the holoenzyme into its constituents is as simple as assumed in reaction 1, the dissociation reaction must obey the differential equation

$$-\frac{\text{d}\,[\text{E-FMN}]}{\text{d}t} = k_{off}'\,[\text{E-FMN}] - k_{on}'\,[\text{E}]\,[\text{FMN}] \qquad \text{or} \qquad (4)$$

$$-\frac{\text{d}\,(a - x)}{\text{d}t} = k_{off}'\,(a - x) - k_{on}'\,x^2 \qquad (5)$$

Solution of this equation between x_i (initial) and x, and t_i and t, yields the integrated rate law for a first-order, second-order equilibrium reaction [24]:

$$k_{off}' \cdot t = \frac{x_e}{2a - x_e} \ln \frac{x\,(a - x_e) + x_e\,a}{a\,(x_e - x)} \qquad (6)$$

Substitution of the experimentally determined values of a, x, x_e and t from kinetic runs into Eqn. 6 should yield straight lines. That this is the case is shown in Fig. 1. The association rate constant (k_{on}') was calculated from k_{off}' and K_d, the latter being calculated according to Eqn. 3.

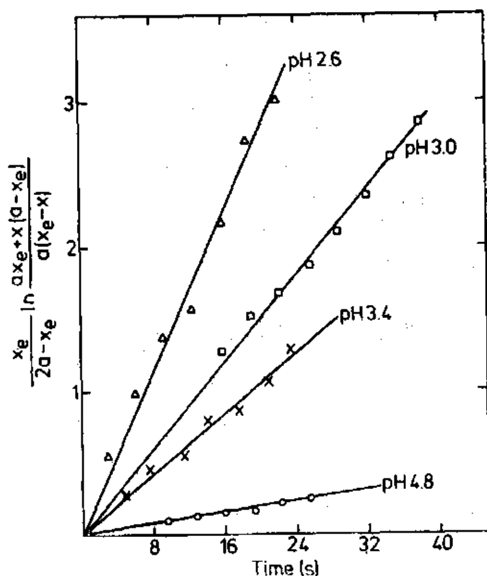


Fig. 1. Effect of pH on the observed k_{off}. Plot of the integrated Eqn. 6 vs. time for four different pH values. Flavodoxin concentration was in all cases 1 μM and I = 22.5, 22°C. (for further details see Materials and Methods).

It was noticed that k'_{off} (Fig. 2A) and K_d depend not only on the pH but also on the ionic strength. Data similar to those shown in Fig. 2A but obtained at higher pH values, indicate that at pH values larger than 4.5 an increase of the ionic strength results in a stabilization of the holoenzyme and thus causes a decrease in k'_{off}. In addition, k'_{off}, rather than being independent of or varying linearly with the ionic strength (I), depends on $\sqrt{I}$ at pH values higher and lower than 3.8–4.2 (Fig. 2A, B). Note that also the slope of the lines changes with pH. As might be anticipated, the value of k'_{on} also depends on the ionic strength and in fact is much more sensitive to it than k'_{off}.

The equation describing the dependence of chemical reaction rates on the ionic strength was first derived by Brönsted and Bjerrum and is at 22 °C (cf. ref. 24).

$$\log k' \approx \log k^0 + 1.0\, Z_A Z_B \sqrt{I} \tag{7}$$

where k' is the measured rate constant at ionic strength I, k^0 the rate constant extrapolated to zero ionic strength, and $Z_A Z_B$ the product of the charges of the reacting species. According to theory, Eqn. 7 is valid only for solutions of low ionic strength (10 mM or less), while it is assumed that the charge is distributed uniformly (spherical symmetry) and that all charges interact with the surrounding ionic atmosphere. It is to be expected therefore that the Brönsted law will be obeyed only qualitatively by proteins, especially at a pH value where the protein carries only a small net charge. Applying Eqns. 2, 3 and 7 to our system, we obtain:

$$\log K'_d = \log K^0_d - 1.0\, Z_E \cdot Z_{FMN} \sqrt{I} \tag{8}$$

and

$$\log k'_{on} = \log k^0_{on} + 1.0\, Z_E \cdot Z_{FMN} \sqrt{I} \tag{9}$$

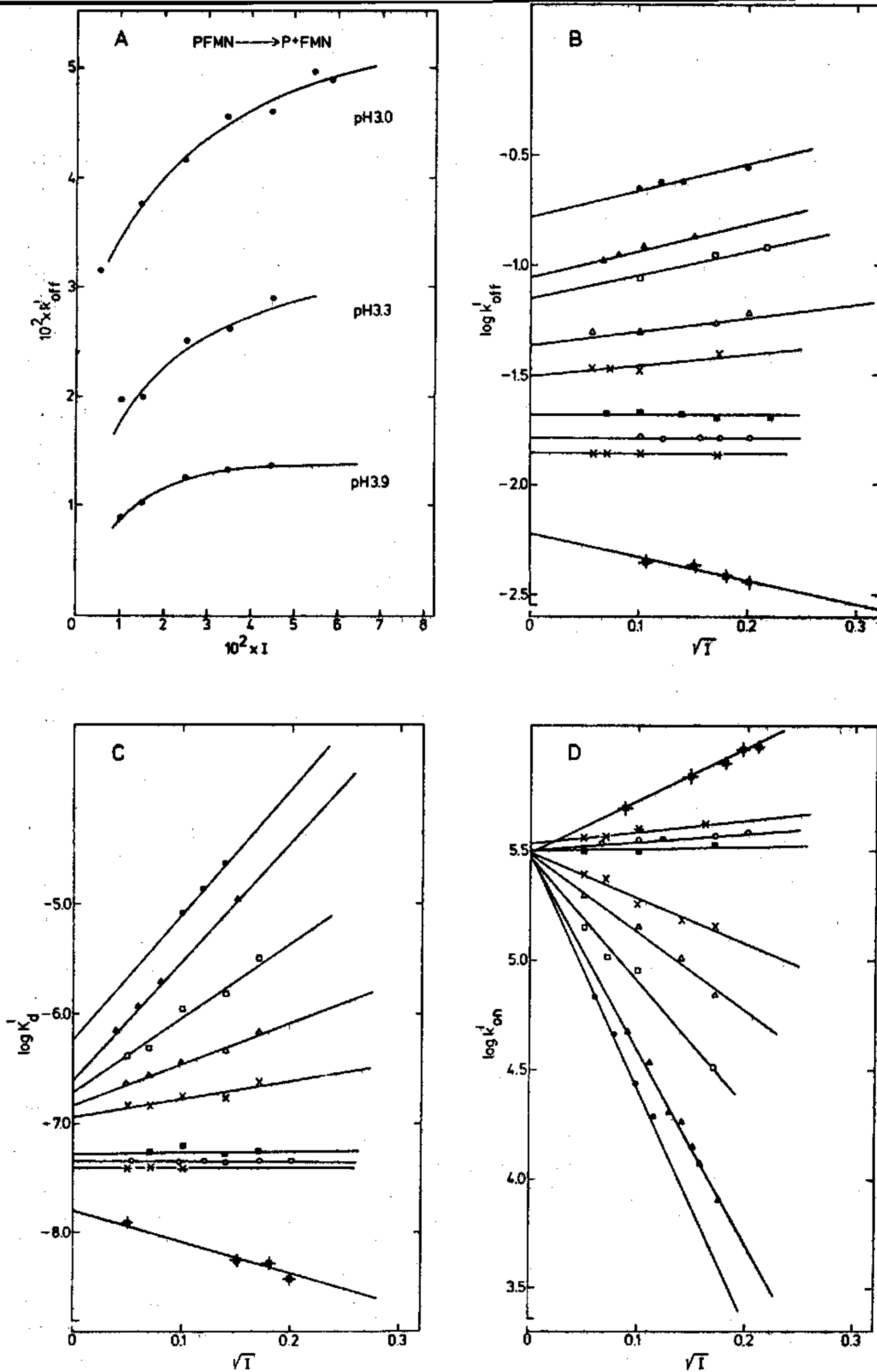


Fig. 2. Dependence of k'_{off}, k'_{on} and K'_d on the ionic strength. (A) Plot of k'_{off} vs. ionic strength. (Experimental conditions as in Fig. 1.) (B) Experimentally determined log k'_{off} vs. $\sqrt{I}$. (C) Experimentally determined log K'_d vs. $\sqrt{I}$. (D) Calculated log k'_{on} vs. $\sqrt{I}$. The various pH values are indicated by the following symbols: ●—●, pH 2.0; ▲—▲, pH 2.6; □—□, pH 3.2; △—△, pH 3.4; ×—×, pH 3.6; ■—■, pH 3.8; ○—○, pH 4.2; ⸸—⸸, pH 4.6; ✚—✚, pH 5.0.

Eqns. 3, 8 and 9 predict that when the logarithms of k'_{on} and K'_d are plotted versus the square root of the ionic strength, the lines should be linear and have opposite slopes. From the relationship between K'_d, k'_{on} and k'_{off} it follows that k'_{off} should be independent of ionic strength. Fig. 2C-D shows that the prediction with respect to K'_d and k'_{on} are fulfilled by the experimental results, and further, that except in the pH region 3.8–4.6, the ionic strength effects measured for K'_d and k'_{off} and calculated for k'_{on} depend in turn on the pH. As the pH is decreased below 3.6, the effects of ionic strength on K'_d and k'_{on} become increasingly pronounced, and in contrast to expectation, a small but similar dependence on $\sqrt{I}$ develops for k'_{off} (Fig. 2B). This last finding is not in accord with the theory. However, it has been found experimentally that most reactions that are predicted to be independent of ionic strength in fact show some ionic strength dependency [24]. The results of Fig. 2B have been used only to evaluate the pH-dependence of k'_{off} at $I = 0$ (see below). Extrapolation of the lines of Fig. 2B-D shows that K'_d and k'_{off} depend on the pH even at zero ionic strength; at $I = 0$, K'_d and k'_{off} increase by factors of 40 and 60, respectively, between pH 5 and pH 2 (Fig. 2B, C); k'_{on}, on the other hand, is pH-independent at $I = 0$ (Fig. 2D), and its extrapolated value is $3.24 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at 22 °C

For reasons discussed earlier, our measurements of K'_d and k'_{off} were not extended above pH 5. However, the limited data at this higher pH indicate that the two constants not only continue to decrease with increasing pH, but that, in contrast to their behaviour in the low pH region, they also decrease with increasing ionic strength. Hence, increasing ionic strength at pH 5 stabilizes the complex of protein and FMN. Fig. 2D shows that the calculated values of k'_{on} increase with ionic strength at pH 5, but the extrapolated value at $I = 0$ remains pH-independent. These calculations are therefore not in accord with earlier direct measurements of k'_{on} which indicated that the association rate constant decreased 3-fold between pH 4.5 and pH 6 in 0.01 M sodium acetate buffer [25]. This discrepancy between the observed and calculated values of k'_{on} near pH 5 is not yet understood, though it should be noted that the earlier measurements were made at a lower temperature, the apoenzyme was diluted from phosphate buffer, which is known to be inhibitory, and the results were not corrected for the change in ionic strength of acetate buffers between pH 4,5 and pH 6.

In addition to the specific ionic strength effects observed in this work, the association of FMN and apoflavodoxin also depends on the type of anions present in solution. For example, Mayhew [25] observed that at pH 7, high phosphate concentrations inhibit the rate of binding, while chloride reverses this inhibition. When, the results of Mayhew (ref. 25, Fig. 6) are replotted in the form of log k'_{on} versus $\sqrt{I}$, surprisingly enough a straight line is obtained for the points between $I = 0.6$ and 0.04, and extrapolation of the line to zero ionic strength gives a value of $1.2 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at 0.5 °C. When this value is corrected for the difference in temperature using an activation energy of 8.3 kcal per mol [25], a value of $3.5 \cdot 10^5\ M^{-1} \cdot s^{-1}$ at 22 °C is obtained, in good agreement with the value of k'_{on} at zero ionic strength given above. This suggests that at zero ionic strength k'_{on} remains pH-independent between pH 2 and pH 7.

It appears that the Brönsted equation is applicable to a number of systems in which proteins interact with smaller charged molecules. Recently Shiga and Tollin [26] have published results on the ionic strength dependence of the association

rates of *Azotobacter vinelandii* apoflavodoxin and FMN. These studies, conducted at pH 8 and in the presence of rather high concentrations of phosphate, phosphate-acetate, Tris and pyrophosphate buffers, revealed that the reaction is inhibited by phosphate and acetate ions, and "activated" by pyrophosphate and Tris ions. When we replot the published data for pyrophosphate according to Brönsted, a straight line is obtained. The line passes through the origin suggesting that pyrophosphate itself is involved in the recombination reaction. Similarly, straight lines are obtained when the results of Gianfreda et al. [27], for the ionic strength- and pH-dependencies of the binding of pyridoxyl- and pyridoxamine phosphate to aspartate amino transferase, are replotted in the form log k'_{on} versus $\sqrt{I}$.

Since the plots of Fig. 2C-D are linear, the Brönsted equation can be used to obtain information about the charges of the reacting species; the slopes of the plots yield an approximate value for $Z_E Z_{FMN}$ (cf. ref. 24). In the pH region 2–5, FMN possesses one negative charge on the phosphate ester group [28]. Since the charge of FMN remains constant in the pH region of the present study, the slopes of the lines of Fig. 2C-D represent the net charges of the apoenzyme. Our results show that k'_{on} is independent of the ionic strength in the pH region 3.8–4.6 (Fig. 2D). In this pH region, therefore, the apoenzyme carries no net charge and is at its isoelectric point, a conclusion that is fully in accord with data obtained from the primary structure of the protein [14]. Below pH 3.8 the slopes of the lines for k'_{on} and K'_d (Fig. 2C, D) increase and approach a maximum between pH 2.6 and 2.0, indicating that in this pH region, flavodoxin attains its maximum positive charge. The calculated value for Z_E in this region is between 11 and 12; again this value is in excellent agreement with the fact that the protein contains 11 basic amino acid residues [14]. The slope of the line for k'_{on} at pH 5 suggests that 2–3 negative charges are involved in the recombination reaction. One of these charges would be due to FMN; the other(s) presumably arise(s) as a result of deprotonation of acidic amino acid residue(s).

The observed pH dependence of the dissociation rate constant was analysed by assuming that all protonations occur rapidly in comparison with the dissociation reaction, and that all acid-base equilibria are also fast. The pH profile of the dissociation rate constants, extrapolated to zero ionic strength, is shown in Fig. 3 for the pH region 2.6–4.6. The maximum slope of the curve of Fig. 3 is 0.78. Furthermore,

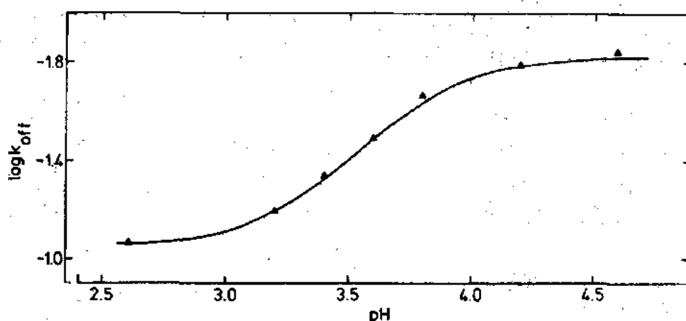
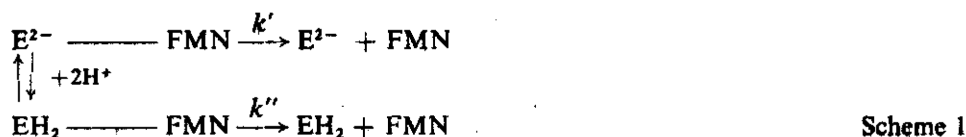


Fig. 3. Effect of pH on k_{off} obtained by extrapolation to zero ionic strength. The points (△) represent values for log k_{off} obtained by extrapolation of the experimental data to zero ionic strength (Fig. 2B); the solid line shows the theoretical curve obtained using Eqn. 10 (see text) and the following values: $k' = 0.015\ s^{-1}$, $k'' = 0.088\ s^{-1}$ and $K^2_{H^+} = 158\ nM^2$.

the curve of Fig. 3 approaches a constant value at the two pH extremes indicating that two rate constants govern the pH-dependent dissociation reaction. From the two extremes of the curve of Fig. 3 we can estimate these rate constants, $k' = 0.015\ s^{-1}$ and $k'' = 0.088\ s^{-1}$, for the high and low pH region, respectively. With this information on hand we attempted to calculate the theoretical curve for the pH-dependent dissociation reaction. Of several possible reaction schemes for the dissociation of E-FMN, involving either one or two protonation steps on the same or different species, the only one that fitted well with the experimental curve was a model with two protonation steps having identical or very similar pH values (Scheme 1).



Scheme 1

According to Scheme I the pH-dependence of the dissosiation rate constant becomes:

$$k_{off} = \frac{k'}{1 + [H^+]^2/K^2_{H+}} + \frac{k''}{1 + K^2_{H+}/[H^+]^2} \qquad (10)$$

where $K^2_{H+} = \frac{[E^{2-} - FMN]\,[H^+]^2}{[EH_2 - FMN]}$

The theoretical curve of Fig. 3 has been calculated with the aid of Eqn. 10 and employing the following values:

$k' = 0.015\ s^{-1}$, $k'' = 0.088\ s^{-1}$ and $K^2_{H+} = 158\ nM^2$.

The excellent agreement between experiment and theory strongly suggests that two protonations are involved in the dissociation reaction. A model of the flavin-binding region of flavodoxin from *P. elsdenii* has been constructed by James et al. [29] based on the three dimensional structure of flavodoxin from *Clostridium* M.P. [12] and the primary structure of *P. elsdenii* flavodoxin [14]. This model predicts that the two glumatic acid residues 60 and 61 are hydrogen bonded to FMN. It can be tentatively assumed that the two ionizations observed in the pH dependence of the dissociation rate constant (Fig. 3) are due to these two glutamic acid residues. This is not an unlikely assumption considering that the two glutamic acid residues could exhibit identical or very similar pK values and thus act highly co-operatively. The high cooperativity could easily be understood if the strain in the flavin-protein interaction becomes intolerable for one glutamic acid residue when the hydrogen bond of the other one is broken.

Last but not least it can be derived from these results that the method of preparing apoenzymes from flavoproteins by applying low pH at high salt concentrations derives its merits from the large decrease of k'_{on} that occurs under these conditions.

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---his head crammed with
theories of probability,
theories of numbers,
theories of error,
a dead brain,a dried-out soul?
What would be left to think with?
What would be left
for learning about life?

ALEKSANDR I. SOLZHENITSYN

The First Circle
chapter 9

THE BINDING OF FLAVINS BY APOFLAVODOXINS FROM PEPTOSTREPTOCOCCUS ELSDENII AND AZOTOBACTER VINELANDII AS STUDIED BY TEMPERATURE-JUMP TECHNIQUE.

Robert Gast and Franz Müller, Department of Biochemistry, Agricultural University, Wageningen, The Netherlands.

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.

It was found that P.elsdenii apoflavodoxin interacts only with flavins possessing five carbon atoms in the N(10) side chain and a terminal phosphate group. Employing a wide range of concentrations of deoxy-FMN 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 (cf. Structure I) have also been investigated. It is shown that 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 (Barman and Tollin, Biochemistry 11,4746 (1972)). It is shown that the published traces are instrumental artifacts.

INTRODUCTION

Flavoproteins can be divided into two 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 is covalently linked to the polypeptide chain (for a review on the subject see (1)).Methods have been developed for the preparation of stable apoenzyme from flavoproteins in the first class (e.g.2,3).Among these flavoproteins there are a number of low molecular weight proteins which are especially suitable for a study of the kinetic parameters of the flavin-apoenzyme interaction. We have chosen to work with Peptostreptococcus elsdenii and Azotobacter vinelandii flavodoxins because their physical and chemical properties have been investigated in detail (4-7).The interaction between FMN 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 techniques used in ref.(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 and Tollin (10) to investigate the kinetics of the interaction of FMN with apoflavodoxins from A.vinelandii and P.elsdenii (10) and also the flavodoxin from Desulfovibrio vulgaris has been studied by this technique (11).The latter enzyme exhi-

bited a single relaxxation 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 which exist between the flavodoxins from A.vinelandii and D.vulgaris (7).Several explanations might account for the two different kinetic patterns observed,the most important of which is that the dissociation constants of flavodoxins from P.elsdenii and from A.vinelandii are very small and 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 so small in fact 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 two-step binding mechanism proposed by Barman and 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 prified according to the method of Hinkson and Bulen (12).The apoenzyme was prepared by the method of Edmondson and Tollin (13).Flavodoxin from P.elsdenii,LC 1,was isolated and purified according to the method of Mayhew and Massey (4).The apoenzyme was prepared by dialysis against 2M KBr in 0.1 M 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°C.Concentrations of holoenzymes were determined using the published extinction coefficients of 10 200 $M^{-1}cm^{-1}$ for flavodoxin from P.elsdenii (4) and 10 600 M^{-1} cm^{-1} for flavodoxin from A.vinelandii (13).

The kinetics of binding of flavin analogs were determined at 22°C (final temperature) at pH 8.5 in Tris-HCl or pyrophosphate buffer (0.1 M) containing 0.1 M 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 G.m.b.H.,Göttingen,Germany) was modified for fluorescence measurements by placing appropriate mirrors behind two of the four cell windows.This modification caused a threefold 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°C.At approximately 4 min. intervals the temperature of the solution was increased 8°±0.3°C by means of a calibrated high-voltage discharge (20kV) from a 0.05 microF capacitor and the resulting relaxation traces were stored in a Data-lab transient recorder DL 905.The data were recorded by plotting the output via a strip chart recorder.Our instrument maintains the highr temperature for about 2 s.After this time interval cooling starts to become noticeable.The exponental heating time was approximately 11 microseconds for a 8.3°C temperature change.

We wish to report here on some factors leading to artifacts using fluorescence detection in temperature-jump experiments when working with flavins and flavoproteins.First,free flavins bearing an hydroxyl group at the 2'-position are subject to intramolecular photoreduction (e.g.15),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 and causes an apparent increase of the fluorescence intensity. This effect occurs within 5 s after the temperature jump under normal viscosity conditions (water, room temperature) 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 into a conventional spectrofluorimeter equipped with a photomultiplier possessing the same sensitivity as the one used in the temperature-jump instrument.

The flavin derivatives (see Structure I) used in this study were prepared according to published procedures; riboflavin 5'-monosulphate (17); N(10)-omega-hydroxyalkyl flavins containing various numbers of methylene groups (n= 2-6) according to (18); the latter compounds were phosphorylated by the method of Flexser and Farkas (19). The analog caboxylic acids were synthesized according to Föry et al. (20). Each of the derivatives gave one spot by thin layer chromatography in at least two different solvent mixtures, and were therefore judged to be pure.

RESULTS AND DISCUSSION

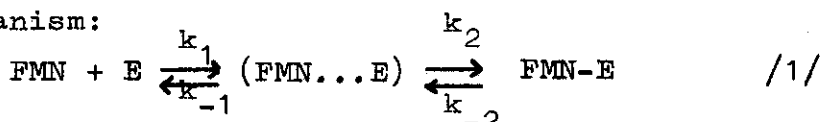
In applying the temperature-jump relaxation technique to study fast chemical reactions,certain conditions have to be fulfilled.For instance in a bimolecular reaction,the interaction between the reactants should not be too strong or too weak.In addition the system must possess a favourable change in enthalpy,so that a measurable displacement of equilibrium occurs by the instantaneous heating of the system.

In the study conducted by Barman and Tollin (10) native flavodoxins from A.vinelandii and from P.elsdenii were employed.It is known (5,13),however that FMN is tightly bound by the apoenzymes of these proteins 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

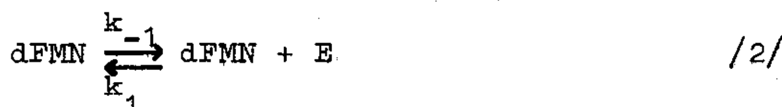
The interaction between the apoenzyme and various FMN derivatives modified at positions 2,3,6 and 8 of the isoalloxazine ring has been studied (21).Further more,from the fact that riboflavin does not bind to

the apoenzyme, it has been concluded (7) that the 5'-phosphate group of the flavin is absolutely required for effective binding. Our measurements with a number of FMN derivatives having side chains of varying length and a variety of terminal groups on the side chain (cf. Structure I) confirm this view. The only compound that interacted with the apoenzyme was dFMN (Id, n = 4) and this was already known (21) to interact with the apoenzyme about three orders of magnitude less strongly than FMN. With this system we tested the proposal of Barman and Tollin (10) that the 5'-phosphate group triggers a conformational change in the apoenzyme (E) and leads to a two-step binding mechanism:



$$\mathrm{FMN} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (\mathrm{FMN} \ldots \mathrm{E}) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{FMN{-}E} \qquad /1/$$

Temperature perturbation of the dFMN-apoenzyme complex yields an increase in fluorescence intensity (Fig. 1). The analysis of the experimental traces which showed 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:



$$\mathrm{dFMN} \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} \mathrm{dFMN} + \mathrm{E} \qquad /2/$$

This has been verified by using a wide range of concentrations of the components of the system. A linear relationship was found between the sum of the concentrations of free dFMN and apoenzyme and the reciprocal value of the corresponding relaxation times

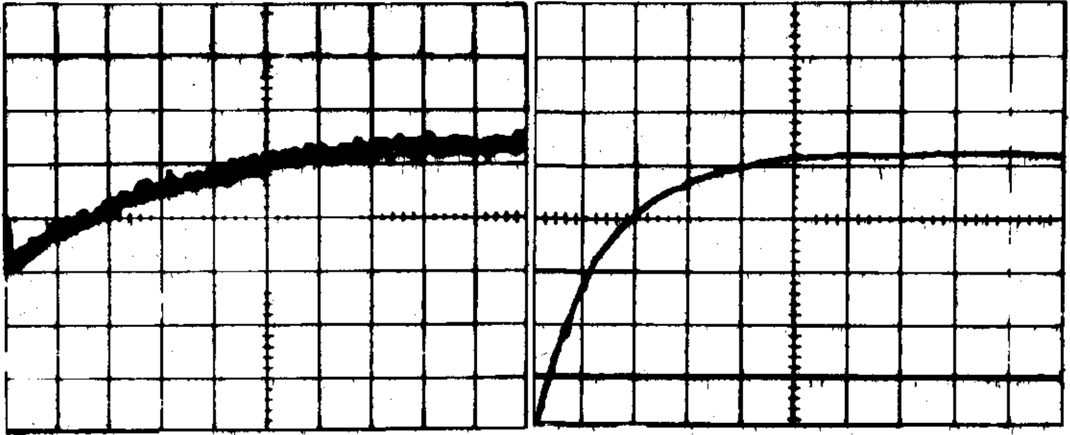


Fig.1: Experimental relaxation traces for the equilibrium displacement of P.elsdenii apoflavodoxin and dFMN in 30 mM P_i buffer, pH 8.5, in the presence of 0.3 mM EDTA and 0.1 M KNO_3, final temperature 22°C. Fluorescence detection has been employed, excitation wavelength was 366 nm. Upward deflection represents an increase in fluorescence intensity.

A) Total protein concentration 2.2 microM, total flavin concentration 2.2 microM. 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 13.3 microM. Time constant 5 ms, 50 mV/large vertical division, 200 ms/large horizontal division.

(Fig.2).The perturbation of equilibrium /2/ follows the equation:

$$1/\tau = k_1 \left(\overline{E} + \overline{dFMN} \right) + k_{-1} \qquad /3/$$

where k_1 and k_{-1} are the bimolecular and monomolecular rate constants,respectively,and $\overline{E}$ and $\overline{dFMN}$ are the new equilibrium concentrations of apoenzyme and free flavin respectively.From the intercept on the ordinate of the plot of Fig.2 a $k_{-1}=0.8\ s^{-1}$ and from the slope a $k_1=3.6 \times 10^5\ M^{-1}s^{-1}$ can be calculated. (It is interesting to note that the bimolecular association rate constants that have been determined with FMN and various apoflavodoxins all fall within the range 10^5 to $10^6\ M^{-1}s^{-1}$ (21)).These values of k_1 and k_{-1} yield a dissociation constant of the system of 2.2 microM.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°C and buffer containing 0.1 M KNO_3.This one and the other values reported below were calculated graphically according to the method of Benesi and Hildebrand (23),assuming a 1:1 complex formation.The dFMN-apoenzyme complex was found to be very weakly fluorescent.The value of 2.2 microM differs by about a factor of five from t that (0.43 microM) published by Mayhew and 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 lies probably in the difference in conditions (ionic strength and temperature).(This is a reasonable assumption considering published results

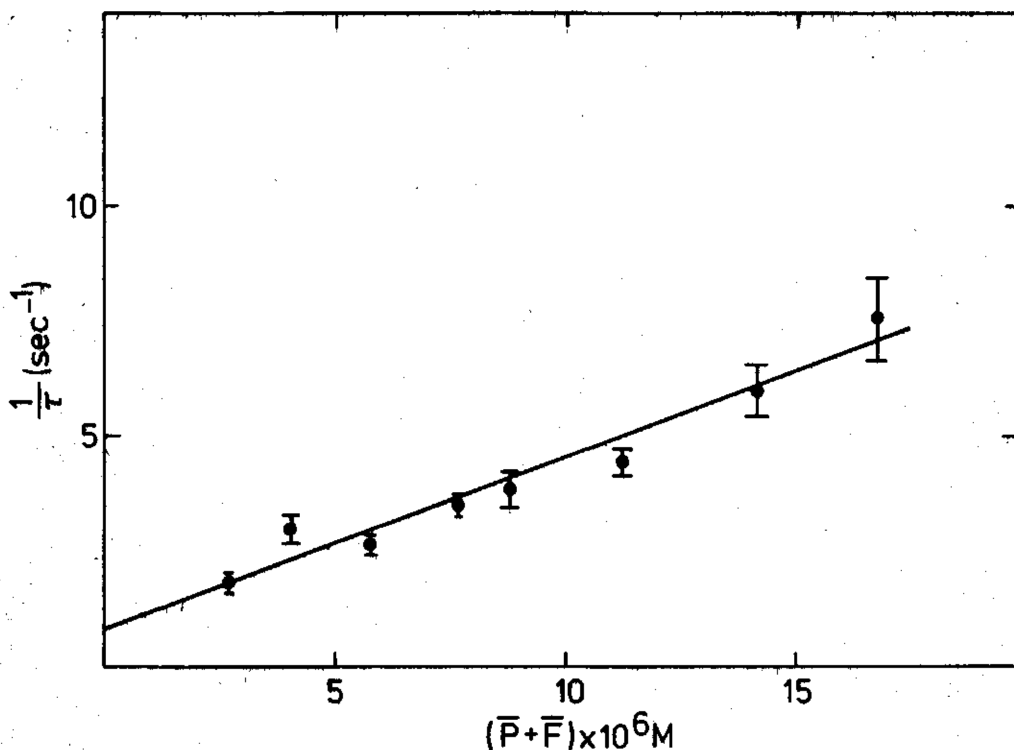


Fig.2:Plot of reciprocal of the observed relaxation times vs. the sum of the concentration of free dFMN and apoflavodoxin from P.elsdenii.The experimental conditions were those of Fig.1.

(8,10,26),which show that the rate constants depend strongly on the salt concentration of the solution).

The association-rate constant for dFMN 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, it is evident that they contribute considerably to the stabilization of the flavin-apoenzyme complex because the dissociation rate constant is about two orders of magnitude greater for dFMN (5).

Our data confirm that the apoenzyme exhibits a high specificity with respect to flavin binding,i.e. side chain consisting of five carbon atoms and a terminal phosphate group are minimal requirements. From these results it is also concluded 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 very reasonable to assume 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 configuration 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 and Tollin (10);i.e. one against two 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 two complexes are very similar (13).Therefore,we performed experiments with native flavodoxin under the same conditions as described in (10),also considering the technical precautions as mentioned in "Materials and Methods".Under these conditions no relaxation was found in the time scale of 1 s.However,when the experiments were carried out in the time range of

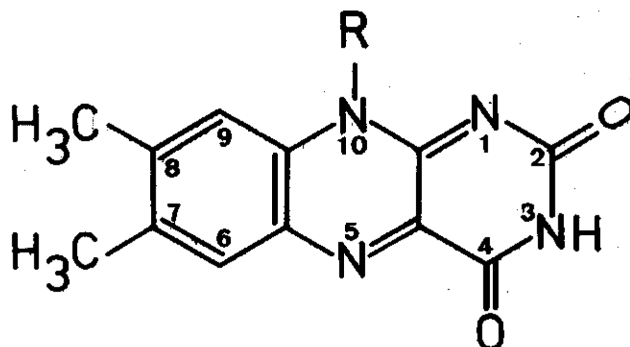
about one minute (cf.ref.10)similar traces were found.Since studies with A.vinelandii flavodoxin have led to the proposal of the two-step mechanism (10) the possible causes for the discrepancy will be discussed below.

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 it was possible with flavodoxin from P.elsdenii.All of the models tested (cf.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 dFMN.The relaxation process observed is in accordance with equation /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 $1/t^2$ against the analytical concentrations of the reactants yielded straight lines in accord with equation /4/ (ref.25)

$$1/\tau^2 = 2k_1k_{-1}\ (F_t + P_t) + k_{-1}^2 \qquad /4/$$

These experiments cannot exclude,however,a conformation change occurring on a time scale longer or shorter than used here.The results are summarized in Table 1.From this table it is seen that all



STRUCTURE I

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

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 for this observation is that dehydration of the terminal ionized group prior to binding is necessary.A similar observation was made by Edmondson and 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

Table 1

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

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

Compound	$k_1 (M^{-1}s^{-1})$	$k_{-1} (s^{-1})$	$K_d (\mu M)$[b)]	$K_d (\mu M)$[c)]
Ib	2.6×10^5	.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

Table 1 continued

a) All experiments were conducted in 0.1 M pyrophosphate buffer, pH 8.5 and 0.1 M 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 value has to be considered qualitatively.

might be due to a phosphate triggered conformational change. The present study does not support this proposal. Nevertheless, from the data presented in Table 1 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 $M^{-1}s^{-1}$ for lumiflavin (10) (cf. Structure I, R = CH_3), which is the least polar molecule among the flavin derivatives discussed here, in the order of 10^6 $M^{-1}s^{-1}$ for riboflavin (10) and its analogs Ic, while in the order of 10^5 $M^{-1}s^{-1}$ 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 given in Table 1 demonstrate another interesting fact.The uncharged derivatives (Ic) gave complexes with dissociation constants in the microM 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 1),thus making temperature-jump studies impossible. However,as the number of methylene groups of the side chain increased (Id,e, n=3,4,5) 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 from A.vinelandii (27) at pH values between 2 and about 7.These studies indicated that two glutamic acid residues are located in the flavin binding region of the proteins.Our results indicate that these glutamic acid residues are involved strongly in the binding of the flavin side chain. Furthermore,it is very likely that the finding of Meighen and MacKenzie (28),who found that the activity of the bioluminescent reaction of bacterial luciferase depended on the number (n more than 2) of methylene groups of the flavin derivatives Id and Ie,is also related to repulsive forces.Although

these authors did not take into account the fluorescence quantum yield of the different flavin derivatives used. Differences in these values could also explain their results.

With three exceptions (Id, n = 4,5 ;Ie, n = 3) the dissociation constants of the complexes investigated are similar (Table 1). The dissociation constants calculated from the given rate constants agree reasonably with those determined by static fluorescence titration experiments (Table 1). Our data also allow us to describe the kinetic parameters which govern the stability of a given complex. Thus it seems that the stability of the complexes between apoflavodoxin and compound Ic is mainly governed by the association rate constants, which vary by a factor of about three, whereas the dissociation rate constants (the inverse of which is related to the lifetime of the complex) are about the same for all four complexes. The association rate constants of the complexes of apoflavodoxin with the charged compounds Ib, Id, Ie vary by a factor of about 60, the stability of these complexes is therefore governed by the rate constant of dissociation. From the results obtained with compounds Id and Ie (Table 1) two 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 native prosthetic group FMN. Second the stability of the complexes formed with

compound 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 about 1 nm between the N(10) atom and the terminal ionizable side chain group of the flavin.

As mentioned above the proposal of Barman and 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 perturbation 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) it seemed possible that these relaxation curves might be artifacts; the following experiments proved that this conclusion was correct. When native flavodoxin was replaced by an identical concentration of egg albumin or by a 0.1 M KNO_3 solution alone the relaxation traces shown in Fig. 3A and B, respectively, were obtained. The shapes of these traces are very similar to those obtained with the native flavodoxins (cf. ref. 10, Figs. 5, 8-10). They are dependent on the incident wavelength and the 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 equilibrium value after about 1 min. The described artifacts are probably caused by straylight from the solutions and/or cell walls. Furthermore, the results obtained from the dFMN-apoflavodoxin complex (cf. Table 1) 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 two-step binding mechanism (10), as opposed to our results, would be accepted strong arguments against the analytical procedures of calculating the rate constants from the published traces (low signal to-noise ratio and cooling effects, cf. ref. 10) can be brought forward. Our recalculation of the relaxation times using the published rate constants (10) and the appropriate equations (25) shows values of $_1^{-1} = 0.164\ s^{-1}$ and $_2^{-1} = 0.05 - 0.07\ s^{-1}$ instead of $0.137\ s^{-1}$ and $0.08 - 0.1\ s^{-1}$, respectively. Furthermore, since both equilibria relax at times not too far apart, the relaxations are coupled to each other and therefore both relaxation times become concentration dependent (25) which has not been considered by Barman and Tollin (10). In addition one has to take into consideration the recent publication of Gafni et al. (29) who showed that the calculated standard deviations of two relaxation times, which differ by a factor of two 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 and Tollin (10) is invalid.

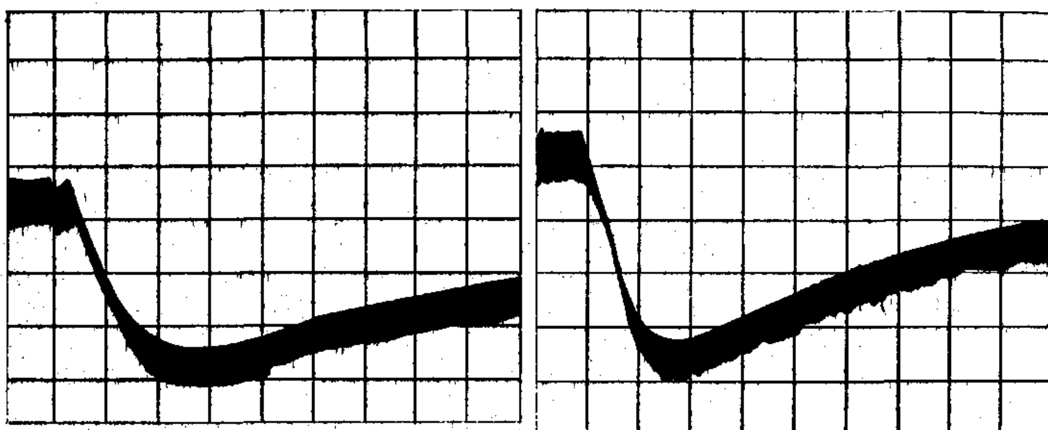


Fig.3: Experimental relaxation traces of "blank" solutions after a temperature-jump of 8.3°C. Final temperature was 22°C. A Schott 485 glass filter was used in the fluorescence detection mode. The time constant was 1 ms. Upwards deflections represent an increase in light intensities. A large horizontal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

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

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

CONCLUSIONS

We concluded from our data that all of the flavins studied were bound to apoflavodoxins from P.elsdenii and A.vinelandii in a simple one-step process.This is in contrast to the conclusion of Barman and Tollin (10) who proposed a two-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 a third flavodoxin,that from D.vulgaris;these authors also conclude that the mechanism of binding involves a single step.The fact that the mechanism of flavin binding in the three 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 three 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 two structures,the flavin binding sites are also not too different.

The kinetics of the interaction between Ib and Id (n = 4) (cf.Table 1) and the apoflavodoxin from A.vinelandii were also studied by independent methods.From stopped flow spectrofluorometry it was found that $k_1 = 5.6 \times 10^5\ M^{-1}s^{-1}$ and $K_d = 0.2 \times 10^{-6} M$ for Ib (10) and from convential fluorescence quenching technique $k_1 = 5.7 \times 10^5\ M^{-1}s^{-1}$ and K_d about

0.01 microM were found for Id (n=4)cf.ref.13.These values are in fair agreement with those given in Table 1 and provide further support for the one-step binding mechanism.It should be noticed however that any conformational change 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.

ACKNOWLEDGEMENTS

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Waar is de weg naar de plaats
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Waar is de weg naar de
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A STUDY ON THE LONG-LIVED INTERMEDIATE IN BACTERIAL BIOLUMINESCENCE

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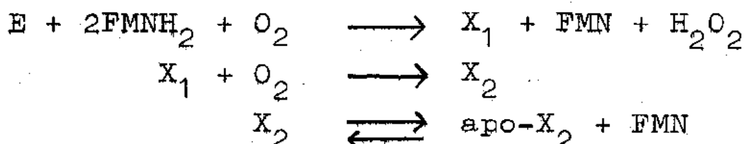
INTRODUCTION

Bacterial luciferase is able to catalyse the oxidation of $FMNH_2$ by molecular oxygen. As a result of this oxidation, a modified form of luciferase is produced, that will cause light emission, if it is reacted with a long-chain aliphatic aldehyde. There is fairly good evidence that this modified luciferase is a real intermediate in the normal in vitro bacterial bioluminescence reaction (1). In this way the luciferase, isolated from the cell type Beneckea harveyi forms an intermediate, which has a lifetime of seconds at 22°C, minutes at 0°C and hours at -20°C (12). However, the intermediate formed with the luciferase from Photobacterium fischeri has a lifetime in the order of only one minute at 0°C and that from Photobacterium phosphoreum is even shorter. With the type of luciferase from Beneckea harveyi, Lee and Murphy (2) identified two intermediates. The first intermediate, which appears after reaction of luciferase with $FMNH_2$ and oxygen, is a reduced luciferase molecule to which one molecule of FMN is bound. This intermediate, which they called X_1, takes up oxygen in a pseudo first-order reaction ($t_{\frac{1}{2}}$=1.3 min, at 5°C) to form another intermediate X_2. Including the stoichiometry of reaction as found by quantum yield data (3) a simplified scheme can be put forward:



$$E + 2FMNH_2 + O_2 \longrightarrow X_1 + FMN + H_2O_2$$
$$X_1 + O_2 \longrightarrow X_2$$
$$X_2 \rightleftarrows apo\text{-}X_2 + FMN$$

In the absence of aldehyde the species apo-X_2 will fall apart into hydrogenperoxide and the free luciferase (E). X_2 contains still one molecule of FMN, which was deduced from its fluorescence and absorption properties. This intermediate can be further

characterized after separation from free FMN.This has been done by molecular sieving of the mixture over a Sephadex G-25 column.Murphy et al. (4) found that in this way essentially all the FMN could be separated from the holoprotein,leaving the apoform of the intermediate X_2.The results presented in this chapter have previously been presented at the 3rd annual meeting of the American Society for Photobiology (5).

MATERIALS AND METHODS

Luciferase was purified from B.harveyi as previously described by Lee (3).The absolute specific activities of the luciferase preparations were in the range of 10 - 40 x 10^{12} photons/s.mg protein.FMN was purified by DEAE-cellulose chromatography,according to the method of Massey and Swoboda (6).$FMNH_2$ was produced by photoreduction in a translucent syringe with EDTA (20mM) present as an electron donor.Apo-X_2 was made by injecting the $FMNH_2$ (0.5 ml),at 0°C,pH 7.0,into a solution of luciferase (a minimum concentration of 80 uM in a volume of 0.5 ml).This mixture was then applied to a Sephadex G-50 column (2 x 40 cm).Fractions of 2 ml were collected,also at 0°C,at a rate of 6 ml/min.For this reason the column was run with a slight overpressure.The column was eluted and pre-equilibrated with 50 mM P_i,pH 7.0.The light producing reaction and the quantum determinations were carried out as described by Lee (3).Absolute light calibration was done by using the well-known luminol chemiluminescence reaction (7).Fluorescence measurements were done on a Cary-14 recording spectrphotometer, equipped with fluorescence accessories.

RESULTS AND DISCUSSION

Our first effort was directed towards optimalisation of the lifetime of the intermediate.From a variety of different experiments it became obvious that the pH played a major role.Thus,storing the intermediate at pH 9.3 a maximum lifetime (half life of 100 min.) was obtained.The influence of the pH on the lifetime of the intermediate is given in Fig.1.The abrupt drop in the lifetime of the intermediate above the optimal pH value of around 9 is probably due to denaturation

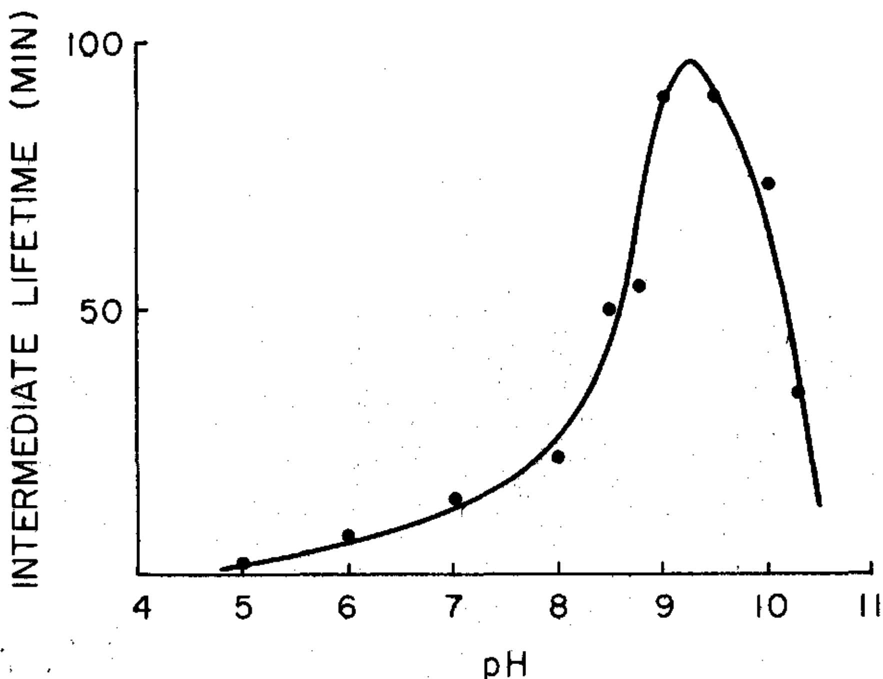


Fig.1 Influence of the pH on the lifetime (half-life) at 0°C of the intermediate in the in vitro bacterial bioluminescence reaction of B.harveyi.

of the protein moiety of the intermediate.A pK for the intermediate with a value of around 9 can be derived from the data.Whether the increase in lifetime at the higher pH values is due to an ionisation of the "active oxygen" group itself or to stabilising effects in the protein structure cannot be decided with the data available at the present.

It should be noted that the stability of the intermediate does not bear any relationship with either the quantum yield or the initial light intensity of the bioluminescent reaction.As has been shown by Lee and Murphy (Fig.5;ref.8),the initial light intensity has its maximum just above pH 6, whereas the quantum yield of the reaction on the other hand is constant over the pH region 6-8.

In Fig.2 both the dependence of the quantum yield of the total reaction and of the intermediate on the pH are shown.As can be seen by the coinci-

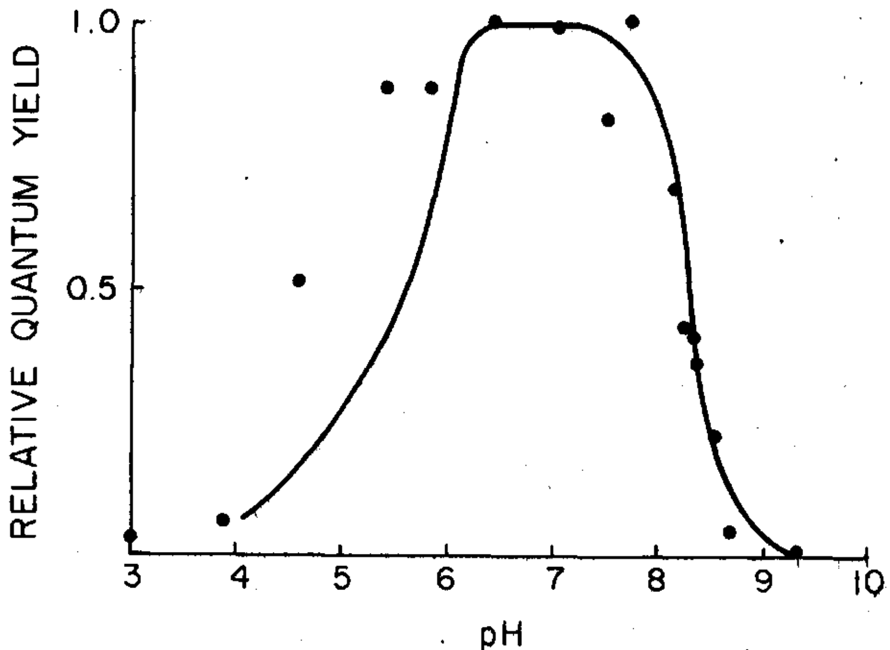
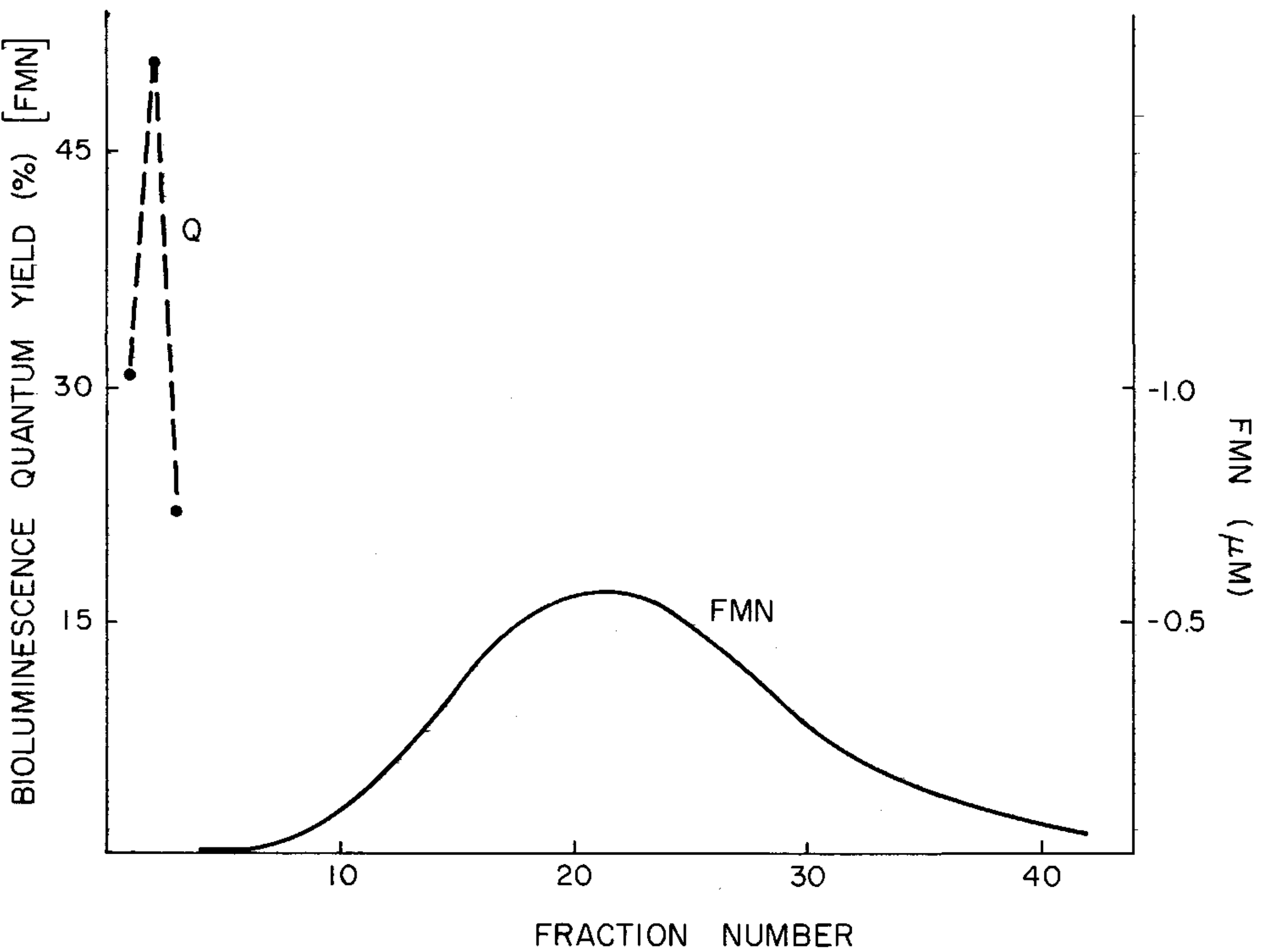


Fig.2 Influence of the pH on the quantum yield
solid line: quantum yield of the enzyme for the total reaction (from ref.8)
dots: quantum yield of the intermediate for the reaction with aldehyde

dence of the dots and the line, the decrease of the total light emission at the higher pH values, is the same for both. The stability of the intermediate reaches its maximum value (Fig.1) even beyond the point (i.e. pH 9), where the quantum yield of the intermediate is already zero (Fig.2). Some factor(s) thus, other than this stability must be involved in decreasing the quantum yield at the high pH side. After the formation of the intermediate, two main processes have to occur in order to finish the complete bioluminescent reaction. First, the reaction with the aldehyde and second the emission of the light. One of these two reactions must have a lower efficiency at the higher pH values, probably due to an acid-base equilibrium. Flavin semiquinone (pK=8.4)



BIOLUMINESCENCE QUANTUM YIELD (%) [FMN]
45
30
15
Q
FMN
10
20
30
40
FRACTION NUMBER
-1.0
-0.5
FMN (μM)

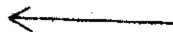


Fig.3 Elution pattern of Sephadex G-50 column for the preparation of the apo-X_2 intermediate.pH 7.0, Initial concentrations:protein 94 μM; $FMNH_2$ 16 μM.

has been put forward by Lee and Murphy (8) as a likely candidate for this equilibrium, but it is at presently difficult to see which role it could play in one of these two processes. A cysteine residue (pK around 8.3) was suggested to play a role in the bacterial bioluminescent reaction by Nicoli et al.(9). Indeed such a group can be involved in the binding of the aldehyde to the luciferase. At the acid side of the curve given in Fig.2, the decline in quantum yield doesn't seem to be as sharp for the intermediate as the drop observed for the total reaction. This can be explained by the possibility that at the acid side (i.e. pH 5), the formation of the intermediate

becomes much less efficient as compared to the efficiency of the rest of the reaction,once the intermediate is formed.

The stability of the oxygenated intermediate X_2 is actually a reflection of the availability of some form of the oxygen for the oxidation of the aldehyde. The "activated oxygen" might be bound to the flavin molecule,as is the case in most flavoproteins (see e.g. ref.10).This situation is proposed for the bacterial luciferase by Tu,Balny and Hastings (11,12), who suggest that the oxygen is bound at the 4a-position of the FMN molecule.Another possibility would be a situation analogous to the photoproteins, one of which can be isolated from the luminous jellyfish Aequorea.For these photoproteins evidence is provided that they contain an oxygenated species, bound to some site on the protein itself (13,14). if such a situation exists in the intermediate X_2, it would be likely that the FMN could be separated from the intermediate,without compulsory destroying the stability of it.A third possibility would be that at some stage in the reaction,i.e. during the formation of X_2,the oxygen forms a bridge between the enzyme and the FMN molecule.In this chapter we present evidence that the FMN can be separated from intermediate X_2.

In order to separate the FMN from the intermediate,the latter was applied to a Sephadex G-50 column as described in the Materials and Methods section.In Fig.3 the elution pattern of this column is shown.The protein is eluted in the first five fractions.Shown as a solid line are the measured concentrations of FMN,as detected by fluorescence. Also plotted in Fig.3 is the bioluminescence quantum yield,which was calculated as the ratio of total light quanta,produced after the addition of decanal and the total number of FMN molecules present.Normally in the reaction,with all the components reacting together,this quantum yield is around 2.5% for the B.harveyi enzyme with decanal (8).However,it is shown in Fig.3,that the quantum yield with respect to FMN greatly exceeds this number in the solutions of the intermediate that are chromatographed through Sephadex G-50.Because in former experiments by Murphy et al. (4) even quantum yields exceeding 100% were obtained,any stoichiometric role for FMN

or a derivative of it, in the light emission process, is excluded.

In view of these results, two possibilities for the emitter are open for discussion. The first possibility would be analogous to the situation as is now known for the bioluminescent earthworm Diplocardia longa, studied by the group of Wampler (15). In this case also a simple aldehyde is one of the reactants, having no visible absorption whatsoever and as yet there is no evidence for visible absorption bands or fluorescent chromophores associated with the luciferase either. It has been suggested that in this case the emission proceeds from a pure transient species, not detectable in either the starting mixture or in the spend reaction mixture. A similar situation could be operating in the in vitro bacterial bioluminescence reaction. The second possibility has been suggested by Murphy et al. (4). They proposed that the in vitro bacterial bioluminescence reaction is a sensitised one, with only FMN as a possible candidate as sensitising agent. We shall see, however, in the following chapters that another sensitising agent indeed can be extracted from the bacteria itself.

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it is that which we do know
which is the great hindrance to our learning
not that which we do not know

Claude Bernard

ISOLATION OF THE IN VIVO EMITTER IN BACTERIAL BIOLUMINESCENCE

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ABSTRACT

A blue fluorescence protein has been isolated and purified from extracts of the luminous bacterium Photobacterium phosphoreum. It is a single polypeptide of molecular weight 22,000 with absorption maxima at 274 nm and 418 nm. If is efficiently fluorescent (ϕ_F 0.45), with a fully corrected spectral maximum (476 nm) and distribution identical to the in vivo bioluminescence from this same type of bacterium. At low concentration this fluorescence red shifts and becomes identical to the in vitro bioluminescence emission. This spectral shift apparently results from a change in the protein pulled by dissociation of the chromophore ($K_d \sim 10^{-7}$ M). If the blue fluorescence protein is included in the in vitro bioluminescence reaction with reduced FMN, oxygen, aldehyde and luciferase (P. phosphoreum), the bioluminescence spectrum is blue shifted from its maximum at 490 nm to one at 476 nm, where it is again identical in all respects to the in vivo bioluminescence spectrum. This is accompanied by an increase in the initial light intensity by an order of magnitude at saturating levels of blue fluorescence protein, and the specific light yield of the luciferase is increased four-fold. It is suggested that the blue fluorescence protein acts as a sensitizer of the bacterial bioluminescence reaction.

In 1953 Strehler (1) reported a stimulatory effect of NAD^+ and NADH on the dim light emission of cell-free extracts of bioluminescent bacteria. Since this demonstration of the *in vitro* reaction of bacterial bioluminescence, the question of what compound actually emits the light has been an intriguing one. Although FMN is the only fluorescent product of the light reaction, it has been pointed out repeatedly that FMN cannot be the emitter due to the clear spectral difference between its fluorescence and the bioluminescence (2,3). Several proposals for the emitting species, without making a distinction between the *in vivo* and the *in vitro* situation, have been made during the last two decades. Following an observation of the chemiluminescence of indoles, it was suggested that the bacterial emitter could be an indole moiety attached to the luciferase (4,5). In 1962 Terpstra (6) reported on a substance isolated from extracts of *Photobacterium phosphoreum* that had a broad fluorescence in the 450 nm region and enhanced the bioluminescence activity of the luciferase in the *in vitro* reaction. Later she reported (7) that the addition of $FMNH_2$ to a luciferase preparation results in the formation of a compound that is transformed by irradiation with UV light (366 nm) into a substance having a fluorescence maximum at 470 nm. She suggested the first compound to be a precursor of the light emitting molecule in bacterial bioluminescence.

The first reports of the absorption spectrum of a solution of crystalline luciferase from *Photobacterium fischeri* showed a shoulder at 415 nm (8) or at 400 nm (9). While contamination by cytochrome is responsible for part of this (10), Cormier and Kuwabara (11) showed that excitation of their luciferase at 420 nm resulted in a fluorescence with a spectral maximum at 515 nm, and further, that on the addition of the proper amount of hydrosulfite, this fluorescence blue shifts so that the maximum closely approaches

that of the bioluminescence. Eley _et al_. (12) had a chromophore associated with their crystalline luciferase preparations that also had a fluorescence maximum at 490 nm and an excitation maximum at 390 nm. In 1969 Cormier _et al_. (13) proposed an NAD^+-aldehyde adduct as the emitter. Model compounds have an absorption at 420 nm and an emission at 515 nm consistent with the earlier observation made on crystalline luciferase (11).

More recently, after Mitchell and Hastings (14) claimed that the emitter must be some sort of flavin-derived species (2), three proposals for its structure have been put forward, supported almost solely by the similarity of the fluorescence of model compounds to the bioluminescence spectra. Eley _et al_. (12) proposed the cation of FMN, McCapra and Hysert (15) a quinoxaline which could be formed as a transient product on ring opening of the FMN molecule, and Balny and Hastings (16) together with Tu and Hastings (17), proposed an $FMNH_2$ molecule substituted in the 4a-position.

It has been tacitly assumed in the past that the mechanism of reaction and identity of the emitter are the same _in vivo_ as _in vitro_. The possibility of a difference has been raised by the recent discovery of a bacterial type emitting at 545 nm (18). In this paper we show that a protein bound chromophore can be isolated from extracts of the bioluminescent bacteria _Photobacterium phosphoreum_ , which is closely associated with the luciferase and which fulfills all the conditions to qualify it as the _in vivo_ emitter.

MATERIALS AND METHODS

The bacterium Beneckea harveyi strain 392 in the classification scheme of Reichelt and Baumann (19), previously designated "MAV", was obtained from J. W. Hastings (Harvard University). The type "A-13" was isolated from the light organ of the "silver macrourid" fish by J. Paxton (Australian Museum) and has been identified as Photobacterium phosphoreum (J. Fitzgerald, private communication). The type Photobacterium fischeri, strain 399, was obtained from F. H. Johnson (Princeton University). The bacteria were grown and the luciferase and FMN were purified as described previously (20,21). The blue fluorescence protein was routinely assayed by its fluorescence intensity at 470 nm when excited at 420 nm, using an Aminco-Bowman Spectrofluorimeter. Luciferase activity was determined by using a digital photometer, designed and constructed by G. J. Faini, which was calibrated for absolute photon sensitivity with the luminol chemiluminescence reaction as a light standard (22). This standard is directly traceable to the National Bureau of Standards Lamp (23) and its calibration has been confirmed by three independent methods (24-26). NADH dehydrogenase was purified from P. fischeri 399 by C. White and was found to couple efficiently with all types of luciferases used. All other chemicals were of the best commercial grades.

Absorption spectra were taken with a Cary 14 spectrophotometer. Absolute fluorescence and bioluminescence spectra were obtained with an on-line computer-spectrofluorimeter system (27) using a band-width of 5 nm. The sample at room temperature (23°C) was contained in a cuvette with a path-length of only 1 mm in the emission direction to minimize corrections necessary for self-absorption. Spectra were also corrected for the spectral sensitivity of the photomultiplier-monochromator system by reference to an NBS Standard of Spectral Radiance. The in vitro bioluminescence emission spectra were all determined

in a reaction mixture containing: potassium phosphate 0.05 M (pH 7), BSA 0.7 mg/ml, NADH 215 μM, FMN 3.2 μM, in a total volume of 1.5 ml at 23°C. To this was added 10 μl of a saturated solution of dodecanal in methanol, 50 μl of NADH dehydrogenase (A_{280} 3.14, specific activity 1.2 μmol NADH min^{-1} mg^{-1}), and luciferase of the type under study in an amount that would give an initial flash height of 10^{12} photons-s^{-1} if assayed using dodecanal by the normal procedure of injection of $FMNH_2$ (20,21).

For the reactions reported in Table 1, optical path lengths of both 5 mm and 1 mm were used. The highest concentrations of blue fluorescence protein used required a correction for self-absorption and re-emission of fluorescence of only 1.3.

Fluorescence lifetimes were determined for the fluorescence at 470 nm and at 490 nm by a single photon counting technique, using an air-gap spark source with most of the excitation at 358 nm, since the sample was contained in a glass cuvette (28).

RESULTS

From extracts of the bacterium type A13 a "blue fluorescence protein" has been isolated. Its association with the luciferase provides a convenient method of purification, by carrying it along through the several stages of luciferase purification detailed elsewhere (20,21). Minor modifications are made here: ammonium sulfate precipitation from the cell lysate, desalting on Sephadex (G-75), adsorption to DEAE cellulose (0.05 M phosphate, pH 7.6) and elution with 0.15 M phosphate (pH 7.6), adsorption on DEAE-Sephadex (A-50, 0.05 M, pH 7.6) with elution by a phosphate gradient (0.05 M - 0.35 M, pH 7.6). Some blue fluorescence protein separates from the luciferase at the Sephadex G-75 and A50 stages but the two fractions can be recombined. Following the A50 step the mixture is subjected to a slow molecular sieving (Sephadex G-75 superfine, 5 x 100 cm), the luciferase elutes in the column front (mol. wt. ~80,000) and the blue fluorescence protein is retarded, consistent with its much lower molecular weight. At this point it has a homogeneity of about 70% and is further purified by repeated gel filtration (Sephadex G-75 superfine, 3 x 85 cm). From 500 g of cell paste the final yield of blue fluorescence protein is about 10 mg. The relative fluorescence 420 → 470 nm is improved about thirty times from the first Sephadex step, based on absorbance at 275 nm.

The homogeneity of the blue fluorescence protein is determined by SDS gel electrophoresis. In Fig. 1C an impurity can be seen just above the heavily staining protein band and is estimated to be about ten percent of the total protein. A 90% purity in this preparation is also supported by the absorption spectrum data. Figure 1B shows the clear difference between the blue fluorescence protein and luciferase, which is a doublet of two non-identical subunits (29), not resolved in this photograph. Luciferase is used as a marker along with carbonic anhydrase and lysozyme, to determine the molecular

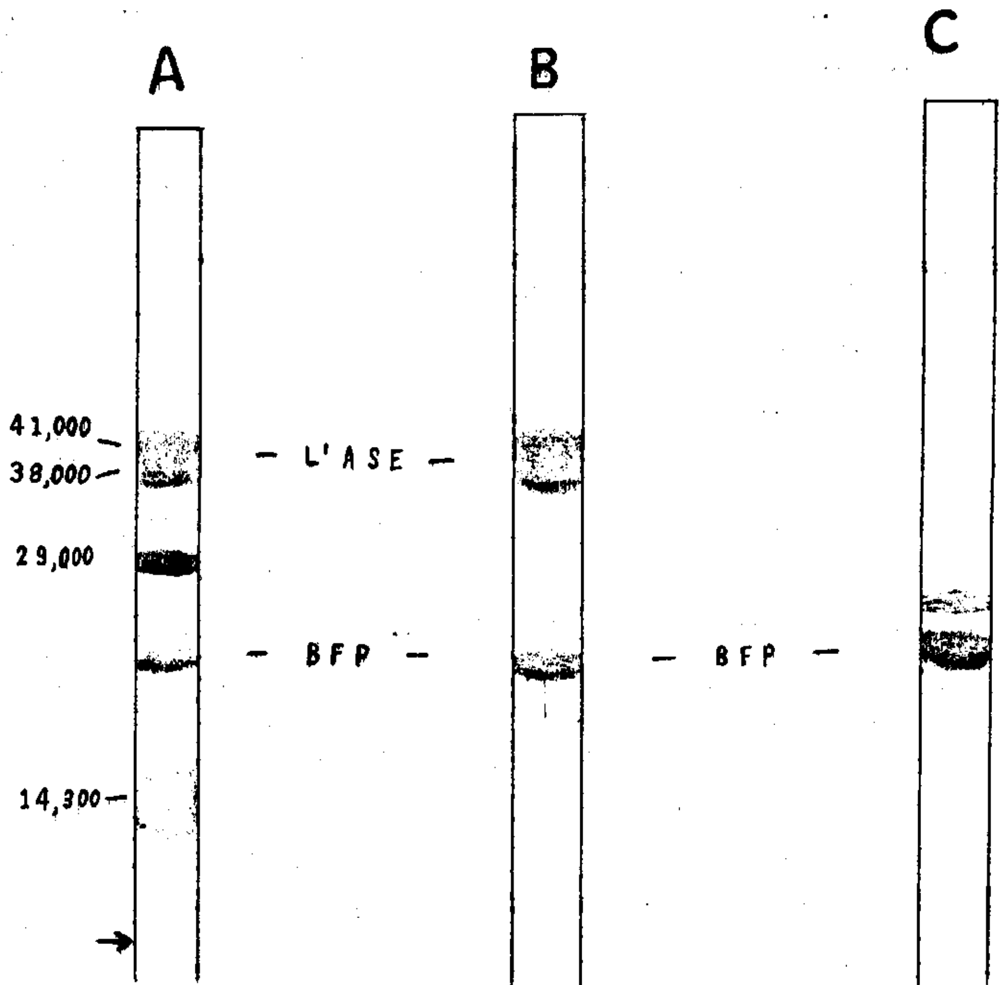


Fig.1 Moleqular weight and homogeneity of blue fluorescence protein by SDS-acrylamide (10%) gel electrophoresis following the method of Weber and Osborn (30).

A)BFP (15 µg) with the markers P.fisoheri luciferase (l'ase,20 µg),carbonic anhydrase and lysozyme;the arrow indicates the dye front.

B)Luciferase and BFP (15 µg).

C)BFP (30 µg).

weight of the blue fluorescence protein, Fig. 1A (30).

The molecular weight of blue fluorescence protein is 22,000, an average of the results from SDS gel electrophoresis, sedimentation equilibrium monitored at 270 nm and 420 nm, and sedimentation velocity (420 nm). Calibrated gel filtration (G-75 superfine) also gives a result consistent with this molecular weight. Since the same molecular weight is obtained by sedimentation and SDS gel electrophoresis, then blue fluorescence protein has a single polypeptide chain.

On alkaline disc gel electrophoresis (31) the blue fluorescence protein undergoes denaturation and aggregation. Before staining however, only one fluorescent band is evident and it corresponds in R_F to the heaviest staining protein band.

Figure 2 is the absorption spectrum of blue fluorescence protein. The protein concentration was determined by the dye binding method of Bradford (32) and the extinction of the chromophore at 418 nm is about 4000 $M^{-1}cm^{-1}$, calculated from the fluorescence yield and lifetime. The mole ratio of the chromophore to protein is therefore 0.9:1.0, consistent with the estimated purity of the preparation and an assumed 1:1 ratio of chromophore to protein.

The fluorescence of the blue fluorescence protein is shown in Fig. 3a. It is of significance that this fluorescence is identical to the _in vivo_ bioluminescence spectrum from the same type of bacterium, Fig. 3b. Both these spectra are similar, if not identical, to the bioluminescence spectrum of _P. phosphoreum_, first published by Spruit-van der Burg (33).

It is relevant to mention here that of all the emitters proposed and listed in the Introduction, none have the skewness, the small width at half-height and the peak position exhibited by the _in vivo_ emission of _P. phosphoreum_ (33,34).

Although the spectral matching shown in Fig. 3 alone makes this newly

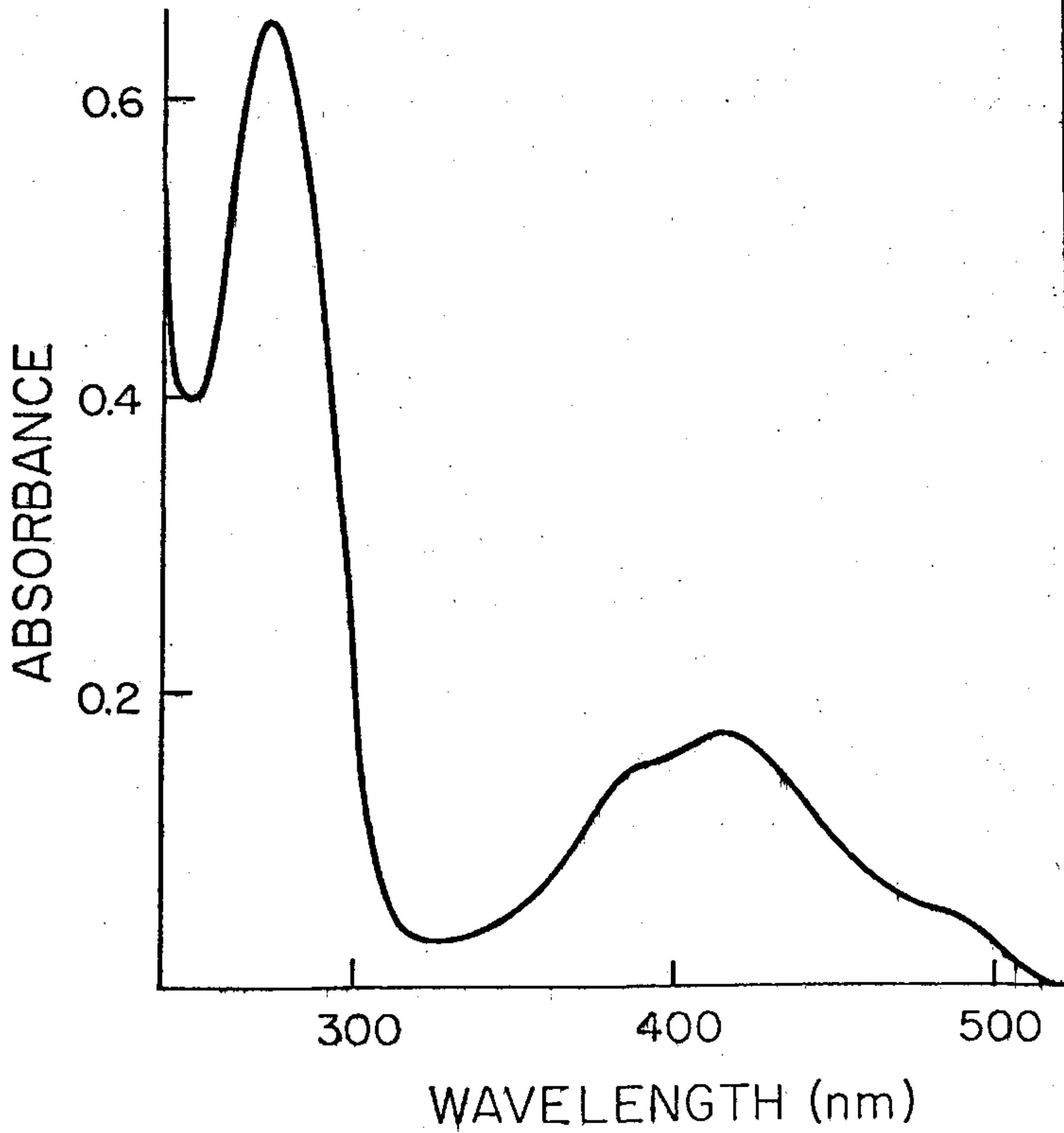


Fig. 2. Absorption spectrum of the blue fluorescence protein (1 mg/ml) in phosphate 0.05 M, pH 7.0, 23°C.

isolated blue fluorescence protein a good candidate for the bioluminescence emitter *in vivo*, a more striking property is the effect of including it in the *in vitro* reaction mixture--the *in vitro* spectrum blue-shifts to become an *exact match* for the *in vivo* spectrum. This is demonstrated in Fig. 4A1 (without) and Fig. 4A2 (with) blue fluorescence protein (~70μM), and Fig. 4A2 is seen to be identical to Fig. 3b. It needs to be recalled here that different types of bacteria have *in vivo* bioluminescence maxima ranging from 472-505 nm and recently one has been isolated at 545 nm (18), but the maxima of the *in vitro* spectra all cluster around 496 nm (18,35). Efforts to shift the *in vitro* maximum by changes in external conditions such as pH, addition of metal ions, temperature, acid denaturation and the chain length of the aldehyde, have all failed (35). We observe that the *in vivo* spectra are similarly unaffected. The fact that the blue fluorescence protein induced shift results in a spectral distribution that exactly matches the *in vivo* bioluminescence favors the idea that the blue fluorescence protein is itself the emitter under these conditions.

The reaction of Fig. 4A uses the luciferase from type A-13, the one from which the blue fluorescence protein was isolated. Fig. 4B shows an attempt at cross-reaction between the blue fluorescence protein from A-13 and the luciferase from another species of bioluminescent bacterium, *B. harveyi*. At the concentration added (~70 μM), the spectrum is certainly altered indicating some cross-reaction, but it is not completely shifted over to the A-13 *in vivo* spectrum. Although we can isolate blue fluorescence protein from other species of luminous bacteria, it is not yet available in sufficient quantity for investigation.

The fluorescence properties of the blue fluorescence protein are easily perturbed by a variety of mildly denaturing conditions, such as dilution, temperature, pH, ionic strength, and urea. At high concentration the fluorescence is the same as shown in Figure 5 for 17 μM but it is seen that as the concentration is reduced down to 1 μM the fluorescence maximum shifts

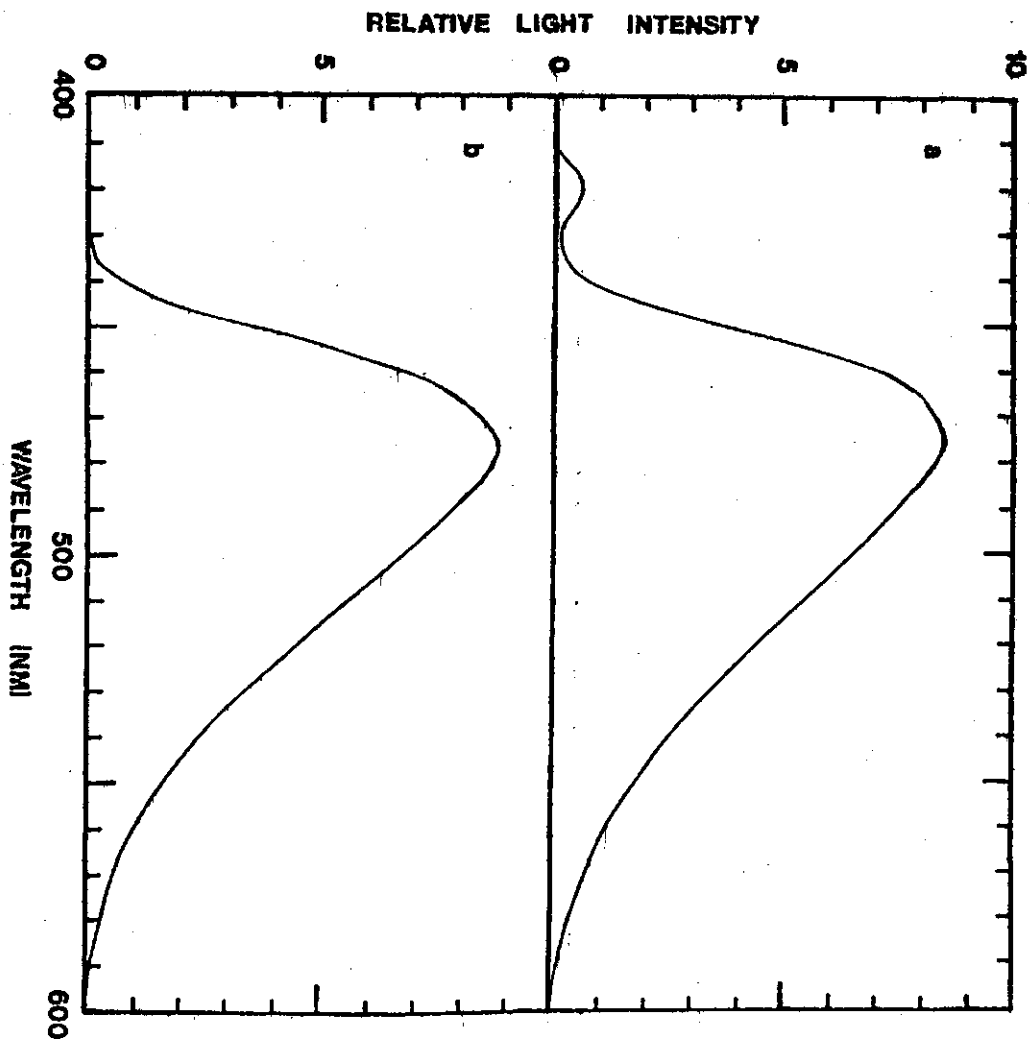


Fig. 3. a) Fluorescence of the blue fluorescence protein (12 μM). Excitation at 420 nm, in phosphate 0.05 M, pH 7.0, 23°C.

from 474 nm to 484 nm with a small reduction in quantum yield. This effect is reversible. Below 1 μM the fluorescence shifts only slightly further to the red where it is now identical to the in vitro bioluminescence, but this is accompanied by considerable loss of fluorescence yield, not entirely recoverable on reconcentration. Also at low concentration (0.5 μM) the fluorescence is completely lost on dialysis, whereas at 10 μM it is quantitatively retained.

The fluorescence quantum yield was measured with fluorescein (N/10 NaOH, ϕ_F 0.9) and quinine (N H_2SO_4, ϕ_F 0.55) as standards. At 17 μM, ϕ_F = 0.45 and the fluorescence lifetime (τ) is 11.1 ns; the extinction coefficient of the chromophore may be calculated by the approximation, $\epsilon = 10^{-4}\phi_F/\tau$ = 4054 $M^{-1}cm^{-1}$ (418 nm). The fluorescence polarization is 0.17, in exact prediction of the Weber-Perrin equation (36). At the low concentration end (0.5 μM) the fluorescence lifetime reduces to 8.4 ns and the polarization drops to around 0.10 ($\pm$ 0.02).

As well as blue-shifting the emission spectrum of the in vitro reaction, the blue fluorescence protein changes the kinetics and increases the total light. The Table shows that at the highest concentration of blue fluorescence protein used the steady-state rate of the light reaction, as measured by the initial light intensity, is increased six-fold. Without luciferase the blue fluorescence protein has negligible bioluminescence activity. Also this stimulation is species specific, since the blue fluorescence protein (from type A13, P. phosphoreum) does not stimulate the activity of the luciferase from two other species, P. fischeri and B. harveyi. Some stimulation of luciferase activity also occurs non-specifically with protein concentration, such as bovine serum albumin, but this effect is small, and much smaller on a weight basis than for blue fluorescence protein. Higher concentrations of bovine serum albumin inhibit. Heat denatured blue fluorescence protein produces approximately the same effect as bovine serum albumin.

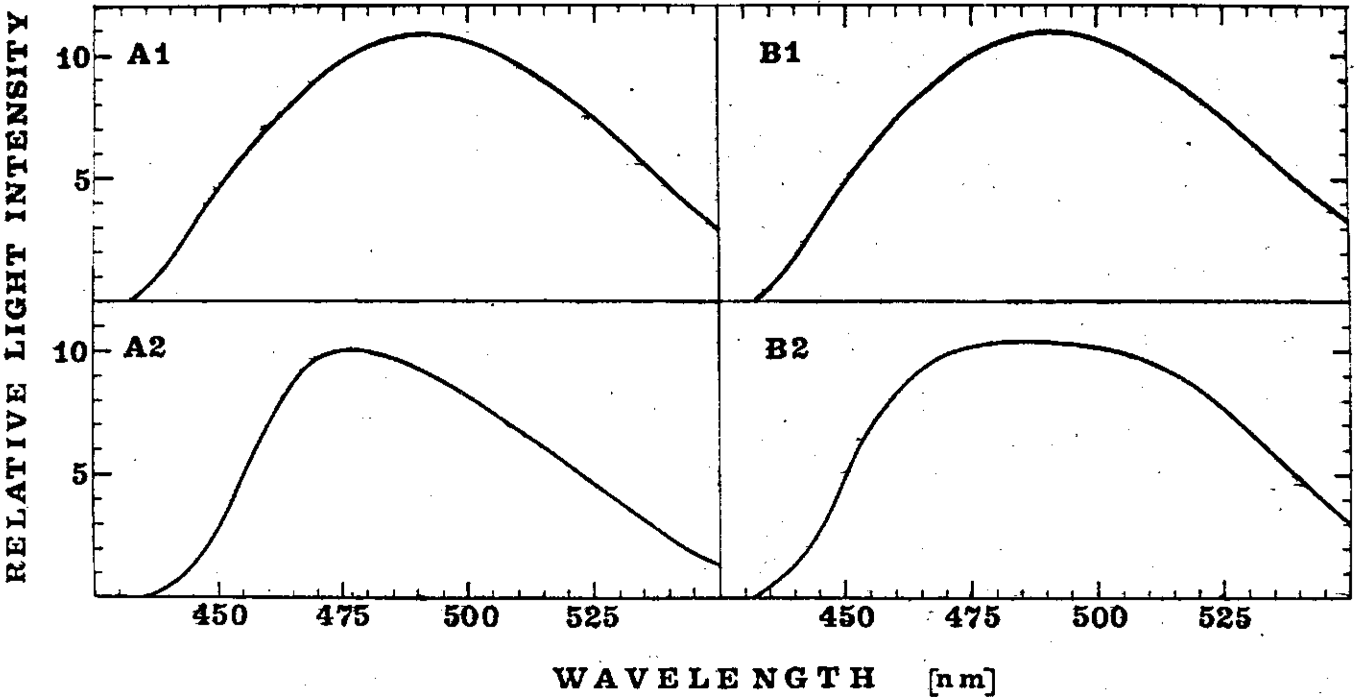


Fig. 4. A1. *In vitro* bioluminescence of luciferase from the bacterium type A-13.

A2. Same as A1, with addition of blue fluorescence protein.

B1. *In vitro* bioluminescence with luciferase from the bacterium type *B. harveyi*.

B2. Same as B1, with addition of blue fluorescence protein.

Table 1. Enhancement of luciferase activity and total light by the blue fluorescence protein.[a]

Blue Fluorescence Protein (μM)	Initial Light Intensity (10^{11} photons s^{-1})	Light Intensity Decay Rate (s^{-1})	Total Light (10^{11} photons)
0	1.8	0.13	13.8
12.6	2.7	0.15	18.0
30	5.0	0.18	27.8
56	5.7	0.17	33.5
101	9.3	0.22	42.3
181	11.2	0.25	44.8
106[b]	0.002	0.10	0.02
BSA[c]	2.7	0.15	17.7

The reaction mixture contained (final concentrations) phosphate 0.05 M, luciferase (type A13) 0.1 mg/ml and blue fluorescence protein. The bioluminescence was initiated by adding 10 µl of a saturated solution of dodecanal in methanol, followed by rapid addition of 0.2 ml of an 80 µM $FMNH_2$ solution. Final volume was 0.45 ml, temperature 23°C, pH 7.0.

[a]all results are an average of 4-6 observations and have a coefficient of variation of ± 10%; [b]no luciferase; [c]bovine serum albumin (BSA) 95 µM (6.5 mg/ml) substituted for blue fluorescence protein.

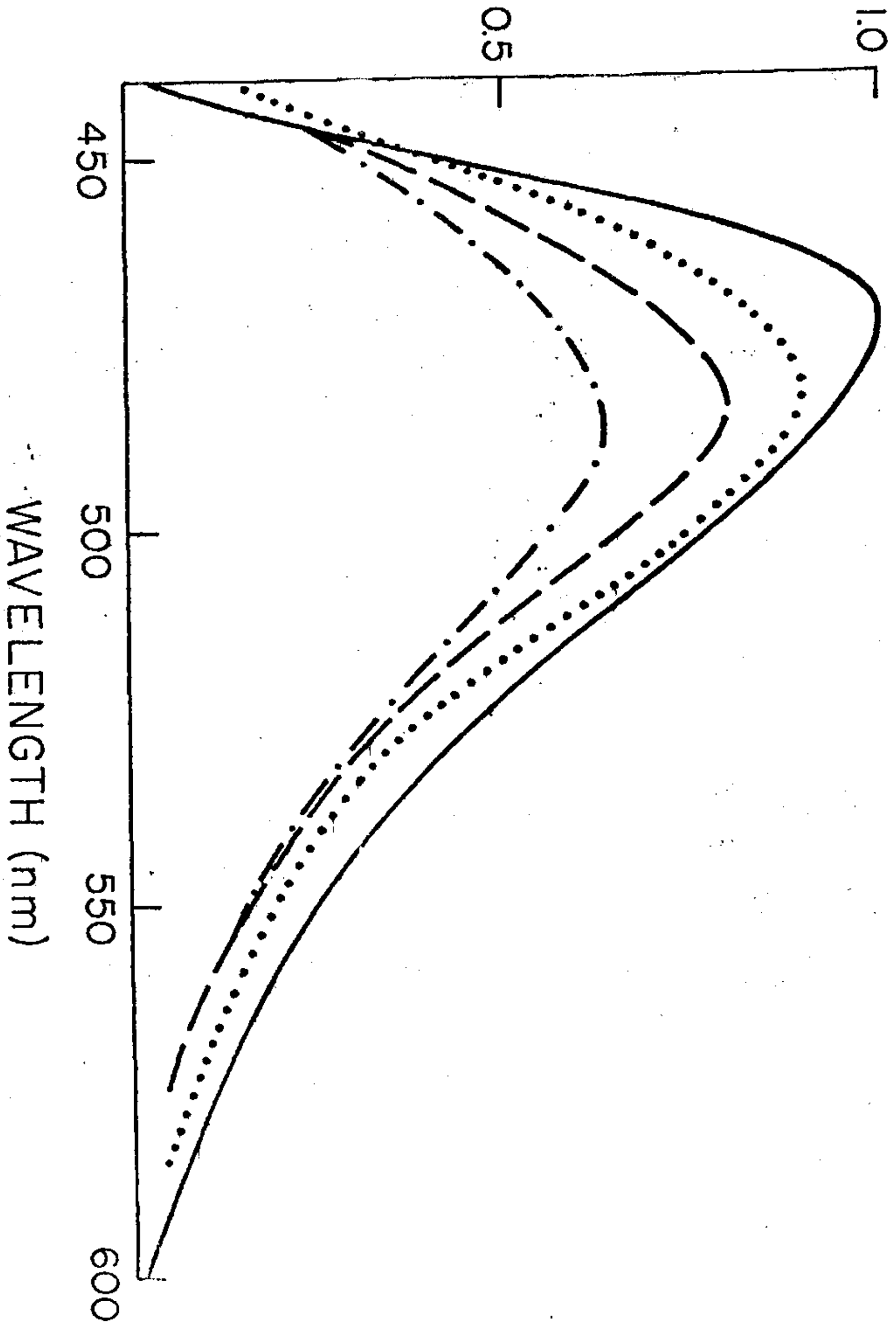


Fig. 5. Fluorescence spectrum of the blue fluorescence protein: (——) 17 μM; (····) 1.75 μM; (– – – –) 0.88 μM; (–·–·–·) 0.44 μM. All in phosphate 0.05 M, pH 7.0, 23°C; excitation at 420 nm.

A reciprocal plot of the data in the Table shows that a maximum stimulation of the initial light intensity of about fifteen times could be achieved at saturating concentrations of blue fluorescence protein. It has a K_m for stimulation of about 60 µM. It is to be noted that the change in spectral distribution on addition of the blue fluorescence protein (Fig. 4A) affects the calibration factor of the photometer, and this has been taken into account in calculating the data.

The blue fluorescence protein also changes the first-order rate of decay of light intensity, by a factor of two at the highest concentration tested here. Combined with the initial light intensity data, the total light is increased four fold at a saturating level of blue fluorescence protein, with a K_m for this interaction of 25 µM.

DISCUSSION

To qualify as the emitter in a bioluminescence system, a chromophore must have a fluorescence spectrum that is the same as the bioluminescence and give evidence of some role in the emission process. Specifically for bacterial bioluminescence, the shift in the emission spectrum on going from the *in vivo* to the *in vitro* reaction, observed for most types of bacteria (35), must also be explained. The new blue fluorescence protein reported here fulfills all these requirements. It has an efficient fluorescence exactly matching the *in vivo* bioluminescence spectral distribution, and the spectrum is readily perturbable by changing conditions such as its concentration, to exactly match the *in vitro* bioluminescence. It is isolated from extracts of luminous bacteria and clearly participates in the emission process, since on addition to the *in vitro* reaction it shifts the *in vitro* bioluminescence spectrum to that characteristic of its own fluorescence, enhances the rate of photon output and rate of decay of light intensity, and increases the specific light yield of the luciferase.

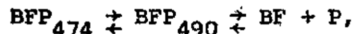
Chemiluminescence reactions divide into two main types, one "direct" where the light emission comes from a fluorescent product molecule formed directly in its excited state, and the other "indirect" or "sensitized" where the primary excited species induces the fluorescence of another chromophore (the sensitizer) already present in the mixture. Since exogenous blue fluorescence protein alters the reaction as described above, it is evident that this is a sensitized chemiluminescence process. Murphy *et al*. (37) made a suggestion that bacterial bioluminescence was a sensitized chemiluminescence when they found that on reaction of bacterial luciferase with $FMNH_2$ and O_2, an intermediate was formed which was separated from all flavin, yet retained full bioluminescence activity on reaction with aldehyde. We have made similar

observations (38). It is also interesting in this regard that Cormier and Kuwabara (11) were able to find a significant bioluminescence activity of reduced neutral red with their crystalline luciferase preparations in the absence of any detectable flavin. Sensitization specifically by energy transfer has recently been proposed by Ruby and Nealson (18) to account for a 545 nm _in vivo_ bioluminescence maximum in a bacterium they isolate, whereas the _in vitro_ maximum was again at 495 nm.

Two other bioluminescence systems that utilize aldehyde as their substrate may also be sensitized. These are the fresh water limpet, _Latia neritoides_ (39) and the earthworm, _Diplocardia longa_ (40). Although in these systems no direct evidence for a sensitizer has been obtained by isolation, oxidation of the aldehyde alone would not be expected to yield a fluorescent molecule. A better characterized sensitized bioluminescent system is that of the coelenterates, where the emission is sensitized by the addition of a "green fluorescent protein" (41). There are clear differences in the properties of these two systems however, the first being that the coelenterate reaction is efficiently sensitized by green fluorescent protein at a concentration about ten times less (42) then for the blue fluorescence protein in the bacterial reaction (Table 1). Although a four-fold increase in light yield occurs in both systems, the overlap of the absorption of blue fluorescence protein with the emission spectrum from the bacterial _in vitro_ reaction is two orders of magnitude less than that of the green fluorescent protein with the coelenterate _in vitro_ bioluminescence, and moreover, the shift in the bacterial case is to a higher energy.

It is also of significance that the fluorescence spectrum of the blue fluorescence protein can itself be suitably perturbed to match either the _in vivo_ or the _in vitro_ bioluminescence. The effect of concentration on its

fluorescence properties can be accounted for by the equilibrium:



$$BFP_{474} \rightleftarrows BFP_{490} \rightleftarrows BF + P,$$

with a dissociation constant 10^{-7}M for the chromophore "BF" from the protein. This chromophore is less stable and non-fluorescent in free solution, and is dialyzable below 0.5 μM, but not at higher concentration. Dilution of the blue fluorescence protein BFP_{474} therefore pulls the equilibrium to the redder form BFP_{490}, where its lowered fluorescence polarization suggests that the chromophore remains protein-bound but is less constrained to rotation that in BFP_{474}. This result is also consistent with the chromophore being more exposed to the water, a more polar environment inducing the red shift. The other perturbing agents (temperature, pH, etc.) produce a similar effect and can be explained by the same model.

The molecular structure of the chromophore in the blue fluorescence protein is not yet known. It does not appear to be derived from flavin (43). There is a similarity to the absorption and fluorescence characteristics of a NAD^+-aldehyde adduct described by Cormier *et al.* (13).

Although our results bear primarily on the nature of the emitter in the *in vivo* reaction, the demonstrated ability of exogenous blue fluorescence protein to sensitize the *in vitro* reaction leads to a reappraisal of the identities both of the primary excited state and of the emitter in the reaction of luciferase alone.

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SEPARATION OF A BLUE FLUORESCENCE PROTEIN FROM BACTERIAL LUCIFERASE

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SUMMARY: Luciferase preparations from two species of marine bioluminescent bacteria, *Photobacterium phosphoreum* and *Photobacterium fischeri*, are shown to contain a low molecular weight protein, containing a blue fluorescence chromophore having an emission maximum in the 470 nm region. A procedure for separating the luciferase and purifying this protein is described. On disc gel electrophoresis the bulk of the protein is observed to migrate along with the blue fluorescence.

The blue emission of bioluminescent bacteria is a broad spectrum with a maximum intensity around 490 nm for most types of bacteria (1). The bioluminescence reaction *in vitro* requires an enzyme, bacterial luciferase, with substrates reduced FMN, oxygen and a long chain aliphatic aldehyde, such as dodecanal. There has been a continuing search over the years for some substance in the reaction having fluorescent properties which would account for the bioluminescence emission, either a chromophore attached to the luciferase (2-6) or one formed by the reaction (4,7,8).

Recently the isolation of a blue fluorescence protein (BFP) from preparations of the luciferase from *Photobacterium phosphoreum*, was announced (9). The fully corrected fluorescence emission spectrum of the BFP was an exact match for the *in vivo* bioluminescence from *P. phosphoreum*, and in the presence of various mildly denaturing agents the spectrum could be shifted 15 nm to longer wavelengths to exactly match the *in vitro* bioluminescence spectrum using *P. phosphoreum* luciferase. The addition of BFP to the *in vitro* reaction also affected the light emission kinetics, increased the light yield and induced a

shift to shorter wavelengths in the bioluminescence emission. These authors suggested that BFP itself was the *in vivo* bioluminescence emitter (9).

In this present work we describe the separation and partial purification of BFP from the luciferase preparations of two species of bacteria, *P. phosphoreum* and *P. fischeri*.

EXPERIMENTAL

The bacteria were *P. phosphoreum* strain "A13" from J. Fitzgerald (Monash University) and *P. fischeri*, strain 399 in the numbering system of Reichelt and Baumann (10). Bacteria were maintained on a solid agar medium and grown in 400 l liquid batches for luciferase preparation (11). Luciferase was assayed by rapid injection of $FMNH_2$ (0.5 ml, 80 μM) to the sample in buffer (1 ml) containing bovine serum albumin (1 mg) and dodecanal (10 μl of a saturated solution in methanol), all at 23°C. The maximum light intensity denotes the luciferase activity, and was measured by a photometer calibrated for absolute photon sensitivity by reference to the luminol chemiluminescence quantum yield standard (12). The BFP was assayed by measuring the fluorescence intensity of a solution in buffer, using an Aminco-Bowman spectrofluorimeter, with the emission wavelength set at 470 nm and the excitation set at the maximum 420 nm for *P. phosphoreum* and 410 nm for *P. fischeri*. The absorbance at the excitation wavelength was adjusted to below 0.1 to avoid attenuation of the fluorexcence signal due to self-absorption. Linearity of fluorescence intensity and concentration of BFP was established and a fluorescein standard solution was used to maintain a constant day-to-day instrumental sensitivity. The concentration of BFP is reported here in arbitrary fluorescence units. Absorption measurements were made on a Cary 14 spectrophotometer. All preparative procedures (see Table) were made at below 5°C, in a buffer of 0.05 M phosphate, 0.3 mM EDTA, pH 7.0. All chemicals were of the best commercial grades. The alkaline disc gel electrophoresis (13) was carried out at 5°C in 7.5% acrylamide containing 1 mM dithiothreitol.

RESULTS AND DISCUSSION

Cells were cultured at room temperature for convenience and harvested in the vicinity of a cell density yielding maximum light per cell. For *P. phosphoreum* a substantial increase in light per cell and yield of BFP can be obtained by growth at 12°C.

The processing of the cell lysate is given in the Table. About 500 g of wet cell cake was suspended in buffer (1 l) containing dithiothreitol (1 mM) and phenylmethylsulfonylfluoride (1 μM) to retard proteolysis, and disrupted by two passages through a French press. Cell walls were removed by centrifugation (60 min, 25000 g) then partial fractionation made by addition over 30 min of ammonium sulfate to 30% saturation, then centrifuging (30 min, 25000 g).

Table. Purification of the Blue Fluorescence Protein (BFP).

A. P. phosphoreum	Total Bioluminescence Activity[a] 10^{12} photons-s^{-1}-ml^{-1}	Total Fluorescence[a] 420 ⟶ 470 nm (arbitrary units)	Total Absorbance at 280 nm[a]
1. Cell lysate	4100	3700[b]	47300
2. Ammonium sulfate fractionation			
30% supernatant	10300	600	59600
80% pellet	7000	540	46600
3. Sephadex G75			
luciferase fraction	7200	124	23600
free BFP fraction[c]	(130)	(210)	(3300)
4. DEAE-cellulose, 0.15 M eluate	7200	30	1700
5. DEAE-Sephadex A50 (0.22 - 0.28 M)	4400	23	780
6. Sephadex G75 super-fine			
luciferase fraction	3500	2	133
free BFP fraction	0.1	6	54
B. P. fischeri			
1. Cell lysate	43400	490	65000
2. Ammonium sulfate fractionation			
30% supernatant	39200	320	38700
80% pellet	31600	250	35200
3. Sephadex G75			
luciferase fraction	30000	44	12500
4. DEAE-cellulose, 0.25 M eluate	24200	26	2000
5. DEAE-Sephadex A50			
0.25 M fraction	5	16.5	354
0.35-0.5 M fraction	26000	0.9	414
6. Sephadex G75 super-fine			
free BFP fraction	6.5	22	223

a. Totals are the luciferase activity/ml x total volume (similarly for fluorescence and A(280). b. This includes a major contribution due to light scattering. c. Not further processed.

The supernatant contained all the luciferase activity (some material in the lysate appears to inhibit the assay) and the pellet was discarded. The supernatant was then made 80% saturation in ammonium sulfate and centrifuged (30 min, 25000 g) after two or more hours.

The amount of true fluorescence in the lysate cannot be estimated due to the major contribution of light scattering. The BFP concentration was therefore estimated here by chromatographing a small sample of the 30% supernatant on a column of Sephadex G75 superfine grade, thereby separating out the BFP from other fluorescent components. About 20% of the fluorescence (Table , line 2), that is about 120 fluorescence units in the 30% supernatant, can be attributed to BFP. For P. fischeri the percentage is about the same.

The third step is desalting by gel filtration in preparation for ion-exchange chromatography. The 80% pellet was redissolved in a minimum amount of buffer (200 ml) and applied to a column of Sephadex G75 (10 x 60 cm). The elution pattern (Fig. 1) shows the luciferase activity at a volume 600 ml almost at the column front, as expected from its molecular weight of 82,000 (5). The fluorescence spectra of fractions in the range 300-1600 ml have maxima around 470 nm corresponding to BFP (9). The material at 2000 ml has a flavin-like fluorescence with a maximum around 520 nm (uncorrected). Some BFP is seen to elute with the luciferase fraction (400-900 ml), possibly an association between these two proteins. However most of the BFP separates and is retarded to around 1400 ml, as expected from its molecular weight of approximately 20,000 (9). An efficient method of purification of this free BFP (1100-1600 ml, Fig. 1) will be described elsewhere as this present work is concerned only with the luciferase-associated BFP. As far as we can tell, the properties of BFP prepared by either route are the same. With P. fischeri (Table B), 95% of the BFP remains luciferase-associated at this stage.

The two anion-exchange steps (4 and 5) were carried out at pH 7.6 for P. phosphoreum and 7.0 for P. fischeri. For P. phosphoreum the luciferase fraction from step 3 (e.g. Fig. 1, 250-1050 ml) was loaded to DEAE-cellulose

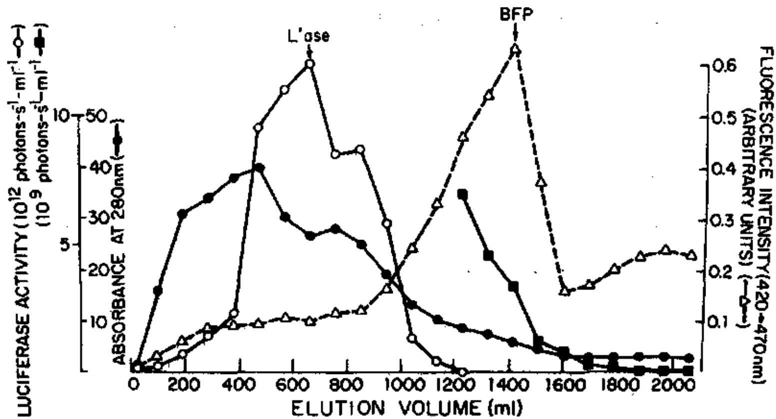

Figure 1. Elution of crude P. phosphoreum luciferase (L'ase) on Sephadex G75 (arbitrary zero), 2 ml/min. Column front is around the 500 ml mark. Some Blue Fluorescence Protein (BFP) co-elutes with the luciferase but most is retarded to around 1400 ml (mol. wt. ~ 20,000).

(DE-32, 6 x 20 cm). The column was washed with buffer (500 ml) and the luciferase/BFP batch-eluted with higher phosphate (0.15 M, 500 ml). This eluate was diluted 1.5-2 fold with distilled water to reduce the salt concentration and loaded to DEAE-Sephadex (A-50, 2 x 20 cm). This column was washed (0.1 M phosphate, 500 ml) and eluted with a linear phosphate gradient (0.15 - 0.3 M, 500 x 500 ml). There was a slight separation between BFP (maximum fluorescence at salt concentration 0.24 M) and luciferase (0.27 M), but the separation was inefficient and all the fractions 0.22 - 0.28 M were therefore combined for filtration on Sephadex G75 superfine grade (4.5 x 100 cm). Elution was made with buffer containing 2-mercaptoethanol (2 mM).

Figure 2 shows that this slow molecular sieving completely separated the P. phosphoreum luciferase which elutes in the front around 250 ml, from the BFP (maximum at 520 ml). The absorbance at 450 nm is maximum at an elution volume of 310 ml and corresponds to a third, highly colored component which, from its characteristic absorption spectrum appears to be a flavoprotein.

The ion exchange steps of P. fischeri required a higher salt concentration, Table B steps 4 and 5. Step 5 in fact provided good separation of BFP in

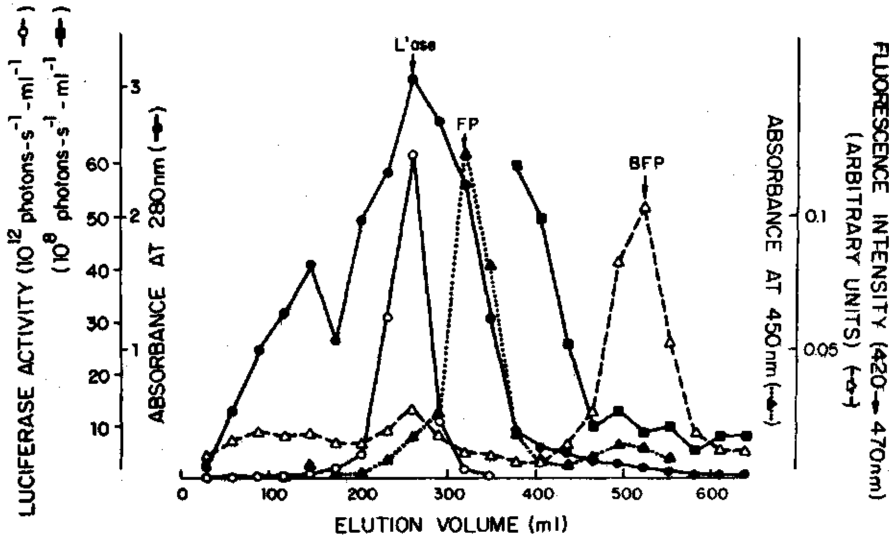

Figure 2. Separation of P. phosphoreum luciferase (260 ml) from blue fluorescence protein (BFP, 520 ml) on Sephadex G75 superfine (10 ml/hr.). A non-fluorescent flavoprotein (FP) appears at 310 ml.

the 0.25 M fraction, from the luciferase. This free BFP was then applied to the slow sieving column, step 6, and eluted in a volume about the same as for P. phosphoreum BFP, suggesting similar molecular weights.

If the figure of 20% of the fluorescence of the 30% supernatant, step 2 in the Table, is used to estimate the BFP in the lysate, then the purification of BFP through this last stage is 50-100 fold, with a yield of 6%, (P. phosphoreum) and 30% (P. fischeri). Two contaminating fluorescent components appear in the BFP preparation, arising from proteins of apparently similar molecular weights. One has a flavin-like fluorescence, the spectrum showing a 520 nm (uncorrected) shoulder when excited at 470 nm. This component is progressively removed by repeated chromatography on Sephadex G75 superfine.

The second fluorescent impurity has a maximum at 420 nm (uncorrected) when excited at 370 nm. It may be a denaturation product of the BFP since its fluorescence intensity increases at the expense of the 420 ⟶ 470 nm fluorescence if the BFP solution is allowed to stand over several days (5°C). If the BFP solution is applied to a DEAE-cellulose column (DE-32) pH 6.5, phosphate

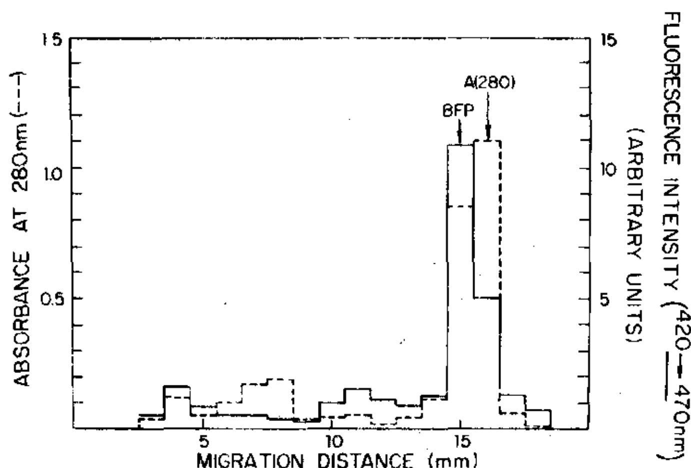

Figure 3. Alkaline disc gel electrophoresis of P. phosphoreum BFP (fraction at 520 ml, Fig. 2).

0.05 M, the BFP can be eluted with 0.1 M phosphate (pH 6.5) and the 420 nm fluorescent material remains bound.

Disc Gel Electrophoresis. Protein from the peak BFP fractions off the Sephadex column Fig. 2 (step 6) was subject to a disc gel analysis, and Fig. 3 shows that the bulk of the protein has the same electrophoretic properties as the 420 ⟶ 470 nm fluorescent material. The gel was sliced into 1 mm sections, extracted into buffer (20 hrs) and then assayed for fluorescence and absorbance against a reference blank gel extract. The maxima of absorbance and fluorescence do not differ significantly and have an average R_F of 0.8. About 70% of the total 280 nm absorbance is included under the fluorescent band. There are no other visible fluorescent protein bands. On further chromatography on the Sephadex G75 superfine, the protein purity is increased to about 90%.

The fluorescence and absorption characteristics of the BFP from P. fischeri suggest a similar degree of purity.

Although the BFP's from these two species of bacteria appear to have similar molecular weights, they differ in that P. fischeri BFP is more tightly

associated with its luciferase and it has a fluorescence excitation maximum shifted about 10 nm to shorter wavelength. Gast and Lee (9) have proposed that BFP has a function in the bacterial bioluminescence reaction. The present evidence for the presence of BFP's having similar properties in two of the four common species of marine bioluminescent bacteria, would appear to add further support to this hypothesis.

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S U M M A R Y

The central theme of this thesis is the interaction of FMN with proteins.For one of the proteins studied,the enzyme luciferase from bacteria,further investigations were done on the process of light emission.

In chapter 2 and 3 studies are reported on the binding of FMN with relatively simple proteins,the flavodoxins.Flavodoxins were chosen,because they are small proteins with a molecular weight not higher than 23 000.They contain only one equivalent of FMN and consist of only one polypeptide chain.No other prosthetic group is known for the flavodoxins.In addition to this they can be obtained in high yields from bacterial cultures.These features make the flavodoxins excellent objects for studies on the binding of flavins to proteins.

In chapter 2 studies on the binding of FMN by apoflavodoxin from Peptostreptococcus elsdenii are reported.Conclusions were drawn from the dependence on the pH and the NaCl concentration.The rate constant of dissociation depends on the pH,even when extrapolated to zero ionic strength.The titration curve of this rate constant can be explained,by the assumption of the involvment of two protonations, that act highly cooperatively.It should be realised that these protonations do not influence the rate

constant of association.But once the complex is formed,the chance of falling apart,if these sites are protonated,is around 6 times higher than without these sites protonated.Furthermore,it was found that the calculated rate constant of association,when extrapolated to zero ionic strength is independent on the pH.At increasing ionic strength this rate constant of association will change.Depending on the pH value,this change is a decrease or an increase in value.At a pH of 3.8 there is almost no change with increasing ionic strength,but above this pH value the rate constant increases,while below this value it decreases with increasing ionic strength. This explains why the combination of a high salt concentration and a low pH is a very effective way of removing the FMN from flavodoxins.This finding might possibly be extrapolated to other flavoproteins as well.By interpreting the results in terms of the Brönsted theory,a net positive charge between 11 and 12 is found on the apoenzyme at low pH.This finding is in agreement with the number of basic amino acid residues in the polypeptide chain.

A series of flavin analogues were synthesised and the kinetic parameters of the interaction with Azotobacter vinelandii apoflavodoxin investigated. These studies are presented in chapter 3.Use was made of a fast kinetic method,the temperature jump relaxation technique.The resolution time of the instrument employed is 11 microseconds.All complexes studied revealed only one relaxation process,indicating that within the time limits studied (11 micro

seconds - ca. 10 seconds),the association of the flavin and the apoenzyme is a one-step process.This finding is in contrast with an earlier publication by other authors,who detected two relaxation processes.It is shown that the earlier published traces are instrumental artifacts.

In chapter 4 the interaction of FMN with an intermediate in the in vitro bacterial bioluminescence reaction is described.The so called "long-lived intermediate",which has been suggested to be an FMN flavoprotein,has been separated into an apoprotein and free FMN.Because of the high quantum yi yields of light with respect to FMN,measured upon reaction of the apoprotein with aldehyde,in vitro bacterial bioluminescence appears to be a sensitised reaction.At a first consideration only FMN could be a likely candidate as a sensitising agent.

However,in chapter 5 it is shown that a novel protein,isolated from the bacteria itself will definitely sensitise the in vitro bacterial bioluminescence reaction.This novel protein (BFP) is efficiently fluorescent (quantum yield of fluorescence 0.45) and has an emission maximum at 476 nm.As a result of these observations,it is called the blue fluorescence protein.By diluting this protein to a concentration of around 1 μM,a spectral shift of the emission maximum is observed.Actually the fluorescence emission spectrum of the protein changes from a spectrum identical to the in vivo bacterial bioluminescence into an emission spectrum identical to the in vitro bacterial bioluminescence emission.Although

this means that the emission of this protein could account for both the in vivo and the in vitro emission spectra,it should be mentioned that investigations learned that the chromophore of this protein is not a product of the in vitro reaction.This blue fluorescence protein is the only one of all the emitters proposed so far,that simulates the bluest of the bacterial emissions exactly.Furthermore,the addition of the blue fluorescence protein to the in vitro reaction affects the light emission kinetics (it acts as a catalyst),increases the light yield and induces a shift to shorter wavelengths in the bioluminescence emission.Together with the fact that the protein is isolated from the bacteria themselves,these features are strong evidence that this protein is the in vivo emitter.

In chapter 6 the purification procedure of the BFP is given in more detail.Furthermore it is shown that it can be isolated from at least two of the four common species of marine bioluminescent bacteria.This suggests that all the bacteria emit their light via the same kind of chemical mechanism.Although the proteins from these two species of bacteria appear to have similar molecular weights,they differ in that the protein from P.fischeri is more tightly associated with the luciferase during the purification procedure than the one from P.phosphoreum and also that its fluorescence excitation maximum is shifted about 10 nm to shorter wavelength.Further investigation should be done in order to learn what the chemical nature of the fluorophore is.

SAMENVATTING

De interactie tussen FMN en eiwitten is het centrale thema van dit proefschrift. Onderzocht is de interactie van FMN (een derivaat van het vitamine B-2) met flavodoxines en met het enzym luciferase uit bacteriën. Flavodoxines zijn vrij kleine eiwitten. Zij katalyseren de elektronenoverdracht tussen andere eiwitten bij een lage redox potentiaal. Als prosthetische groep bezitten ze meestal slechts één molecuul FMN en zijn o.a. daarom erg geschikt voor de bestudering van FMN eiwit interacties. Het luciferase is een enzym, dat de lichtproduktie in lichtgevende bacteriën katalyseert. Voor de activiteit ervan in vitro is $FMNH_2$ vereist.

In hoofdstuk 2 wordt een studie beschreven over de binding van FMN aan het apoflavodoxine van Peptostreptococcus elsdenii. Onderzocht is de afhankelijkheid van de pH en van de NaCl concentratie. De berekende associatie snelheidsconstante blijkt onafhankelijk te zijn van de pH, indien de waarde ervan geëxtrapoleerd wordt naar een ionsterkte van nul. Toepassing van de Brönsted theorie levert een netto lading op van +12 voor het apoflavodoxine bij lage pH. Deze waarde komt overeen met de waarde die afgeleid kan worden uit de primaire structuur van het flavodoxine. Veel apovormen van flavoproteinen kunnen worden gemaakt door het enzym bij lage pH tegen een hoge zoutconcentratie te dialyseren. Uit de resultaten van het

onderzoek beschreven in hoofdstuk 2,kan worden afgeleid dat dit veroorzaakt wordt door de grote verlaging van de associatie snelheidsconstante bij deze omstandigheden.De dissociatie snelheidsconstante is wel afhankelijk van de pH,ook indien naar een ionsterkte van nul wordt geëxtrapoleerd.Deze afhankelijkheid kan verklaard worden door aan te nemen dat er twee protonen aan het eiwit adderen.Door deze addities wordt de waarde van deze snelheidsconstante verhoogd van 0,015 s^{-1} tot 0,088 s^{-1}.

Een serie flavine analogen werd gesynthetiseerd en de kinetische parameters van de interactie met de apoflavodoxines van Azotobacter vinelandii en Peptostreptococcus elsdenii werden bepaald.Dit onderzoek is beschreven in hoofdstuk 3.Er werd gebruik gemaakt van een snelle kinetische methode,de "temperatuursprong" relaxatie.Het oplossend vermogen in de tijd is 11 microseconden voor het instrument waarmee de bepalingen gedaan werden.Alle complexen die bestudeerd zijn,vertonen slechts één relaxatie,zodat geconcludeerd kan worden,dat de binding van het FMN aan het apoflavodoxine een 1-staps proces is.Onafhankelijk bepaalde evenwichtsconstanten komen overeen met uit de kinetische bepalingen berekende waarden, indien een 1-staps proces wordt aangenomen.Dit is in overeenstemming met de eerder getrokken conclusie.

In hoofdstuk 4 wordt de interactie van FMN met een tussenprodukt van de in vitro bacteriële bioluminescentie reactie beschreven.Het blijkt dat FMN gescheiden kan worden van het eiwitgedeelte.Doordat nu zeer hoge lichtquantum opbrengsten t.o.v. FMN geme-

ten werden,moest geconcludeerd worden,dat de bacteriële bioluminescentie reactie,in vitro van het "indirecte type" is.M.a.w. het luminescentie emissie spectrum is dat van een ander fluorescerend molecuul in de oplossing en dus niet van het produkt van de reactie.

Hoewel op het eerste gezicht alleen FMN deze rol van acceptor zou kunnen vervullen,wordt in hoofdstuk 5 een nieuw ontdekt eiwit beschreven,dat in alle opzichten voldoet om als uiteindelijke lichtemittor te kunnen fungeren.Het is sterk fluorescerend en heeft een emissie maximum bij 476 nm.Door het eiwit te verdunnen tot een concentratie van rond de 1 μM,wordt een verschuiving van dit emissie maximum naar langere golflengten waargenomen.In feite verandert het emissie spectrum van het eiwit door de verdunning van een in vivo type naar een in vitro type spectrum.Deze eigenschap van het eiwit zou dus een verklaring kunnen geven voor het verschil dat er bestaat tussen het in vivo en het in vitro emissie spectrum.Dit nieuw ontdekte eiwit is de enige van alle voorgestelde emittors,waarvan het fluorescentie emissie spectrum identiek is aan het meest-blauwe bacteriële emissie spectrum.Voorts is er een groot effect op de in vitro reactie.Toevoeging van dit eiwit aan het reactiemengsel,versnelt de lichtemissie, verhoogt de lichtopbrengst en verschuift het emissie maximum naar kortere golflengten.Het eiwit fungeert dus ook als katalysator.Samen met het feit dat dit eiwit uit de bacteriën zelf geisoleerd kan worden, vormen deze eigenschappen een bewijs,dat dit eiwit

in vivo de emittor is.

In hoofdstuk 6 wordt nader ingegaan op de zuivering van het eiwit met de blauwe fluorescentie. Ook wordt aangetoond,dat het eiwit geisoleerd kan worden uit tenminste twee van de vier groepen van bioluminescente zoutwater bacteriën,te weten Photobacterium phosphoreum en Photobacterium fischeri.Hoewel de eiwitten van deze twee bacteriën een zelfde molecuulgewicht (22 000) blijken te bezitten,verschillen ze in zoverre van elkaar dat het eiwit van de laatstgenoemde,tijdens de zuivering minder gemakkelijk van het luciferase te verwijderen is.Hoewel het fluorescentie excitatie maximum voor dit laatste eiwit 10 nm naar kortere golflengte ligt, mag worden aangenomen dat het om hetzelfde fluorophoor gaat.Verder onderzoek moet leren wat de chemische structuur van dit fluorophoor is.

CURRICULUM VITAE

Overeenkomstig de wens van het college van dekanen van de Landbouwhogeschool volgt hier een korte beschrijving van mijn levensloop. Op 8 juli 1945 werd ik te Langedijk geboren. Het kleuteronderwijs volgde ik in Alkmaar en het lager onderwijs genoot ik in Utrecht. Na in 1962 het eindexamen H.B.S.-b te hebben afgelegd aan het Eindhovens Protestants Lyceum, begon ik in hetzelfde jaar aan de Vrije Universiteit te Amsterdam met mijn studie scheikunde. Het kandidaatsexamen (letter: e) werd in juni 1967 afgelegd. De studie werd voortgezet met als hoofdvak biochemie (Prof.Dr.R.J. Planta), bijvak fysiche chemie (Prof.Dr.Ir.C.McLean) en tweede bijvak missiologie (Prof.Dr.J.Verkuyl). Het doctoraalexamen werd afgelegd in december 1970. Van april 1967 tot augustus 1970 was ik tevens, als vakleraar scheikunde, verbonden aan "De Eikenhof", school voor M.A.V.O. te Bloemendaal. Gedurende de jaren 1971-1974 was ik in dienst bij de Nederlandse Organisatie voor zuiver-wetenschappelijk Onderzoek (ZWO), als wetenschappelijk medewerker aan het Laboratorium voor Biochemie van de Landbouwhogeschool, bij een werkgroep o.l.v. Prof.Dr.C.Veeger, van de "Werkgemeenschap Eiwitten" van de Stichting Scheikundig Onderzoek Nederland (SON). Gedurende 1975-1976 was ik als "Research Associate" verbonden aan de University of Georgia (Department of Biochemistry) Athens, Ga, U.S.A., op een plaats gesubsidieerd door de National Institutes of Health en de National Science Foundation in subsidies toegekend aan Prof.Dr.John Lee. In 1976 werd de zomercursus van het Summer Institute of Linguistics, verbonden aan de University of North Carolina at Charlotte, Charlotte, N.C., U.S.A., gevolgd.

Van de volgende publikaties ben ik (mede-)auteur;

- Müller, F., Jarbandhan, T., Gast, R. en Grande, H.J. (1975) in Reactivity of Flavins (Yagi, K., ed.) p.51 University of Tokyo Press, Tokyo; ook verschenen als Proc. Japanese Biochem. Soc. 46, 17 (1973)
- Gast, R. en Müller, F. (1978) Helv.Chim.Acta inpress; hoofdstuk 3 in dit proefschrift
- Grande, H.J., Gast, R., v.Schagen, C.G., v.Berkel, W.J.H. en Müller, F. (1977) Helv.Chim.Acta 60, 367
- Gast, R., Valk, B.E., Müller, F., Mayhew, S.G. en Veeger, C. (1976) Biochim.Biophys.Acta 446, 463; hoofdstuk 2 in dit proefschrift

-Gast,R. en Lee,J. Abstract MAM-B3,p.43 third annual meeting of the American Society for Photobiology; hoofdstuk 4 in dit proefschrift (1975)

-Gast,R. en Lee,J. (1978) Proc.Natl.Acad.Sci.U.S. in druk;hoofdstuk 5 in dit proefschrift

-Gast,R. en Lee,J. (1976) Isolation of the bacterial bioluminescent emitter,Abstract at the 7th International Congress on Photobiology,p.324

-Gast,R.,Neering,I.R. en Lee,J. (1978) Biochem. Biophys.Res.Commun. 80,14;hoofdstuk 6 in dit proefschrift

-Gast,R. en Müller,F "On flavoquinone-water interaction",manuscript in voorbereiding.

Na terugkomst in Nederland was ik gedurende enkele maanden werkzoekend.Sedert 1 augustus 1977 ben ik werkzaam als leraar klinische en fysiologische chemie aan de H.T.S./S.L.P. te Hengelo.Het onderzoek,beschreven in dit proefschrift is uitgevoerd in de periode van oktober 1972 - december 1976.Het onderzoek beschreven in de hoofdstukken 2 en 3 zijn in Wageningen uitgevoerd onder de dagelijkse leiding van Dr.F.Müller;het onderzoek beschreven in de hoofdstukken 4,5 en 6 is uitgevoerd in Athens,Ga. onder verantwoordelijkheid van Dr.John Lee.Mijn adres is:v.Heemskerkstraat 63 te 7622 JH Borne,T.05409-4369.

In analyzing or evaluating an object,we think and judge from a particular point of view.The psychologist,economist,and chemist pay attention to different aspects of the same object.Such is the limitation of the mind that it can never see three sides of a building at the same time.The danger begins when,completely caught in one perspective, we attempt to consider a part as the whole.In the twilight of such perspectivism,even the sight of the part is distorted.What we cannot comprehend by analysis,we become aware of in awe.When we "stand still and consider",we face and witness what is immune to analysis.

Abraham Joshua Heschel

God in Search of Man:
a philosophy of judaism

New York,1976,p.75