On the Structure of Flavin-Oxygen Intermediates Involved in Enzymatic Reactions

Sandro GHISLA, Barrie ENTSCH, Vincent MASSEY, and Mazhar HUSEIN

Fachbereich Biologie der Universität Konstanz, and Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan

During the catalytic reactions of flavoprotein hydroxylases and bacterial luciferase, flavin peroxides are formed as intermediates [see Massey, V. and Hemmerich, P. (1976) in *The Enzymes*, 3rd edn (P. Boyer, ed.) pp. 421–505, Academic Press, New York]. These intermediates have been postulated to be C(4a) derivatives of the flavin coenzyme. To test this hypothesis, modified flavin coenzymes carrying an oxygen substituent at position C(4a) of the isoalloxazine ring were synthesized. They are tightly bound by the apoenzymes of D-amino acid oxidase, p-hydroxybenzoate hydroxylase and lactate oxidase; the resulting complexes show spectral properties closely similar to those of the transient oxygen adducts of the hydroxylases. The optical spectra of the lumiflavin model compounds were found to be highly dependent on the solvent environment and nature of the substituents. Under appropriate conditions they simulate satisfactorily the spectra of the transient enzymatic oxygen adducts. The results support the proposal that the primary oxygen adducts formed with these flavoproteins on reaction of the reduced enzymes with oxygen are flavin C(4a) peroxides.

Flavoprotein oxidases and monooxygenases are redox enzymes which catalyze the reaction of molecular oxygen with a variety of substrates [1–3]. Characteristic of this catalysis is the reaction with oxygen of the reduced flavoenzyme in the presence of substrate or product. It is the reduced flavin which is involved in the activation of oxygen. With monooxygenases the result is the incorporation of one atom of the molecule of oxygen in the substrate, and the reduction of the other one to water. First insights into the mechanism of the rapid reaction of reduced flavin with O_2 [4,5] were obtained from its kinetics, which indicated the reversible formation of a Fl_{red}-O_2 complex prior to the actual electron transfer [6,7]. Direct spectral evidence for the transient occurrence of a covalent flavin-oxygen adduct was obtained later in studies with the enzymes meliotate hydroxylase [8], p-hydroxybenzoate hydroxylase [9], phenol hydroxylase [2] and very recently with salicylate hydroxylase [10]. In the case of p-hydroxybenzoate hydroxylase a detailed kinetic investigation lead to the discovery of at least three sequential intermediates arising from the reaction of the reduced enzyme with oxygen [11]

during the process of hydroxylation of a substrate. Comparison of the characteristic spectra of two of the three intermediates [11] with those of models [12,13] suggested the structure of covalent flavin adducts at C(4a), C(4a)-OOH and C(4a)-OH.

The possible structure and function of flavin oxygen intermediates, however, has been a matter of some controversy, and most flavin functions, e.g. positions C(4a), N(5), C(6), C(8), C(9a) and C(10a), have been proposed as the position of a covalent link to a catalytically active oxygen peroxide [1,2,4,5, 14–19], but as yet none of these structures has been verified in biological systems.

The aim of the present work was the structure elucidation of the enzymatic Fl-O_2 intermediates mentioned above by comparison of their spectral properties with those of suitable, enzyme-bound modified coenzymes of unambiguous structure. Derivatives of the 4a,5-dihydro-isoalloxazine at the FAD and FMN level, which carry an oxygen function at C(4a), were first chosen as models as their spectra [13,20] bear considerable similarity to those of the transient intermediates [11]. The chromophores which can be derived from other isomeric forms of the reduced flavin have spectra with considerably different properties [21]. 4a-OR flavins are accessible only upon blocking of N(5) of the isoalloxazine through alkylation [12]. A 4a-OH substituent rather than a 4a-OOH substituent was chosen because the two

intermediates with the proposed structures of $-OOH$, and $-OH$ flavin adducts have almost identical spectra [11], and because a rapid exchange of an $-OOH$ substituent by $-OH$ arising from aqueous solvent has been shown to occur [20].

EXPERIMENTAL PROCEDURE

Spectroscopic Methods

Absorption spectra were recorded with a Cary model 17 or 118 spectrophotometer. Fluorescence emission and excitation spectra were recorded at the temperature indicated with a ratio-recording fluorimeter, which will be described elsewhere (Ballou, D. P. and Ford, G., unpublished), the fluorescence excitation spectra are thus corrected for shape and λ_{max}.

The rapid reaction experiments were carried out with a Gibson-Milnes stopped-flow spectrophotometer, also equipped for fluorescence observation.

Enzymes, Apoenzymes and Materials

Alkaline phosphatase from *E. coli* was obtained from Worthington. *Naja naja* venom (phosphodiesterase) was obtained from the Ross Allen Reptile Institute (Silver Spring, Florida). FAD and FMN were from Sigma Chemical Co. and were used without further purification for the chemical syntheses. The other chemical compounds used were from commercial sources and were usually purified by recrystallization. 5-Ethyl-flavoquinonium perchlorate was synthesized as described elsewhere [12].

The following enzymes and apoenzymes were prepared by published procedures: flavodoxin was from *Peptostreptococcus elsdenii* [22] and apoflavodoxin [23], D-amino acid oxidase from pig kidney [24] and apo D-amino acid oxidase [25], lactate oxidase from *Mycobacterium smegmatis* [26] and apo lactate oxidase [27], melilotate hydroxylase [28], and apo-melilotate hydroxylase [25]; p-hydroxy-benzoate hydroxylase from *Pseudomonas fluorescens* was prepared by the procedure of Howell *et al.* [29], as modified by Entsch *et al.* [11]. Phenol hydroxylase from *Trichosporon cutaneum* [30] was a generous gift from Dr H. Neujahr; the apoenzyme [31] was prepared by dialysis against KBr by the procedure described [25].

Apo-p-hydroxybenzoate hydroxylase, as used in the experiments reported in this paper, was prepared by the following dialysis technique. A solution of $25-30$ μM enzyme was dialysed at $2-4$ °C against 50 vol. of a solution of 1 M KBr, 1 M urea, 0.5 mM EDTA, 0.3 mM p-hydroxybenzoate, and 0.1 M potassium phosphate, pH 7.9 to 8.0. The dialysis fluid was changed every 12 h until the enzyme activity of

the apoprotein solution was 2% or less of the total active enzyme present after reconstitution with FAD. The apoprotein solution then was dialysed with three changes against a solution of 0.3 M p-hydroxybenzoate, 0.5 mM EDTA, and 0.1 M potassium phosphate, pH 7.5. A recovery of $70-90\%$ of the starting enzyme activity was achieved. The apoprotein solution lost 5% or less re-constitutable activity after 4 weeks at 0 °C. The solution could be conveniently concentrated with a semipermeable membrane (*e.g.* Amincon Diaflo membrane PM-30).

Chemical Syntheses

5-Ethyl-4a-hydroxy-4a,5-dihydro-FAD (4a-OH-FAD). Solid FAD (50 mg) was dissolved in 5 ml of 2.0 M potassium phosphate (a molar ratio of 9/1 for dibasic/monobasic phosphate) and 5 ml of dimethylformamide was added. The mixture was stirred vigorously under nitrogen and 200 mg of sodium dithionite was added. The solution changed to a pale orange-yellow when reduction of the mixture was complete. 1 g of ethyl iodide was added, the reaction mixture was stirred, and held at $10-12$ °C. The alkylation of FAD was usually complete in 3 h. A subsequent addition of 0.5 g ethyl iodide was necessary after $80-90$ min if the reaction was run under a stream of nitrogen. The reaction was followed spectrophotometrically. An aliquot of the upper (aqueous) layer was added to 1 M acetic acid and shaken quickly. The unchanged, oxidized FAD was measured by the absorbance at 450 nm ($\varepsilon = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$). The degree of alkylation was also determined on the same aliquot as follows. The pH was raised to 5.5 by adding 5 M NaOH and the solution shaken again. The absorption at 585 nm was followed to maximum formation of the blue colour characteristic of the neutral radical of 5-ethyl-FAD ($\varepsilon = 4.0 \text{ mM}^{-1} \text{ cm}^{-1}$). The synthesis was stopped when oxidized flavin was less than 5% of the total. The aqueous phase of the reaction mixture was adjusted at room temperature to pH 5.0 with 5 M phosphoric acid. Much of the phosphate present precipitated and was separated mechanically. The yellow solution was extracted three times with 50 ml of chloroform and once with 50 ml of ether. The aqueous phase turned black due to the blue radical formed by oxidation with oxygen. Without delay, the pH was adjusted (if necessary) to $4.9-5.0$ with acetic acid or NaOH, and the solution cooled to 0 °C. $80-100$ μmol of $NaNO_2$ (in solution) was added to the product, which was then kept for 10 min at 0 °C. The solution turned dark orange-red upon oxidation of the radical form. At $2-4$ °C this solution ($5-7$ ml) was passed through a column of Bio-Gel P-2 ($200-400$ mesh in a column of 2.6×40 cm, equilibrated with 50 mM potassium acetate, pH 4.9). The desired product separated from an orange-brown

band on the column, and was recognized by its characteristic absorption spectrum (Fig. 2). Small amounts of the blue radical form were frequently eluted with the product as detected by long-wavelength absorbance at pH 6.0 – 7.0. The radical present was conveniently oxidized before use by titration with a solution of potassium ferricyanide at 0 °C and pH 7.0. The band of product was followed on the column by unchanged FAD, which was recognized by its absorption at 450 nm and by its green fluorescence; the product was not fluorescent. The peak fractions of highest purity (at least 95 % having the 4a-hydroxy-5-ethyl-isoalloxazine structure) were collected and stored for use at −70 °C. The product was stable at this temperature for at least 3 months. Aliquots could be kept at 0 °C for several hours (pH 5.0) with negligible decomposition. The yield was approximately 30 % of the starting FAD on a molar basis.

5-Ethyl-4a-hydroxy-4a,5-dihydro-FMN (4a-OH-FMN) was prepared from purified 4a-OH-FAD by incubation of the latter with phosphodiesterase (*Naja naja* venom) and separation over a Bio-Gel P-2 column equilibrated with 0.5 M sodium acetate buffer pH 5.0. The corresponding 4a-OH-riboflavin may be prepared in a similar fashion by incubation with phosphatase.

4a-OH-flavins at the FAD, FMN and riboflavin level are well separated by thin-layer chromatography on silica gel plates in butanol/acetic acid/water (12/3/5). The spots can be visualized by conversion to the red cations by spraying with 1 M HCl.

5,8-10,11-Tetramethyl-1,2dihydro-8 H-benz[g]-oxazolo [2,3-e]-pteridin-4,6-dione, (4a,5-oxapropano-4a,5-dihydro-3-methyllumiflavin *cf.* structure insert in Fig. 1), was synthesized as will be published elsewhere [32a], m.p. 202 − 204 °C. The structure is confirmed by X-ray crystallography [32].

RESULTS

Synthesis and Chemical Properties of Flavin Models

The synthesis of some 4a,5-dihydroflavins at the lumiflavin level as reported earlier [12], involves drastic reaction conditions (pH > 12), which would decompose FAD, or requires organic solvents, in which FAD or FMN are not soluble. These difficulties have been circumvented by the use of a biphasic solvent mixture (dimethylformamide/water) strongly buffered at pH 8, where the reduced isoalloxazine is monoionized; N(1)-H, pK 6.5. Under these conditions reduced FAD was found to be alkylated practically selectively at N(5), the other nucleophilic functions (*e.g.* adenine) remaining unaltered, as indicated by the binding studies with apoenzyme detailed below. FAD analogues, in which the adenine moiety is
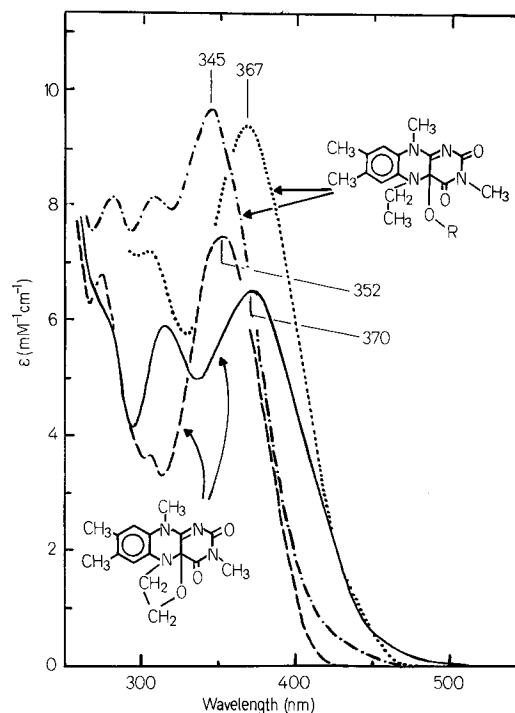

Fig. 1. *Effect of C (4a) substituent and of solvent on the 4a,5-dihydroflavin chromophore.* A solution of the 4a,5-bridged, lumiflavin model compound (see inserted structure), 4.0 mM in absolute dioxane was diluted with 99 vol. cyclohexane (– – – –), or 99 vol. 1.0 M NaCl solution (———). The flavin 4a-OH adduct (–·–·–·) (see inserted structure, R = H) is formed by addition of 10 μl of a 10 mM potassium carbonate solution to 1.0 ml of a solution of 5-ethyl-lumiflavinium perchlorate, 50 μM in absolute acetonitrile. The base is required for the neutralisation of ClO$_4^-$. The 4a-OOH adduct (·····) (R = OH) is formed analogously by addition of 10 μl of 85 % H$_2$O$_2$ containing 10 mM potassium carbonate. Kemal and Bruice [20] have reported a similar spectrum of 5-methyl-4a-peroxy-lumiflavin in methanol

modified, bind only very weakly to the apo enzyme of D-amino acid oxidase [33 − 35]. The purity of the modified OH-FAD is best estimated from the degree of conversion of the neutral species into the alloxazinium cation (Fig. 2) [12], *e.g.* the ratios of the absorbancies at 257 and 357 nm (pH 7), *versus* 437 or 550 nm, at low pH and from the ratios of the cation absorbancies at 550 and 320 nm (= 3.2) for the cation. The purity was also checked by thin-layer chromatography in the system already described, where 4a-OH-FAD runs as a single spot, well separated from the FMN and riboflavin forms and from decomposition products. The 4a-OH-isoalloxazine structure was confirmed by its behavior towards reductants which is analogous to that reported for lumiflavine models [12,36].

The reversible removal of a solvent molecule from position C(4a) of 4a-OH-FAD and 4a-OH-FMN occurs with a pK 3.7. This pK compares with a pK of 4.1 for the corresponding lumiflavin derivative [12].

The corresponding pK for the C(4a), N(5)-oxapropano-bridged lumiflavin model compound (*cf.*
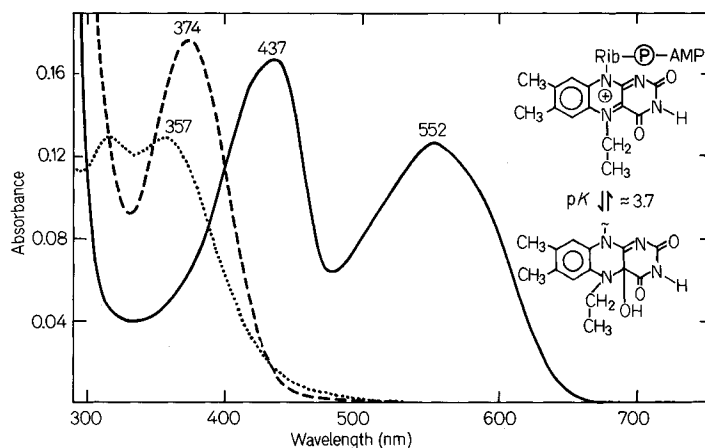
Fig. 2. *Absorption spectrum of 4a-OH-FAD, free in solution, and bound to apo D-amino acid oxidase, and release of the modified coenzyme from the holoenzyme.* Curve ($\cdots\cdots$) shows the spectrum of 4a-OH-FAD 16.7 μM in 0.1 M phosphate buffer, pH 6.5. A solution of apoenzyme (0.30 ml of 0.21 mM) was added to a solution containing 10 equiv. of 4a-OH-FAD, to give 1.5 ml of 40 mM potassium acetate, 0.10 M potassium phosphate, and 0.4 mM EDTA, pH 7.1 at 0 °C. The solution was incubated at 25 °C for 6 min, cooled to 0 °C, and passed through a column of Sephadex G-25, equilibrated at 2 − 4 °C with 50 mM potassium phosphate and 0.4 mM EDTA, pH 7.5. Curve (— — — —), absorption spectrum of the eluted protein fraction. 1.0 ml of this solution was acidified with 80 μl 5 M HCl, incubated at 25 °C for 4 min and the protein precipitate centrifuged at 4 °C. Curve (———) represents the spectrum of the supernatant solution. The spectrum of the same solution after neutralization with 80 μl 5 M NaOH and addition of ferricyanide (5 μM final), and subtraction of the ferrycyanide absorption contribution, is closely similar to curve ($\cdots\cdots$)

structure, Fig. 1) is lowered to 1.25, and reflects reversible addition of the terminal hydroxyl to position C(4a).

Absorption Spectra of 4a-OR-5-R-4a,5-dihydroflavins

The spectra of 4a,5-dihydroflavins are characterized by the presence of two or three bands of strongly varying intensity at $\lambda > 270$ nm (Fig. 1) and by a lowest energy transition with λ_{max} ranging from 327 to 386 nm [12,13].

Thus any structural interpretation based on spectral comparisons with 4a,5-dihydroflavin models will be inherently ambiguous, unless the factors affecting the chromophore such as solvent, substituents, geometry, and also the internal mobility of the molecule have been deliniated. Such effects are well illustrated on Fig. 1. The 4a,5-bridged 4a,5-dihydro-flavin was chosen as a model, as it should be largely rigid as compared to other dihydroflavins, and thus simulate a restrained mobility possibly induced by enzyme active sites. In fact it approximates, in apolar medium, the spectral shape of the enzymatic intermediates [11]. X-ray crystallography shows that the benzene, pyrazine and pyrimidine subnuclei form an only slightly distorted plane, whereby the plane formed by the $-CH_2-CH_2-O-$ bridge is close to being perpendicular to it [32]. Major spectral effects are observed on variation of the C(4a) substituent, $e.g.$ $-N-R_2$, $-O-R$, $-O-CO-R$, $-CH_2-R$. $(R = $ alkyl or H). The three absorption bands at $\lambda > 270$ nm vary in their intensity by a factor up to two, in some cases the 'second band' ($\lambda_{max} \approx 310$ nm) being of higher intensity

than the lowest energy one, and its λ_{max} varying by $\pm$ 20 nm (not shown).

Binding of 4a,5-Dihydro-FAD Models to Apo D-Amino Acid Oxidase and Properties of the Complexes

Apo D-amino acid oxidase binds 4a-OH-FAD tightly. The process of binding is accompanied by the appearance of a fluorescence emission maximal at about 500 nm and by a marked change in the absorption spectrum in the 360−400-nm region (Fig. 2). 4a-OH-FAD does not exhibit detectable fluorescence free in solution at room temperature.

The spectrum of the resulting complex (Fig. 2) is characterized by a single symmetric absorption band in the near ultraviolet with a maximum at 374 nm. The rate of binding, as monitored by the absorption and fluorescence changes (Fig. 3, upper part) is biphasic for the first 3 − 4 min. While the first phase is concentration-dependent, the second slower phase is fairly well described as a first-order process with $k = 1.43$ min^{-1}. This behavior is analogous to that found for the biphasic binding of normal FAD, as measured by fluorescence quenching, where the second reaction step has $k = 0.7$ min^{-1} under similar conditions [25]. After the first two comparatively rapid phases, the absorption of the complex was found to undergo very small changes (Fig. 3, lower part), which were consistent with a species with a longer wavelength maximum changing to a final complex with $\lambda_{max} = 374$ nm. In contrast to this minor absorption change, the fluorescence emission decreases, after the

first rapid increase, to a quarter of its maximal value with $t_{1/2} \approx 15$ min. During this process the excitation and emission spectra, which were recorded at constant $\lambda_{exc.} = 382$ nm, and $\lambda_{emiss.} = 498$ nm, changed only in their intensity but not in their shape or λ_{max}. Further-
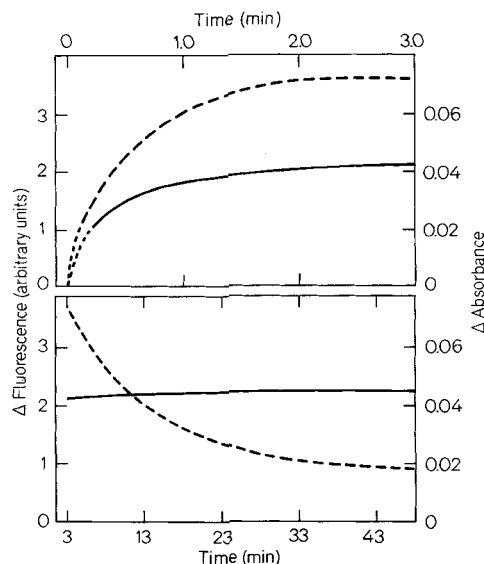

Fig. 3. *Changes in fluorescence and absorbance upon binding of 4a-OH-FAD to apo D-amino acid oxidase.* The figure represents a typical experiment in which the binding process was initiated by addition of concentrated solution of modified FAD (final concentration 16 μM) to the apoprotein (20 μM) in 40 mM potassium phosphate buffer pH 7.55 at 25 °C. Curve (------) represents the fluorescence changes occurring with time, excitation 382 nm, emission 495 nm, and curve (———) the absorbance changes recorded at 385 nm

more, while the fluorescence excitation and emission spectra show temperature-dependent changes in their wavelength maxima (Table 1), the absorption spectrum is largely unaffected. Corrected fluorescence excitation spectra (Table 1) do not coincide with the absorption spectra (Fig. 2) showing a red shift of the excitation maximum. This suggests the presence of a single fluorescent species and that the dominant absorbing species is not identical with the fluorescent one.

When a solution of apoenzyme is titrated with increments of 4a-OH-FAD no deviation from linearity is evident in a plot of the observed spectral changes *versus* the amount of added FAD derivative (Fig. 4). Similarly a sharp titration plot is shown for the appearance of fluorescence (not shown) recorded at the time of its maximal formation. Both fluorescence and absorption titrations indicate a $K_d < 10^{-7}$ M, and thus a better binding of 4a-OH-FAD as compared to normal FAD ($K_d = 2.2 \times 10^{-7}$ M [37]). The end point of titrations such as in Fig. 4 corresponds to 1.09 equivalents of 4a-OH-FAD bound per apoenzyme flavin binding site; from this $\varepsilon_{374} \approx 9400$ M^{-1} cm^{-1} can be estimated for the complex.

That no chemical changes of 4a-OH-FAD occur upon binding to the protein is shown by the experiment outlined in Fig. 2. Release of the modified coenzyme by acid denaturation of the protein leads to quantitative formation of the 5-ethyl-flavinium cation of FAD (Fig. 2). Based on the reasonable assumption that the ε_{437} and ε_{550} values are constant for 5-ethyl-flavoquinonium cations modified only in the N(10) side chain (lumiflavin or riboflavin derivatives) this experiment indicates an $\varepsilon_{374} \approx 9500$ M^{-1} cm^{-1} for the

Table 1. *Fluorescence properties of 4a,5-dihydro-flavins bound to apoflavoproteins*
The samples were prepared essentially as described in the legends of Fig. 2 and 5

| Complex		Temperature	Excitation λ_{max} ($\lambda_{emission}$)	Emission λ_{max} ($\lambda_{excitation}$)
flavin	enzyme			
		°C	nm	
4a-OR-Lumiflavin [13] (free)		−196	381	486
4a-OH-FAD	D-amino acid oxidase	25	382	498
		3	388	503
4a,5-propano-FAD	D-amino acid oxidase	25	385	518
4a-OH-FAD	p-hydroxybenzoate hydroxylase	3	320; 357	494
4a-OH-FAD + p-hydroxybenzoate	p-hydroxybenzoate hydroxylase	3	(505) 372	(372) 505
			(460) 357	(350) 499
			(550) 382	(390) 511
4a-OH-FAD + 5-aminonicotinate	p-hydroxybenzoate hydroxylase	3	360	470
4a-OH-FMN	lactate oxidase	25	320; 400	(400) 530
4a-OH-FMN	FMN-luciferase[a]	25	375	490

[a] Ghisla, S., Massey, V. & Hastings, W. (unpublished results).
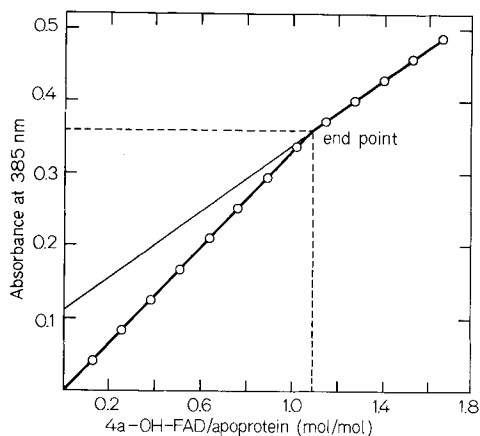
Fig. 4. *Stoichiometry of binding of 4a-OH-FAD to apo D-amino acid oxidase.* Freshly prepared apoenzyme 42 μM in 1.10 ml buffer pH 7.55 (5 mM sodium pyrophosphate, 35 mM potassium phosphate) was titrated at 25 °C with increments of the modified FAD 1.18 mM in 55 mM potassium acetate and 88 mM potassium phosphate, pH 7.1. The absorbance changes at 385 nm were measured after complete equilibration of the system had occurred ($\approx$ 4 min) and are corrected for dilution

Fig. 5. *Absorption spectra of 4a-OH-FAD bound to apo* p-*hydroxybenzoate hydroxylase in the presence and absence of* p-*hydroxybenzoate.* 1.0 ml of 55 μM apoprotein solution was added to 5 equiv. of 4a-OH-FAD to give a final solution of 1.4 ml of 80 mM potassium phosphate, 15 mM potassium acetate, 0.4 mM EDTA, and 0.25 mM p-hydroxybenzoate, pH 6.9 at 0 °C. The solution was incubated at 25 °C for 15 min and cooled to 0 °C. Excess derivative and p-hydroxybenzoate were removed by passage through Sephadex G-25 equilibrated with 0.05 M potassium phosphate buffer pH 7.5 containing 0.4 mM EDTA, at 4 °C. Curve (————) represents the spectrum of the complex (22 μM) in the absence of p-hydroxybenzoate at 3 °C. Curve (— — — —) shows the same complex at 25 °C after addition of p-hydroxybenzoate or of 6-aminonicotinate to final concentrations of 0.85 mM and 0.62 mM respectively. The curve (O·····O) represents the spectrum of intermediate I (peroxy adduct of reduced flavin). Reduced enzyme complex with p-hydroxybenzoate was reacted with oxygen in a stopped-flow spectrophotometer at 3.5 °C. The final solution contained 17.7 μM enzyme, 0.5 mM p-hydroxybenzoate, 0.66 mM oxygen, 50 mM potassium phosphate, 25 mM EDTA (Na salt), pH 6.6. The open circles represent the experimentally measured absorbance of the solution at the wavelength, 20 ms after the end of flow. The dead time of the instrument was between 2 and 2.5 ms. Curve (— · — · —) represents the fluorescence excitation spectrum (arbitrary units on the vertical scales) of the p-hydroxy benzoate ternary complex recorded with $\lambda_{emiss.}$ = 550 nm

protein-bound 4a-OH-FAD, in good agreement with the value obtained by direct titration. Neutralization of the acid solution of the modified FAD released from the protein restores the spectrum of 4a-OH-FAD. Hence the spectral changes resulting upon binding to apo-D-amino acid oxidase result from the specific interaction of the chromophore with the apoenzyme, and from protein conformational changes, but not from chemical modifications. It should be noted that the derivative is stabilized substantially on binding to D-amino acid oxidase; no significant decay of the protein-bound flavin was observed over 24 h at 0 °C. As expected from the altered chemical properties of the derivative no catalytic activity is observed with the modified enzyme. Apo D-amino acid oxidase similarly binds 4a-5-propano-4a,5-dihydro-FAD [38], and forms a fluorescent complex (Table 1). In this case, though, the spectral changes observed upon binding are comparatively small: the complex has an absorption maximal at 382 nm ($\varepsilon \approx 6500$ M^{-1} cm^{-1}), and a shoulder at 325 nm, which compares to $\lambda_{max} = 380$ nm ($\varepsilon \approx 6500$ M^{-1} cm^{-1}) for the same FAD model, free in solution.

Binding of 5-Ethyl-4a-hydroxy-4a,5-dihydro-FAD to Apo p-Hydroxybenzoate Hydroxylase and Properties of the Complex

As in the case of D-amino acid oxidase, the apoenzyme of p-hydroxybenzoate hydroxylase was found to bind 4a-OH-FAD tightly. Qualitative estimations indicate that one molecule of derivative is bound per molecule of apo-enzyme with a slightly higher K_d than
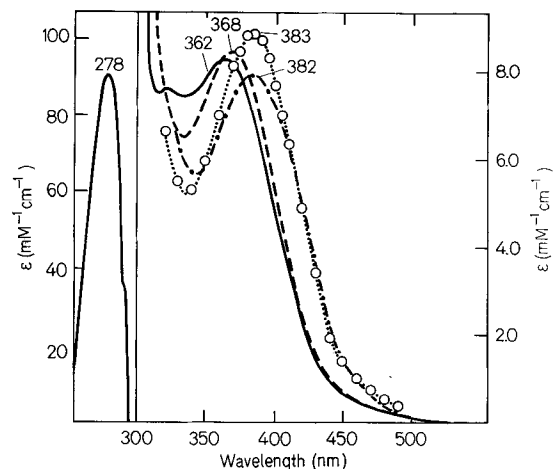
that for normal FAD. With this hydroxylase a detailed binding study was not possible due to the presence of inactive apoenzyme and the instability of the complex. The enzyme reconstituted with modified FAD had a small residual activity ($\approx 1.5\%$) attributable to the presence of residual FAD. The preparations of apoenzyme used in this study showed consistently the presence of approximately 30% inactive enzyme. Reconstitution with normal FAD indicates binding of one equivalent per apoenzyme unit, with an activity recovery of $\approx 70\%$. Similarly the apoprotein appears to bind one equivalent of 4a-OH-FAD. As in the case of D-amino acid oxidase, no catalytic activity was found. In the absence of the normal enzyme substrate, p-hydroxybenzoate, the complex has an absorption spectrum (Fig. 5) similar to those of 4a,5-dihydroflavins in aqueous solution. p-Hydroxybenzoate is

bound to the complex with a K_d of 3.5×10^{-5} M, estimated from absorbance changes, which compares to 3.2×10^{-5} M for the binding to 'normal' oxidized holoenzyme [29], and also similar to that found for the reduced holoenzyme (2.1×10^{-5} M) [11]. In the ternary complex the absorption of the lowest energy band appears red-shifted, and the shape of the spectrum is similar to that of the D-amino acid oxidase complex (Fig. 2), or to that of 4a,5-dihydro-flavins in apolar medium (Fig. 1). 6-Aminonicotinate (2-aminopyridine-5-carboxylic acid), a competitive inhibitor and effector of the enzyme, also binds very tightly, and causes an almost identical spectral shift. The absorption coefficient at 368 nm for these ternary complexes was estimated as approximately 8400 M^{-1} cm^{-1} by acid release of the modified FAD in an experiment similar to that described in Fig. 2 for the apo D-amino acid oxidase complex.

The complex of apoenzyme and 4a-OH-FAD exhibits fluorescence ($\approx 30\%$ that of native enzyme) with an excitation spectrum (Table 1) very similar to that of its absorption spectrum (Fig. 5). In the ternary complex with the substrate p-hydroxybenzoate the fluorescence excitation, when recorded at the emission maximum of 505 nm (Table 1), and the absorption spectrum are similar and equally red-shifted (Fig. 5). However, when the excitation of this complex is recorded with $\lambda_{emiss.} = 460$ and 550 nm, two different excitation spectra are obtained (Table 1, and Fig. 5), clearly showing that two species with quantum yields of similar magnitude are present. These two curves probably do not represent the absolute spectra of each of the two possible forms, as some overlap of the corresponding emission spectra exists at 460 and 550 nm. The absorption spectrum of the ternary complex ($\lambda_{max} \approx 368$ nm, Fig. 5) may be simulated reasonably well when the two spectral curves with $\lambda_{max} \approx 382$ and $\lambda_{max} \approx 357$ nm, as obtained from the excitation spectra (Table 1, and Fig. 5), are taken in a ratio 2:1. Thus the species with $\lambda_{max} \approx 382$ nm, which best correlates the absorption of the transient oxygen intermediate (Fig. 5), most probably will constitute the main component in the complex.

A similar, but clear-cut result is obtained from the ternary complex with the inhibitor 6-aminonicotinate. The fluorescence yield is now increased by about 120% relative to native enzyme and only one fluorescent species is detectable. The emission maximum is blue-shifted to 470 nm (not shown). The excitation spectrum closely resembles the absorption spectrum of the binary complex of 4a-OH-FAD and apoenzyme (Fig. 5). The noncoincidence of absorption and excitation spectra of the complex with 6-aminonicotinate indicate the presence of (at least) two species, one fluorescent and one nonfluorescent.

These experiments also clearly demonstrate that substrate and inhibitor are bound by the two forms of

complex between 4a-OH-FAD and p-hydroxybenzoate hydroxylase, and that in one form complexation does not lead to substantial spectral alterations of the complex. In contrast to this, with the other form a major spectral alteration occurs upon binding, and the occurrence of fluorescence depends on the chemical structure of the aromatic carboxylic acid bound. The very close similarity should be noted between the excitation spectrum of the complex with excitation maximum at 382 nm, and the spectrum of the flavin peroxide formed transiently when reduced native enzyme complexed with p-hydroxybenzoate reacts with oxygen (Fig. 5). Similar transient spectra have been reported earlier for a number of enzyme-substrate complexes [11].

In contrast to the results obtained with D-amino acid oxidase, the 4a-OH-FAD bound to p-hydroxybenzoate hydroxylase is markedly less stable; the characteristic absorbance at 370 nm decreases at pH 7.5, 25 °C, at a rate approximately one fifth that of the compound in free solution.

One product of this process is enzyme-bound neutral radical as indicated by the absorption at about 500 nm, and by the fact that the normal spectrum of the enzyme derivative can be restored partially by oxidation with ferricyanide. Further products probably represent compounds in which the pyrimidine moyety of the isoalloxazine has been hydrolyzed (Ghisla, S., unpublished).

Rapid Reaction
of Reduced p-*Hydroxybenzoate Hydroxylase —*
6-Hydroxynicotinate Complex with Oxygen;
Search for Fluorescence from a Transient Intermediate

The finding of a fluorescence emission from 4a-OH-FAD bound to apohydroxybenzoate hydroxylase suggested a further experimental approach to verify the chemical constitution of the observed intermediates [1]. The enzyme effector 6-hydroxynicotinate was selected, because in this case only one oxygen intermediate with an absorbance maximal at 383 nm had been observed [11]. At 388 nm the observed oxygenated intermediate and oxidized enzyme have an isosbestic point; thus at this wavelength only the formation of the intermediate from reduced enzyme will be detected ($k = 30$ s^{-1} with 0.13 mM oxygen, Fig. 6). At 460 nm the intermediate has the same absorbance as reduced enzyme, so the change will represent formation of oxidized enzyme only (final rate = 10.6 s^{-1}). The time course of the fluorescence excitation changes observed at 380 nm and 460 nm for reaction of the reduced complex with O_2 parallel the absorbance changes at 460 nm due to formation of oxidized enzyme (Fig. 6), indicating that the intermediate (57% formed at the maximum under these conditions) has no detectable fluorescence compared to that of oxidiz-
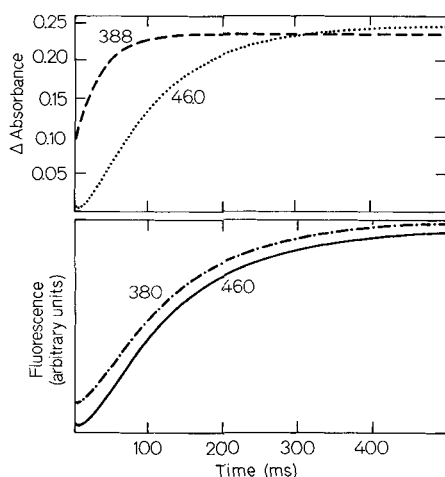
Fig. 6. *Comparison of absorption and fluorescence emission changes occurring during the reaction of reduced* p-*hydroxybenzoate hydroxylase complexed with the effector 6-hydroxynicotinate with oxygen.* The time course of absorbance changes were recorded with a stopped-flow instrument at the wavelengths indicated at 5 °C, and the fluorescence emission changes with excitation at the indicated wavelengths at 1.5—2.0 °C. For fluorescence measurements, when the excitation wavelength was 460 nm a filter (Corning CS-370) which transmits light only at wavelength > 500 nm was placed between the reaction cell and the photodetector. For 380-nm excitation either this filter or one (Corning CS-373) which transmits at wavelengths greater than 410 nm was used. The final reaction solution contained 17.6 µM enzyme, 10 mM nicotinate, 0.13 mM oxygen, 20 nm EDTA, and 50 mM potassium phosphate, pH 6.55. The time course of fluorescence changes was identical with either filter

ed enzyme. If the intermediate had fluorescence greater than 10% that of oxidized enzyme the time course of fluorescence change should have been observedly faster with 380-nm excitation than with 460-nm excitation, and should have depended on the wavelength range of fluorescence emission which was monitored.

Binding of 5-Ethyl-4a-hydroxy-4a,5-dihydro-FMN to Apo Lactate Oxidase

The behavior of 4a-OH-FMN with apo lactate oxidase is comparable to that found, with the two previously described enzymes. One derivative equivalent is bound per apoprotein molecule, and the complex resulting has an absorption spectrum with maxima at 320 and 375 nm, and a fluorescence emission which is considerably red-shifted compared to that of the other apo enzyme complexes (Table 1). The excitation spectrum does not correspond to the absorption spectrum in that its maximum is shifted to 400 nm, again indicating the presence of two chromophores. As judged from the decrease of the absorption spectrum occurring in the 350—400-nm region over a period of a few hours, the complex was not stable,

and thus more detailed binding studies could not be carried out.

Binding Behavior of 5-Ethyl-4a-hydroxy Derivatives of FAD and FMN to Other Apoflavoproteins

The binding studies detailed above were qualitatively extended to a series of apoflavoproteins available to us. Within the flavodoxins, the apoprotein from *Anacystis nidulans* did not bind 4a-OH-FMN or its reduced form, 5-ethyl-1,5-dihydro-FMN. Upon incubation of 4a-OH-FMN with the apoenzyme of *Peptostreptococcus elsdenii*, the formation of a blue colour was observed, and the absorption spectrum of a Sephadex G-25 protein eluate indicated the presence of a protein-bound mixture of 5-ethyl-FMN radical and 5-ethyl-1,5-dihydro-FMN. After 15 min at 25 °C the mixture had converted quantitatively to protein-bound 5-ethyl-FMN radical. Further, much slower changes followed, as shown by the disappearance of the radical spectrum; they did not lead to the 4a-OH-FMN derivative spectrum, but to yet unidentified species; these are probably similar to the decay products of 4a-OH,5-R-isoalloxazines, which will be described elsewhere (Ghisla, S., unpublished results). The apoenzyme of phenol hydroxylase and melilotate hydroxylase yielded upon incubation with 4a-OH-FAD considerable amounts of blue radical, which was not bound to a detectable extent by the apoprotein, i. e. did not cochromatograph with the protein fraction on Sephadex G-25.
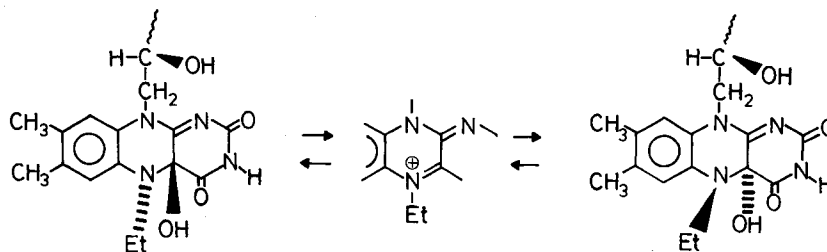
DISCUSSION

Spectra of transient flavin peroxide have been reported for four enzymes, p-hydroxybenzoate hydroxylase [11], melilotate hydroxylase [8], phenol hydroxylase [2] and bacterial luciferase [39]. Within this group the flavin peroxide varies in wavelength maximum from 370 to 395 nm, and in absorption coefficient from 8000 to 10000 cm^{-1}. In all these cases the intermediate, being short-lived, has precluded characterization by chemical trapping or by other spectroscopic methods. Hence, structural elucidation has to rely on comparison of the spectral properties with those of known flavin derivatives. On the basis of such comparison it has been suggested that in all cases the oxygenated intermediate is a C(4a)-flavin peroxide. This assignment is not unambiguous, however, since considerable spectral variation is found in C(4a)-substituted dihydroflavins, depending on the nature of the substituents and on the solvent (Fig. 1). In the case of p-hydroxybenzoate hydroxylase, the sequential occurrence of three oxygen-containing intermediates [11] has been shown. Two of these, intermediates I and III, have almost identical spectra which were attributed to the 4a-OOH and 4a-OH adducts re-

spectively. Therefore it must be concluded that with the enzyme intermediates, in contrast to the free system (Fig. 1), the main effect on the spectral properties of the substituted flavin comes from the environment imposed by the protein structure. Hence evidence obtained from binding studies to apoproteins with C(4a)-hydroxy flavins at the FMN and FAD level should be satisfactory for deductions basing on spectral properties of the enzyme intermediates. 4a-OH-FAD bound to apo D-amino acid oxidase and apo p-hydroxybenzoate hydroxylase indeed was found to yield complexes with spectra closely similar, in all respects to those of the catalytic oxygen adducts (Fig. 2 and 5). While keeping in mind the restrictions inherent to the system and the experimental limitations outlined above, we consider that the results presented here clearly support the C(4a)-OOH and C(4a)-OH structure for the intermediates in question.

This type of intermediate might well occur during the process of oxygen activation with the flavoprotein oxidases, where the corresponding intermediates could have escaped detection for kinetic reasons.

The noncoincidence of the fluorescence excitation and absorption spectra indicate that two species are present in the complex between 4a-OH-FAD and apo D-amino acid oxidase. It is improbable that these two forms represent 4a-OH-FAD complexes with apoenzyme of different activity, since the same preparations of apoenzyme could usually be reconverted to fully active enzyme with normal FAD. The linearity of the titration curves and the stoichiometry of binding, which was found to be close to unity, suggest that a single molecular type is bound. On the other hand, 4a-OH-FAD can be represented in the two following enantiomeric forms, which can interconvert in solution (over its symmetric flavin cation) and could both bind with similar K_d values to the apoenzyme, giving rise to different properties of each form in the asymmetric enzyme matrix:

and apo D-amino acid oxidase might represent slow equilibration of the two enantiomers, after preferential binding of one, either in the enzyme complex, or upon slow dissociation from the complex. A similar interpretation would also account for the finding of at least two 4a-OH-FMN complexes with apo lactate oxidase. With p-hydroxybenzoate hydroxylase the experimental results are compatible with the presence of two types of complexes: 4a-OH-FAD bound to active ($\approx 70\%$) and inactive enzyme ($\approx 30\%$). Theoretically, however, the possible combinations would be four, the two enantiomers of 4a-OH-FAD bound to active and inactive enzyme. In this case one enantiomer may bind preferentially, as in the case of apoflavodoxin [40], or less probably, the enzyme-bound enantiomers could have similar spectral properties.

The variation in light absorption and fluorescence properties of the complexes is not surprising in view of the variety of factors affecting the properties of 4a,5-dihydroflavin models (Fig. 1). Thus the drastic effect on the fluorescence found upon binding of the effector 6-aminonicotinate can be interpreted as enhancement of the fluorescence of the inactive enzyme complex and quenching of the active enzyme complex with 4a-OH-FAD. Similarly, the lack of detectable fluorescence in the catalytic oxygen intermediate as compared to the ternary complex of 4a-OH-FAD, enzyme and substrate might originate in small differences in the coenzyme-protein interactions. For example, specific hydrogen bridges to position N(5) of the reduced flavin might not be possible in N(5)-substituted coenzyme complexes; such bridges are likely to constitute a major factor in fluorescence quenching. In fact N(5)-substituted 1,5-dihydroflavins have a much higher fluorescence yield as compared to unsubstituted models at low temperature in rigid medium [13], and a very strong increase in fluorescence intensity has been observed upon N(5)-substitution of reduced lactate oxidase FMN [13].

Support for this concept comes from the recent finding that the two stable enantiomers of 4a-propyl-4a,5-dihydro-FMN can be resolved and shown to bind to apoflavodoxin rather weakly, though with K_d differences larger than 100-fold [40].

The slow secondary fluorescence changes occurring after formation of the complex between 4a-OH-FAD

The conclusions which can be drawn from the present studies apply for the intermediates of types I and III [11], observed within the hydroxylases [2, 8, 11], and to the bacterial luciferase oxygen adduct [39]; clearly they do not support, in our opinion, the proposal by Müller et al. [17] that these intermediates are 9a-flavin adducts. As binding of flavin chromo-

phores to apoflavoproteins in general causes batho-chromic shifts of absorption λ_{max} and fluorescence emission λ_{max} (Fig. 2, 5, and Table 1 [42,43]), Müller's models, which in solution already have the lowest energy transitions at $\lambda > 400$ nm (absorption λ_{max} = 372 — 460 nm, fluorescence emission λ_{max} = 530 — 572 nm [17,41]), would show λ_{max} shifted still further to the red upon binding to apoproteins.

The present work neither supports nor disproves the interesting hypothesis put forward by Hemmerich [4,44] that the high-absorption intermediate II of the p-hydroxybenzoate hydroxylase reaction has a 1,9a-dihydro structure. A discussion of this possibility and its mechanistic relevance and implications will be found elsewhere [4,11,44].

REFERENCES

1. Flashner, M. S. & Massey, V. (1974) *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.) pp. 245—283, Academic Press, New York.
2. Massey, V. & Hemmerich, P. (1976) in *The Enzymes*, 3rd edn (Boyer, P., ed.) pp. 191—252, Academic Press, New York.
3. Bright, H. J. & Porter, D. J. T. (1976) in *The Enzymes*, 3rd edn (Boyer, P., ed.) pp. 421—505, Academic Press, New York.
4. Hemmerich, P. (1976) in *Progress in the Chemistry of Organic Natural Products*, vol. 33 (Grisebach, H., ed.) pp. 451—526, Springer-Verlag, Wien.
5. Bruice, T. C. (1976) *Progress in Bioorganic Chemistry*, vol. 4, (Kaiser, E. T. & Kezdy, E. J., eds) pp. 1—87, Wiley, New York.
6. Gibson, Q. H. & Hastings, J. W. (1962) *Biochem. J. 68*, 368—377.
7. Massey, V., Palmer, G. & Ballou, D. P. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., eds) pp. 25—49, University Park Press, Baltimore.
8. Strickland, S. & Massey, V. (1973) *J. Biol. Chem. 248*, 2953—2962.
9. Spector, T. & Massey, V. (1972) *J. Biol. Chem. 247*, 7123—7127.
10. Presswood, R. & Kamin, H. (1976) *5th Int. Symp. on Flavins and Flavoproteins* (Singer, T. P., ed.) pp. 145—154, Elsevier, Amsterdam.
11. Entsch, B., Ballou, D. P. & Massey, V. (1976) *J. Biol Chem. 251*, 2550—2563.
12. Ghisla, S., Hartmann, U., Hemmerich, P. & Müller, F. (1973) *Liebigs. Ann. Chem.* 1388—1415.
13. Ghisla, S., Massey, V., Lhoste, J.. M. & Mayhew, S. G. (1974) *Biochemistry, 13*, 589—597.
14. Hemmerich, P. & Müller, F. (1974) *Ann. N. Y. Acad. Sci. 212*, 13—26.
15. Yamasaki, M. & Yamano, T. (1973) *Biochem. Biophys. Res. Commun. 51*, 612—619.
16. Hamilton, G. A. (1971) *Progress in Bioorganic Chemistry*, vol. 1 (Kaiser, E. T. & Kezdy, E. J., eds) p. 83, Wiley Interscience, New York.
17. Müller, F., Grande, H. J. & Jarbandhan, T. (1976) *5th Int. Symp. on Flavins and Flavoproteins* (Singer, T. P., ed.) pp. 38—50, Elsevier, Amsterdam.
18. Orf, H. W. & Dolphin, D. (1974) *Proc. Natl Acad. Sci. U.S.A. 71*, 2646—2650.
19. Mager, H. I. X. & Berends, W. (1974) *Tetrahedron, 30*, 917—927.
20. Cemal, K. & Bruice, T. C. (1976) *Proc. Natl Acad. Sci. U.S.A. 73*, 995—999.
21. Massey, V. & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446—465.
22. Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 794—802.
23. Mayhew, S. G. (1971) *Biochim. Biophys. Acta, 235*, 289—302.
24. Brumby, P. E. & Massey, V. (1968) *Biochem. Prep. 12*, 29.
25. Massey, V. & Curti, B. (1966) *J. Biol. Chem. 241*, 3417—3423.
26. Lockridge, O., Massey, V. & Sullivan, P. A. (1972) *J. Biol. Chem. 247*, 8097—8106.
27. Choong, Y. S., Shepherd, M. G. & Sullivan, P. A. (1975) *Biochem. J. 145*, 37—45.
28. Strickland, S. & Massey, V. (1973) *J. Biol. Chem. 248*, 2944—2952.
29. Howell, J. G., Spector, T. & Massey, V. (1972) *J. Biol. Chem. 247*, 4340—4350.
30. Neujahr, H. Y. & Gaal, A. (1973) *Eur. J. Biochem. 35*, 346—400.
31. Neujahr, H. Y. & Gaal, A. (1975) *Eur. J. Biochem. 58*, 351—357.
32. Bolognesi, M., Ghisla, S., Incoccia, L. & Ungaretti, L. (1976) *Proceedings of the Yugoslav Centre of Crystallography II*, A154—A156.
32a. Bolognesi, M., Ghisla, S. & Incoccia, L. (1977) *Acta Crystallogr.* in press.
33. McCormick, D. B., Chassy, B. M. & Tsibris, J. C. M. (1964) *Biochim. Biophys. Acta, 89*, 447—452.
34. Harvey, R. A. & Damle, S. (1972) *FEBS Lett. 26*, 341—343.
35. Koberstein, R. (1976) *Eur. J. Biochem. 67*, 223—229.
36. Müller, F., Brüstlein, M. Hemmerich, P., Massey, V. & Walker, W. (1972) *Eur. J. Biochem. 25*, 573—580.
37. Massey, V. & Ganther, H. (1965) *Biochemistry, 4*, 1161—1173.
38. Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H. & Walsh, Ch. T. (1976) *Biochemistry, 15*, 1791—1797.
39. Hastings, J. W., Balny, C., Le Peuch, Ch. & Douzou, P. (1973) *Proc. Natl Acad. Sci. U.S.A. 70*, 3468—3472.
40. Scola-Nagelschneider, G., Brüstlein, M. & Hemmerich, P. (1976) *Eur. J. Biochem. 69*, 305—314.
41. Müller, F., Jarbandhan, T., Gast, R. & Grande, H. J. (1975) *Reactivity of Flavins* (Yagi, K., ed.) pp. 51—70, University of Tokyo Press, Tokyo.
42. Müller, F., Mayhew, S. G. & Massey, V. (1973) *Biochemistry, 12*, 4654—4662.
43. Becvar, J. (1973) Ph. D. Thesis, The University of Michigan, Ann Arbor, Mi., U.S.A.
44. Hemmerich, P. & Wessiak, A. (1976) *Flavins and Flavoproteins* (Singer, T. P., ed.) pp. 9—22, Elsevier, Amsterdam.

S. Ghisla, Fachbereich Biologie der Universität Konstanz, Postfach 7733, D-7750 Konstanz, Federal Republic of Germany

B. Entsch, Department of Developmental Biology, Research School of Biological Sciences, Australian National University, P.O. Box 4, Canberra City, A.C.T., Australia 2600

V. Massey and M. Husein, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan, U.S.A. 48104