Use of riboflavin-binding protein to investigate steric and electronic relationships in flavin analogs and models

(4a,5-ring-opened isoalloxazine/pyrimidopteridine/charge transfer/nonplanar flavins/flavin hydroxylase models)

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ABSTRACT We have examined the affinity of two recently synthesized flavin analogs for the isoalloxazine binding site of riboflavin-binding protein (RBP). The results showed that pyrimidopteridines could bind to RBP (K_d 160–250 μ M). This suggested that, at the FMN or FAD level, these analogs might also bind to other apoflavoproteins, thereby providing a high potential probe for flavin enzymology. In contrast, 4a,5-ring-opened isoalloxazines did not bind to RBP. However, 1,10a-ring-opened flavins bind with considerable avidity (K_d about 40 nM). Evidence is presented which indicates that the 4a,5-ring-opened species adopted a nonplanar configuration which, in turn, was responsible for the lack of affinity to RBP. Steric and electronic consequences of a 4a,5 ring opening are discussed in relation to flavin-dependent phenolic hydroxylases.

Riboflavin-binding protein (RBP) is a protein of small molecular weight, whose primary physiological function is storage of vitamin B₂ (riboflavin, formula I in Scheme 1) (1).

SCHEME 1.

Similar to other flavoproteins (2), its “active site” is able to bind not only the native “cofactor” but also various analogs in which the isoalloxazine nucleus of riboflavin has been modified (3–8). In contrast to other flavoproteins, in which the N(10) side chain of the cofactor (ribityl phosphate in case of FMN, ribityl diphosphate adenosyl for FAD) has to be highly preserved to accomplish binding and catalytic function, RBP tolerates modifications of the ribityl side

chain. It therefore binds a variety of molecules that resemble flavins in their steric and electronic characteristics (3, 4, 6, 9). Due to that feature, the isoalloxazine binding site of RBP has been well characterized by physical methods and a variety of probes (10–13). It is considered to be hydrophobic, and a tyrosine and a tryptophan are thought to be part of it. Their interaction with flavins and other probes is considered to be responsible for the fluorescence quenching of bound molecules. “Sandwiching” the flavin between them may account for the preferential binding of molecules with flat geometries. Weak binding of negatively charged molecules is ascribed to the presence of a negative charge in the flavin-binding site of the protein.

Recently, two additional types of flavin models have become available for investigation (Scheme 1). The 4a,5-ring-opened isoalloxazines (6-[[2-(dimethylamino)-4,5-dimethylphenyl]methylamino]-5-oxo-3H,5H-uracils) IIa ($R^3 = \text{CH}_3$) and IIb ($R^3 = \text{H}$) were synthesized (14–16) to compare their spectral properties to those of enzyme-bound intermediates, observed during the hydroxylation reaction of flavin-dependent phenolic hydroxylases (17). Pyrimidopteridines (2,3,4,6,7,8,9 - heptahydro - 2,4,6,8 - tetraoxopyrimido - 5, - 4g-pteridines), III (R^3 or $R^7 = \text{H}$ or CH_3), were designed to provide flavin cofactor analogs with more negative redox potentials than the native FMN of FAD (18). This would enable us to gain mechanistic insights by studying the reverse reactions of flavoenzymes with high redox potentials.

The UV/visible spectra of the compounds II free in solution and those of enzyme-bound intermediates turned out to be quite dissimilar (14–16). It therefore became of interest to determine whether the effect of apoprotein interactions on the physical properties of compounds II might account for the differences. Unfortunately, the susceptibility of compounds II to hydrolysis made the synthesis of N(10)-ribityl derivatives and their further conversion to the FMN or FAD level difficult and impractical. Thus, reconstitution of the flavoprotein hydroxylases with compounds II is not possible. We therefore sought to approximate the hydroxylase case by studying the interaction of compounds II directly with RBP. We were not able to find any indication of binding of compounds II to RBP, and we ascribe this to steric hindrance between N(5)(CH_3)₂ and C(4a)=O that prevents the two rings of this compound from becoming coplanar. We have investigated the importance of a flat conformation in the N(5)/C(4a)/C(4) region for binding of flavin models to RBP. The equilibrium between carboxyureide (IVa) and spirohydantoin (IVb, Scheme 2) (refs. 21, 22 and references therein, and 23) provides a measure of the effect of a 1,10a-ring opening.

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Abbreviations: RBP, riboflavin-binding protein; 2,6-TNS, 6(N)-toluidinonaphthalene-2-sulfonate.

SCHEME 2.

On the other hand, 6(*N*)-toluidinonaphthalene-2-sulfonic acid (V in Scheme 3; 2,6-TNS) (24) is structurally analogous to the 4a,5-ring opened compounds II, except that the steric interactions between the phenyl ring and the naphthalene ring (25) should be less constraining than those between the benzene and pyrimidine parts in compounds II. Despite having little resemblance to riboflavin, 2,6-TNS binds to RBP.

SCHEME 3.

Steric and electronic consequences of a possible 4a,5-ring-opening mechanism for phenolic hydroxylases are discussed with respect to these results.

In contrast to compounds II, the compounds III have been obtained on the riboflavin level (refs. 26–28 and this communication). Probably due to their permanent negative charge due to ionization of N(1) (18–20), it was not possible to convert them into FMN and FAD derivatives by use of the enzyme extract from *Brevibacterium ammoniagenes*, which is generally applied for this conversion (29). To assess the usefulness of a more cumbersome chemical synthesis, a preliminary investigation of the interactions of compounds III with an isoalloxazine-binding site became desirable. Compounds III show weak binding to RBP despite their negative charge. We think, therefore, that the chemical synthesis of their FMN and FAD derivatives is worthwhile.

METHODS AND MATERIALS

Synthesis of the 5-oxo(3*H*,5*H*)uracils IIa and IIb is described in refs. 15 and 16, respectively.

Preparation of compound IIIa is described in refs. 26–28. By the same method, IIIb and IIIc were obtained by the condensation of 3-methyl-5-amino-6-D-ribitylamino-uracil (28) with alloxane (for IIIb) or methylalloxane (for IIIc) in a yield of about 70%. TLC [$\text{CHCl}_3/\text{MeOH}/32\%$ (aq) AcOH, 12:9:4 (vol/vol)] on silica gel showed a single fluorescent blue spot with R_f values of 0.45 (IIIb) and 0.53 (IIIc). IIIb, mp $>320^\circ\text{C}$. $[\alpha]_D^{23} = +10.6^\circ$ (0.123 g/100 ml of 0.05 M NaOH). Calculated for $\text{C}_{14}\text{H}_{16}\text{O}_8\text{N}_6 \cdot 3\text{H}_2\text{O}$: C, 37.34; H, 4.92; N, 18.66. Found: C, 37.42; H, 4.01; N, 18.62. IIIc, mp $>320^\circ\text{C}$, $[\alpha]_D^{23} = +32.9^\circ$ (0.126 g/100 ml of 0.05 M NaOH). Calculated for $\text{C}_{15}\text{H}_{18}\text{O}_8\text{N}_6 \cdot 3\text{H}_2\text{O}$: C, 38.80; H, 5.20; N, 18.10. Found: C, 38.84; H, 4.62; N, 18.45. The hydrogen analyses were 0.91% and 0.58% less than calculated. The hydrogen number of IIIb and IIIc was carefully counted by ^1H NMR in freshly opened 99.9% completely deuterated dimethyl sulfoxide to support the written molecular formulae of IIIb and IIIc.

Carboxyureide IVa, synthesized as described in ref. 21, was a generous gift of the late Prof. P. Hemmerich. 2,6-TNS was a gift from Irwin Goldstein (University of Michigan). All other reagents and buffers were reagent grade (purchased from Aldrich) and used without further purification. RBP

was isolated as described in refs. 3 and 4; 2.46 mM and 593 μM stock solutions (stored at -10°C) were used for titrations. The concentrations of the protein solutions were standardized by titrating a known amount of riboflavin and monitoring the fluorescence decrease upon binding of protein (3, 4). Dissociation constants were determined as described in refs. 3 and 4 for the tight binding carboxyureide IVa and the moderately tight binding 2,6-TNS. Dissociation constants for the weakly binding pyrimidopteridines were determined by Benesi-Hildebrand plots (30).

Fluorescence spectra were determined on a ratio-scanning temperature-controlled spectrofluorometer built by D. P. Ballou and G. Ford (31). Absorbance spectra were recorded on a Cary model 17, double-beam, temperature-controlled, recording spectrophotometer.

RESULTS

4a,5-Ring-Opened Flavins (IIa and IIb). Since compounds II hydrolyze in protic solvents, they were kept in acetonitrile stock solutions (HPLC grade), which were diluted 100 times with buffer for use. The reaction temperature was kept at 4°C for these experiments to slow down hydrolysis. Dilution of a 1 mM stock solution of IIa with 100 mM phosphate buffer, pH 7 (giving a concentration of 10 μM in IIa) resulted in a shift of the wavelength of the lowest energy transition from 342 nm, in acetonitrile, to 333 nm, in buffer (extinction coefficient in buffer of $7400 \text{ M}^{-1} \text{ cm}^{-1}$). Due to hydrolysis, this peak diminished slowly. No change in the wavelength of maximum absorption, extinction, or rate of hydrolysis was observed when apo-RBP (123 μM) was present in the buffer (spectra were taken against a standard-containing buffer, acetonitrile and apo-RBP in the same concentrations). This observation suggests that IIa does not bind to apo-RBP. The lack of binding of IIa to apo-RBP was further indicated by filtration of the reaction solution through a Sephadex G-25 column. Compound IIa separated cleanly from apo-RBP. These results were confirmed in similar experiments with 20 μM IIb, which had a half-life of 340 min (0.1 M potassium phosphate buffer, pH 7) in the presence or absence of apo-RBP (36.9 μM). Compound IIb (in 100 mM phosphate buffer, pH 7) was found to have the same half-life in the presence or absence of 2% bovine serum albumin (to which 3-methyl-lumiflavin binds) or 129 μM apo-flavodoxin. Only in the presence of bacterial luciferase (4.4 mg/ml) was the hydrolysis marginally slowed down. In summary, the 4a,5-ring-opened flavin models IIa and IIb seem to be rather remarkable in their lack of interaction with apoproteins known to bind a variety of flavin compounds.

Carboxyureide (IVa) and Spirohydantoin (IVb). To establish the importance of the geometry around the N(5)/C(4a)/C(4) region of riboflavin, use was made of the slow equilibrium between carboxyureide IVa and spirohydantoin IVb (Scheme 2). At pH 7 and 25°C , the ring-opened form IVa predominates (about 95% as judged from the absorbance at 400 nm), while at pH 8.5 the ring-closed spirohydantoin IVb is the thermodynamically more stable species. This equilibrium has been investigated by Guttman and Platke with a related molecule under different conditions (35°C), in which a larger proportion of the corresponding spirohydantoin seems to be present at neutral pH (23). An explanation for the equilibrium is probably the lower ionization constant of IVb. Since carboxyureide IVa (absorption maxima at 400 nm, extinction $7600 \text{ M}^{-1} \text{ cm}^{-1}$, and 330 nm, extinction $10,800 \text{ M}^{-1} \text{ cm}^{-1}$) and spirohydantoin IVb (absorption maximum at 305 nm, extinction $8100 \text{ M}^{-1} \text{ cm}^{-1}$) differ greatly in their spectral properties, their interconversions are easy to follow.

Carboxyureide IVa bound to apo-RBP with a dissociation constant of 40 nM (determined by fluorescence quenching), which is marginally tighter than that of lumiflavin (47 nM) (3,

4). The tight binding resulted in a shift of the absorption maxima to higher wavelength (340 nm, extinction $8200 \text{ M}^{-1} \text{ cm}^{-1}$ and 410 nm, extinction $6900 \text{ M}^{-1} \text{ cm}^{-1}$) as shown in Fig. 1. This tight binding provided a means of determining the concentration of the saturated carboxyureide stock solution ($29.2 \mu\text{M}$).

On the other hand, spirohydantoin IVb did not bind to apo-RBP. This could be demonstrated in several ways. A $26.3 \mu\text{M}$ solution of carboxyureide in 10 mM potassium pyrophosphate buffer, pH 8.5, was found to convert to the spirohydantoin with a half-life of 4 min. The reaction was followed by the disappearance of absorbance at 400 nm. Addition of apo-RBP (in a small volume, to give a solution $12.3 \mu\text{M}$ in apo-RBP) to this solution of spirohydantoin resulted in no immediate spectral change, but within 2 hr approximately 40% of the spirohydantoin had been converted into RBP-bound carboxyureide as judged by the return of absorbance at 410 nm. Further addition of apo-RBP to give a $60 \mu\text{M}$ final concentration resulted in complete conversion of all the spirohydantoin present to the ring-opened carboxyureide with a half-life of 95 min. The rate-determining step for the conversion was presumably the ring opening of spirohydantoin free in solution to give an equilibrium amount of the carboxyureide. This was followed by rapid binding of the carboxyureide by the RBP. Thus, at pH 8.5 and 25°C , the equilibrium ratio of spirohydantoin to carboxyureide free in solution should be given by the ratio of the observed half-life values, 95/4, a value in reasonable agreement with the 6% residual absorbance at 400 nm when equilibrium is established. On addition of a small excess of apo-RBP, this equilibrium is shifted almost completely in favor of the carboxyureide, due to the preferential binding of the latter. Nonbinding of spirohydantoin IVb was confirmed by incubation of a $26.3 \mu\text{M}$ solution of spirohydantoin (pyrophosphate buffer, pH 8.5) with apo-RBP (final concentration $60 \mu\text{M}$) for 1 min, followed by gel filtration over Sephadex G-25 (previously equilibrated with the same buffer). Only the minor carboxyureide constituent eluted with protein, while the spirohydantoin eluted separately. Tight binding of carboxyureide IVa under the same conditions was shown in a separate Sephadex G-25 gel filtration experiment.

FIG. 1. Titration of carboxyureide IVa with apo-RBP. At 25°C , 0.9 ml of a saturated carboxyureide solution was mixed with 0.1 ml of 1 M potassium phosphate buffer, pH 7, and titrated with apo-RBP (2.46 mM). Curve 1 is the free carboxyureide. Curves 2–4 are after the addition of 4, 8, and $12 \mu\text{l}$ of stock apo-RBP. No further changes occurred on addition of more protein.

Binding of 2,6-TNS. While spirohydantoin IVb was a probe whose steric constraints were more severe than those expected in the 4a,5-ring-opened compounds II, 2,6-TNS seemed to be an analog with the same stereorelationships as compound II (phenyl and naphthalene rings interact in a way comparable to the benzene and pyrimidine rings in compounds II; cf. Scheme 3) (32). Due to the small size of the hydrogens (compared to the dimethylamino and $\text{C}(4a)=\text{O}$ groups), the energy barrier resisting a flat conformation should be less high with 2,6-TNS than with the ring-opened flavins II. Nevertheless, x-ray analysis of 2,6-TNS crystals has shown a nonplanar conformation (25). Despite the limited structural similarity of 2,6-TNS to riboflavin, compared to the close similarity of compounds II, 2,6-TNS showed moderate binding (K_d of about $30 \mu\text{M}$ at pH 7 and 4°C) as judged from the appearance of an intense fluorescence (excitation maximum at 333 nm, emission maximum at 465 nm), when a practically nonfluorescent solution of 2,6-TNS (in 100 mM phosphate buffer, pH 7) was titrated with apo-RBP. A binding stoichiometry of 1:1 was confirmed by titration in the concentration range of the dissociation constant. The fluorescence due to 2,6-TNS could be quenched to its initial level by addition of an amount of riboflavin equimolar with the RBP present. This indicates that the 2,6-TNS was displaced from the RBP by riboflavin. We are aware of only one other case where the binding of a fluorescence indicator similar to 2,6-TNS has been reported for a flavoprotein. Bacterial luciferase binds 8(N)-anilinonaphthalene-1-sulfonate with a low dissociation constant ($23 \mu\text{M}$ at 23°C) and 1:1 stoichiometry (33). In this case, however, it has been shown that 8(N)-anilinonaphthalene-1-sulfonate does not bind to the flavin site. Although we cannot rule out binding at a different site and allosteric interaction with riboflavin, the simplest explanation for monomolecular binding and complete displacement by riboflavin is binding to the same site. Fluorescence enhancement instead of the usually encountered quenching does not argue against an interaction at the flavin-binding site, since fluorescence enhancement was also observed upon the binding of 8-N(CH_3)₂-riboflavin to apo-RBP (results not shown).

Binding of the Pyrimidopteridines IIIa, -b, and -c. All three pyrimidopteridines are permanently ionized in the neutral pH range due to the low ionization constant of their nitrogens N(1) or N(9) [$\text{p}K_a$ of 1.16 for an N(10)-methyl substituent] (20, 21). Apo-RBP is known to bind anionic flavin derivatives less tightly, and in the cases of 8-OH and 8-SH flavins ($\text{p}K_a$ of 4.8 and 3.8, respectively) preferential binding of the protonated forms to RBP was clearly seen from the UV/visible absorbance spectra. Binding led to a shift of the $\text{p}K_a$ for the bound form to be about 9 in each case (34, 35). Despite their unfavorable charged state, all pyrimidopteridines show weak binding to apo-RBP (IIIa, $\text{R}^7 = \text{R}^8 = \text{CH}_3$; very weak, K_d not determined; IIIb, $\text{R}^7 = \text{CH}_3$; K_d about $250 \mu\text{M}$; IIIc, $\text{R}^7 = \text{R}^8 = \text{CH}_3$; K_d about $160 \mu\text{M}$) in 100 mM phosphate buffer, pH 7. If this were due to selective binding of the unionized form and a resultant $\text{p}K_a$ shift for the bound form, then lowering the pH by 2 units should lead to lowering of the apparent K_d by two orders of magnitude. Since no such shift was seen when the K_d of IIIc was determined in 100 mM potassium acetate buffer, pH 5, we conclude that the pyrimidopteridines are bound in their negatively charged form.

DISCUSSION

Compounds II are generally drawn for convenience as "flat" (see Scheme 1). Molecular models immediately show that there should be serious steric constraint in a flat structure, and that their conformation is best represented by the twisted or nonplanar formula as in formula II of Scheme 3. Compounds II were synthesized as models for an intermediate

that appears between the hydroperoxy and hydroxy species during the hydroxylation reaction catalyzed by flavin-dependent phenolic hydroxylases (17). This intermediate has been postulated to be a 4a,5-ring-opened species (17) in which N(5) is substituted by two hydrogens instead of the methyl groups, as in compounds **II** (14–16). Steric aspects of a possible 4a,5-ring-opening mechanism have up to now not been considered explicitly. The 4a,5-ring-opening mechanism is best considered in analogy to the reaction of amines with carbonyl compounds to form carbinolamines and the reversal of this reaction. Nucleophiles attack carbonyls from “out-of-plane” (36). If monooxygen transfer from 4a-hydroperoxyflavin in phenol hydroxylases were to involve N(5)—C(4a) ring opening, then a low-energy pathway might well involve a twisting movement of the benzene ring relative to the pyrimidine ring. As our negative results with compounds **II** have shown, such extensive movement demands a compatible apoprotein. The considerable binding energy available for a flat conformation bound to apo-RBP was not enough to overcome the energy barrier against flattening compounds **II**.

Since we were unable to bind compounds **II** to apo-RBP, we have no information on what changes in physical properties (in particular the UV/visible absorbance and fluorescence properties) could be manifested in 4a,5-ring-opened flavins by specific apoprotein interactions. If the absorbance spectra of N(5)-alkylated models such as **IIa** and **IIb** are good approximations to the enzyme-bound case [in which N(5) bears two hydrogens], then the ring-opening mechanism of hydroxylation seems to be wrong, since the absorbance spectra of **IIa** and **IIb** are quite different from the spectrum of the enzyme-bound intermediate (14–16). An alternative interpretation of the results would be that compounds **II** have such severe steric constraints, due to the methyl groups on N(5), as to be unsuitable models. The flexible, nonplanar 2,6-TNS binds to apo-RBP even though the latter demands a flat conformation. In comparing the binding of 2,6-TNS to the inability of **IIa** and **IIb** to bind, the advantage of the former may be due to the possession of a lyophobic naphthalene ring in place of a polar 5-oxo-3*H*,5*H*-uracil ring, to greater steric hindrance by the N(5)(CH₃)₂ functions in **IIa** and **IIb** or to both. Different conformations of 2,6-TNS and related molecules [which are very popular as fluorescence indicators of protein conformation and solvent interactions (32)] have been invoked to explain changes in the photophysical properties—i.e., via “twisted intra-molecular charge transfer states” (37). As noted above, the spectra of **IIa** and **IIb** do not resemble (particularly in extinction coefficients) the spectra of intermediate **II** of the phenol hydroxylases. Intermediate **II** could have the opened structures shown by **IIa** and **IIb** only if its spectrum were greatly perturbed by intramolecular charge transfer [possibly allowed by the absence of the N(5)(CH₃)₂ substituents of **IIa** and **IIb**] or by apoprotein and juxtapositioning of substrate anions. Further experimental approaches should be designed to assess these possibilities.

With respect to the binding (albeit weak) of the pyrimidopteridines to an isoalloxazine site, the obvious conclusion is that there is good reason to believe that phosphorylation of these models to provide FMN analogs would allow their functioning as artificial cofactors in flavoproteins for which most of the flavin-binding energy is provided by the side chain of the flavin—i.e., with almost all flavoproteins.

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