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THE INTERPLAY BETWEEN BASICITY, CONFORMATION, AND ENZYMATIC REDUCTION IN BILIVERDINS *

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SUMMARY: Biliverdins with extended conformations are reduced by biliverdin reductase (BvR) at higher rates than biliverdins with helical conformations. To find out the molecular basis for this important feature of BvR mechanism, helical and extended biliverdins were titrated for their acid-base equilibria in a protic solvent (methanol). It was found that the basicity of biliverdins increases with the stretching of the conformation. Biliverdin IX γ (*all-syn*) has a pKa = 3.6; 5,10,15-*syn,syn,anti*-biliverdin has a pKa = 3.7; 5,10,15-*syn,anti,syn*-biliverdin has a pKa = 6.1; 5,10,15-*syn,anti,anti*-biliverdin has a pKa = 6.4; and 5,10,15-*all-anti*-biliverdin has a pKa = 7.9. The increase in basicity with progressive stretching of conformations closely parallels the increase in the reduction rates by BvR. A biliverdin constrained by a four carbon chain to a helical conformation and which is a very weak base (pKa = 0.4) is not reduced by BvR. Nucleophilic additions of 2-mercaptoethanol at the C_{10} in biliverdins closely parallel their basicities, as can be expected if the formation of a positive mesomeric species at C_{10} is linked to the basicity (i.e., the ease of protonation) of the N_{23} on the pyrrolenine ring.

Biliverdin reductase (BvR), the enzyme that reduces biliverdin IX α (**1a**, Fig. 1) to bilirubin IX α, catalyzes in mammals a critical step in heme catabolism and its malfunction could be the origin of severe pathologies such as the bronze baby syndrome (1), and the green jaundice (2). Although its substrate in mammals is usually biliverdin IX α, BvR is unusual in that it has a very broad substrate specificity and readily reduces not only the four biliverdin IX isomers (α, β, γ, δ) but also a large number of bilitrienes substituted with both polar and non-polar side-chains (3). BvR is a NADPH-dependent enzyme and reduces the 5,10,15-bilitriene (biliverdin) at its C_{10} meso carbon, thus affording a 5,15-bilidiene (bilirubin). We have recently shown (4) that biliverdins with extended (or "stretched") conformations (such as **2-5**, Fig.1) are more readily reduced by BvR than the helical conformers (such as **1**). The reduction rates increase with the progressive stretching of the conformations. We have further explored the possible molecular basis for this important feature of BvR mechanism.

Biliverdins usually have the helical *all-syn* conformation 5Z,10Z,15Z-*all-syn* (as in **1**, Fig. 1) due to an efficient intrachromophoric hydrogen bond system based on the N_{24}—H···N_{23}···H—N_{22} bonding network (5) (Fig. 1). A disruption of the latter drives the tetrapyrrole chain into an extended

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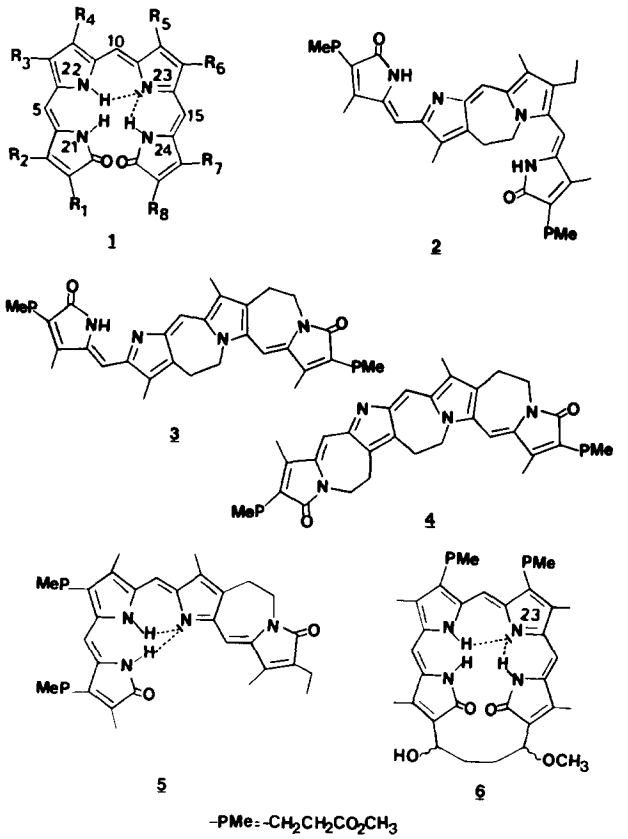


Figure 1: Structures of the biliverdins used in this study. **1a**: biliverdin IX α dimethyl ester: R_1=R_3=R_6=R_7=methyl; R_4=R_5=methyl propionate; R_2=R_8=vinyl. **1b**: biliverdin IX γ dimethyl ester: R_2=R_3=R_5=R_7=methyl; R_1=R_8=methyl propionate; R_4=R_6=vinyl.

conformation; a geometry which can be achieved under different experimental conditions. Whereas the N-methylation at N_{21} and N_{24} of an alkyl bilitriene does not affect the overall helical conformation, the N_{23}-methyl derivative adopts a partially extended conformation (6). If compensation for the N_{23}···H—N_{24} hydrogen bond is provided by other optimally oriented donor groups such as those present in certain covalently bound biliverdin-tripeptides, then the tetrapyrrole chain goes into an extended conformation (7) (very likely by rotation around the C_{14}-C_{15} bond (8)). Protonation of the pyrrolenine nitrogen N_{23} has a similar effect (9); in the monoprotonated N^+_{23}–H species the central hydrogen bond system is destroyed and the helical conformation changes to a more extended one, presumably by rotation around the C_9-C_{10} bond. There is therefore enough evidence to suggest that upon disruption of the central hydrogen bonding system in helical biliverdins extended conformations become favored. In the C-phycocyanines, the large number of hydrogen bonds and salt linkages between the bilitriene moiety and the apoprotein are responsible for the stretching of the chromophore (5).

When mapping the active center of BvR we advanced a possible mechanism for the enzyme's action (10). It was based on experimental data which showed the presence of a cationic ε-amino lysine residue in the vicinity of the central pyrrole rings of the biliverdin, very close (~ 3 Å) to a thiol

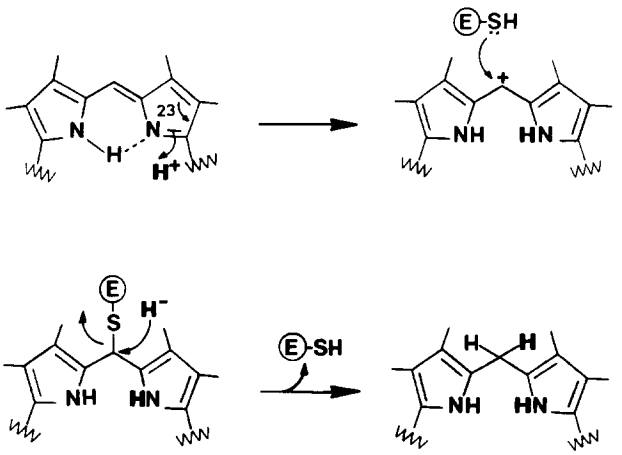


Figure 2: Outline of the mechanism involved in the enzymatic reduction of biliverdins to bilirubins.

residue which is essential for the catalytic activity of the enzyme. According to the proposed mechanism, the cationic residue protonates N_{23} with a concurrent formation of a mesomeric species positively charged at C_{10}; this is then attacked by the nucleophilic species, first by the thiol residue and then by the hydride anion released from the NADPH (Fig. 2). It has been recently reported (9) that upon protonation of N_{23} the ^1H NMR resonance of H_{10} in **1a** shifts downfield by 1.2 ppm; this could also be expected from the sequence of events depicted in Fig. 2. Moreover, if this mechanism were correct, a stretching of the bilitriene oligopyrrole should favor the protonation of its pyrrolenine N_{23} nitrogen. This in turn should increase the readiness of the substrate for reduction by the NADPH-dependent BvR. The following protonation study carried out on biliverdins **1-5** confirms that indeed the basicity of biliverdins increases when their conformations are changed from helical to more extended geometries, and that this increase in basicity parallels an increase in the enzymatic reduction rates by BvR.

MATERIALS AND METHODS

Materials. The dimethyl esters of biliverdins IX α **1a** and IX γ **1b** were obtained by the coupled oxidation of hemin IX and were separated as described elsewhere (11). The extended biliverdins **2-5** were obtained as their methyl esters using the published synthetic procedure (12). The cyclic biliverdin **6** was obtained as described (13) and comprises a mixture of two racemic diastereoisomers rapidly interconverting at room temperature. NADPH was from Sigma Chem. Co.. 2-Mercaptoethanol, sodium borohydride and all other chemicals and solvents used were of the purest analytical grade from Merck.

Methods. The UV-vis spectra were recorded at 25 °C in 98% methanol using a Hitachi U-2000 spectrometer. Titrations were carried out using either 0.1N HCl-methanol or 0.01N HCl-methanol solutions for low pH determinations and 0.1N or 0.01N NaOH-methanol solutions for higher pH measurements. pH determinations were carried out using a glass electrode and a digital pH meter (Chemcadet) at 25 °C. Values of pKa were determined by plotting

$$pH = pKa + \log \frac{[\text{Neutral form}]}{[\text{Protonated form}]}$$

and measuring the ordinate to the origin. The ratios between both concentrations were calculated from the extinction coefficients at the corresponding wavelengths:

$$\frac{[\text{Neutral form}]}{[\text{Protonated form}]} = \frac{A_i\,\varepsilon_{aii} - A_{ii}\,\varepsilon_{ai}}{A_{ii}\,\varepsilon_{ni} - A_i\,\varepsilon_{nii}},$$

where A_i and A_{ii} are the absorbances at the vis_{max} wavelengths of both forms, ε_{ai} and ε_{aii} are the extinction coefficients of the protonated biliverdins at the vis_{max} wavelengths of both forms, and ε_{ni} and ε_{nii} are the extinction coefficients of the neutral biliverdins at the vis_{max} wavelengths of both forms. The titrations were usually carried out by going from the protonated (HCl) form to the neutral form, using alkaline solutions. In the case of biliverdin **5** (which showed two pKa, see Table I), titrations were also carried out by going from the alkaline pH to the acid pH. The methyl esters of **1b-6** were converted into the acids as described in reference (4).The molecular form 1 of rat liver biliverdin reductase was obtained and purified as described elsewhere (10).

Assay of biliverdin reductase. The incubation mixture contained, in a final volume of 200 µl: 20 mM potassium phosphate buffer (pH 7.4), 100 µM NADPH, the indicated biliverdin at a 13 µM concentration and the enzyme (1-2 µg of protein). Incubations were carried out at 37 °C for 7 min. Blanks were run in which the coenzyme was omitted. After incubation, the volume was completed to 500 µl and the decrease in the vis_{max} of the assayed biliverdins was measured. Biliverdin disappearance was further checked by measuring bilirubin formation at their vis_{max}. The ε values of the **1b-6** biliverdins and their respective bilirubins were used to calculate their concentrations.

RESULTS

The basicity of helical and extended biliverdins. For biliverdin IX γ **1a** (Fig. 1) a pKa = 3.6 ± 0.3 was determined in methanol. This value was estimated by the spectrophotometric titration (Fig. 3A) of the equilibrium:

$$\text{N}_{23}\text{H}^+ \rightleftarrows \text{N}_{23} + \text{H}^+ .$$

Helical biliverdins have pKa values of ca. 3.0 - 4.7 (9,14,15), due to the relative weak basic character of the pyrrolenine nitrogen in these conjugated systems. The spectrophotometric titrations of the biliverdins **2- 5** (Fig. 1) are shown in Fig. 3, the corresponding data are summarized in Table I. The ratio of UV/vis extinction coefficients strongly decrease upon protonation of N_{23} (Table I), an effect which has been attributed to a conformational change from helical to extended forms (9). Biliverdin **2** (dihydrophorcabilin), which has a fixed extended geometry at C_{10}, was also protonated at the pyrrolenine ring. By titration back to the neutral form the spectra showed two isosbestic points (Fig. 3B) which allowed us to calculate its pKa = 6.1 ± 0.3 (Table I). The extended conformation therefore results in a marked increase in the basicity of the biliverdin. No significant changes in the $R_{UV/vis}$ values when going from the neutral to the acid form were observed, as could have been expected from an already extended geometry. The hexacyclic biliverdin **3** is kept in a still more rigid conformation than **2** (compare the $R_{UV/vis}$ values, Table I), and upon titration of its acid-base equilibrium (Fig. 3C) we measured a pKa = 6.4 ± 0.3 for this compound. The fully extended biliverdin **4** could also be titrated from its monoprotonated form back to its neutral form (Fig. 3D), and a pKa = 7.9 ± 0.3 was determined (Table I). It is the most basic of all biliverdins assayed, and a striking confirmation that upon stretching the bilitriene backbone there is a strong increase in the basicity of the pyrrolenine nitrogen. A peculiar situation was found for the partially extended

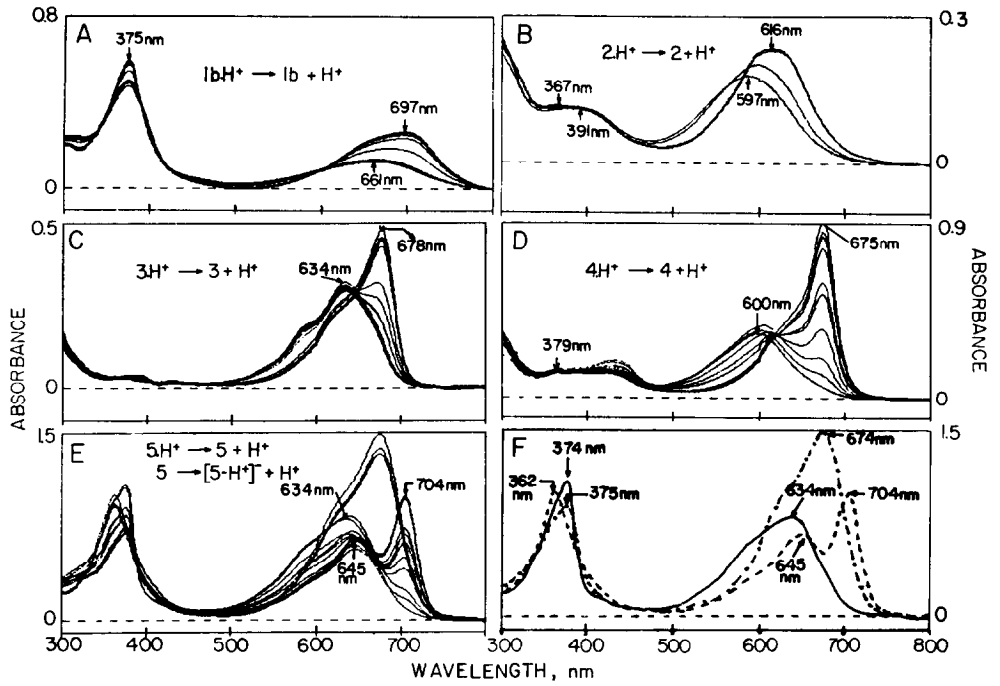


Figure 3: Spectrophotometric titration of the hydrochlorides of biliverdins **1b, 2, 3, 4**, and **5** with increasing concentrations of NaOH in methanol at 25 °C. Series of UV/vis spectra of: **A)** biliverdin **1b** (9 μM); **B)** dihydrophorcabiline **2** (7 μM); **C)** biliverdin **3** (15 μM); **D)** biliverdin **4** (36 μM); **E)** biliverdin **5** (23 μM); **F)** UV/vis spectra of the neutral (——), protonated (- · - ·), and deprotonated (-----) forms of biliverdin **5**.

biliverdin **5** which showed several isosbestic points in the visible region. On passage from the protonated to the neutral form a change in the conformation of the bilitriene took place (Figs. 3E and 3F), similar to that observed for the helical biliverdin **1b** (Table I). A pKa = 3.7 ± 0.3 was calculated, indicating that its basicity was similar to that of **1b**. At higher pH a form absorbing at 704 nm appeared for which a pKa = 7.1 ± 0.3 was determined (Table I). We attribute this bathochromic shift to the deprotonation of the N_{22}-H center. Although such deprotonations usually take place at higher pH (5), the deprotonation of bilindiones at the acidic NH residues are strongly dependent of the structural details of the conjugated systems.

The basicities of the helical and extended biliverdins show a close relation to their enzymatic reduction rates (Fig. 4). Biliverdins **1b** (pKa = 3.6) and **5** (pKa = 3.7) were reduced by BvR at similar rates; these rates increase considerably in the cases of biliverdins **2** (pKa = 6.1) and **3** (pKa = 6.4), and are even faster in the case of **4** (pKa = 7.9).

Reduction and nucleophilic additions to the obligate helical biliverdin **6**. The reduction rates of biliverdins by BvR increase with the progressive stretching of their conformations; this stretching also increases the basicity of the corresponding bilitrienes. We therefore analyzed the enzymatic reduction of the obligate helical biliverdin **6** (Fig. 1) which has a fixed (Z,Z,Z-*syn,syn,syn*) conformation. The helical biliverdin **6**, in its diacid form, was not reduced by BvR (Fig. 4) even after long reduction

TABLE I

UV/vis spectral parameters of biliverdins **1b-5** and pKa values of their monoprotonated forms

Biliverdin	λ (nm),(ε, $mM^{-1}cm^{-1}$)	$R_{UV/vis}$	pKa
1b (pH 7.6)	374 (51.0) 662 (15.2)	3.35	-
1b.H⁺ (pH 2.4)	375 (60.1) 698 (28.9)	2.08	3.6 ± 0.3
2 (pH 7.6)	391 (16.1) 597 (32.2)	0.54	-
2.H⁺ (pH 2.4)	367 (16.1) 617 (37.0)	0.43	6.1 ± 03
3 (pH 7.6)	379 (2.2) 634 (19.8)	0.11	-
3.H⁺ (pH 2.4)	379 (2.4) 677 (29.6)	< 0.10	6.4 ± 0.3
4 (pH 9.0)	379 (2.2) 600 (8.4)	0.26	-
4.H⁺ (pH 2.4)	377 (2.0) 675 (22.8)	< 0.10	7.9 ± 0.3
5 (pH 6.0)	374 (45.7) 634 (34.6)	1.32	-
5.H⁺ (pH 2.4)	361 (39.5) 675 (58.9)	0.67	3.7± 0.3
5 -H⁺ (pH 9.0)	376 (4 2.2) 645 (37.5) 704 (50.0)	0.84	7.1 ± 0.3

Data were calculated from the spectra shown in Fig. 3. pKa values were calculated as explained in Methods.

times (1h). Since it is a very weak base (pKa = 0.4 (9)) it is unlikely that its N_{23} could be protonated by any cationic residue of the protein. It is therefore not surprising that **6** only undergoes nucleophilic additions at C_{10} to a very low extent. While a 5 mM mercaptoethanol solution afforded 60% of the

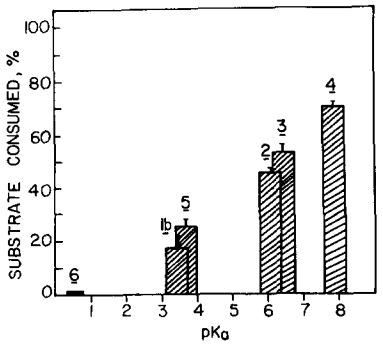
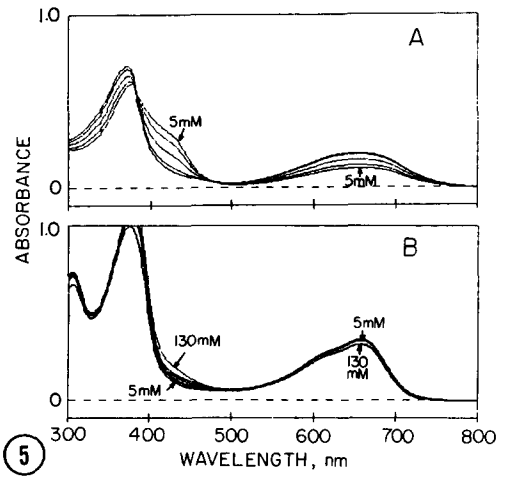


Figure 4: Enzymatic reduction of biliverdins **1b-6** as a function of their pKa's. The incubation mixture and conditions are as described in Methods. The results are means±SD of four independent experiments.



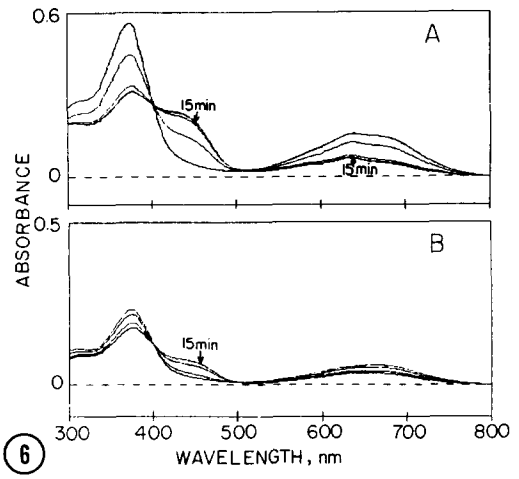


Figure 5: UV/vis spectra of the thiobilirubinoid adducts formed by addition of 2-mercaptoethanol to: **A**) biliverdin **1a** (13 μM), and **B**) the obligate helical biliverdin **6** (18 μM). Thiol concentrations are given in the Figure.

Figure 6: Reduction rates with $NaBH_4$ (4 mM) of : **A**) biliverdin **1a** (8 μM), and **B**) the helical biliverdin **6** (5 μM) .

C_{10} rubinoid adduct with biliverdin IX α dimethyl ester **1a** (Fig. 5A), a 130 mM mercaptoethanol solution only gave 10% of the C-S adduct with the dimethyl ester of **6** (Fig. 5B). Since the formation of a C_{10}-S bilirubinoid adduct (λ_{max} 420 nm) is the first step in the BvR catalyzed reduction of biliverdin (10), the lack of enzymatic reduction of **6** is readily explained.

Reduction of biliverdins by the NADPH-dependent BvR involves the transfer of an hydride atom from the reduced nucleotide to the electrophilic center at C_{10}. Reduction with sodium borohydride ($NaBH_4$) should then mimic the enzymatic reaction, and indeed extended biliverdins are more readily reduced by $NaBH_4$ to bilirubins than the helical conformers (4). This analogy breaks down however, when the chemical reduction of **6** by $NaBH_4$ is compared with that of **1a**. Biliverdin IX α **1a** is reduced by $NaBH_4$ to bilirubin. A 4 mM solution of the reductant in methanol reduced in 15 min (at 25 °C) ca. 55% of the biliverdin **1a** present (Fig. 6A). To determine the analogous reduction of **6**, the biliverdin was first reduced with an excess of $NaBH_4$ to its bilirubin derivative (λ_{max} 385 nm , ε = 56.9 $mM^{-1}cm^{-1}$; λ_{max} 430, ε = 20.4 $mM^{-1}cm^{-1}$). It was then possible to estimate that a 4 mM $NaBH_4$ solution in methanol reduced ca. 40% of the biliverdin **6** present in 15 min (Fig. 6B). Whatever the reason for this discrepancy between the enzymatic and the chemical reduction of **6**, it is obvious that the former is governed by the tetragonalization step at C_{10} due to the addition of the thiol residue of the enzyme. The S-C_{10} bond is then cleaved by the hydride released from the reduced nucleotide, as shown in Fig. 2. Analogous mechanisms operate in several well known NADPH-dependent dehydrogenases (16).

DISCUSSION

The helical conformation is predominant for biliverdins dissolved in common solvents. The presence of extended populations can only be shown by fluorescence spectroscopy, but are not

detectable by UV/vis or NMR methods (5). The possible relationship between protonation at the pyrrolenine nitrogen and the enhancement of populations with extended conformations was adumbrated from the changes in the $R_{UV/vis}$ ratios in the protonated forms (9). But analogous changes could also be due to other factors which produce bathochromic and hyperchromic shifts on protonation of the chromophore (17). It was therefore important to secure unambiguous evidence that the basicity of a biliverdin is linked to its geometry. Biliverdins with fixed conformations such as **2-5** offered the possibility of correlating the protonation of the basic pyrrolenine nitrogen atom to the geometry of the tetrapyrrole. The titrations were carried out under conditions where monoprotonation takes place (9); i.e., in a protic solvent such as methanol and at concentrations which avoid the formation of aggregates. Under these conditions, it was found that the basicity of biliverdins increases with an increasing stretching of the configurations. The fully extended biliverdin **4** has a basicity at least four orders of magnitude higher than the helical conformer **1** (Table I). The enzymatic reduction rates of the biliverdins by BvR could be correlated with their basicities (Fig. 4): a favored protonation of the pyrrolenine basic nitrogen by the enzyme favors its reduction by the coenzyme (NADPH), whereas upon hindering the protonation of the basic nitrogen (as in **6**) no enzymatic reduction takes place. The fact that the facile thiol addition to the C_{10} in extended biliverdins can be correlated with their enzymatic reduction rates (4), while in the obligate cyclic biliverdin **6** the thiol addition was considerably hindered, supports the mechanism outlined in Fig. 2 for the BvR mode of action.

Protonation of biliverdins **2-4** produced bathochromic and hyperchromic shifts of their visible absorption bands (ca. 20 nm in **2**, 40 nm in **3**, 75 nm in **4** (Table I)). Their $R_{UV/vis}$ ratios however, experienced only slight changes. Similar effects were also found for other biliverdins where geometrical changes were improbable (17,9) and should be attributed to changes in the chromophores due to protonation.

The increase in the pKa values reflects an increase in the localization of the pyrrolenine nitrogen lone pair in these series. An alkyl substituted dipyrrylmethene is a relatively strong base (pKa = 8.5 (14)) due to the basicity of its pyrrolenine ring. Upon increasing the delocalization of the nitrogen's lone pair of electrons by extension of the conjugated system, the basicity of the pyrrolenine ring decreases (to pKa ca. 6.0 in tripyrrinones (15), pKa ca. 4.0 in biliverdins, pKa ca. 2.5 in pentapyrrins (18)). It is evident that the insertion of the pyrrolenine ring in a tricyclic ring system in **2-4** subtracts it from the bilindione conjugated system and makes it more basic. In **4** the extended conjugated system present in **1** is almost completely interrupted, and therefore the pyrrolenine basicity is again on the same order of magnitude as in a dipyrrylmethene.

A special case is biliverdin **5**. The pyrrolenine nitrogen is still held by hydrogen bonding in the *syn* -conformation (as shown by its ^1H NMR spectrum (19)). Its pKa is similar to that of **1** and upon protonation stretching takes place. At higher pH the visible band shifted bathochromicly by 70 nm (to 704 nm). Such dramatic shifts are known for helical biliverdins at pH $\approx$ 11, and were attributed to the deprotonation of the pyrrole $N_{22}H$ (20). The pKa = 7.1 measured for **5** is therefore too low, and the increased acidity of the pyrrole ring should be attributed to the presence of the fused ring system in **5**.

The established correlation between the basicity of the pyrrolenine ring and the enzymatic reduction at C_{10} in the biliverdins suggests that biliviolins, urobilins and stercobilins, which are more

basic than the verdins (14), could also be substrates of BvR. We will address this subject in forthcoming reports.

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