A comparison of the initial rate kinetics for human biliverdin-IXα reductase and biliverdin-IXβ reductase with a series of synthetic biliverdins with propionate side chains “moving” from a bridging position across the central methene bridge (α isomers) to a “γ-conformation” reveals characteristic behavior that allows us to propose distinct models for the two active sites. For human biliverdin-IXα reductase, as previously discussed for the rat and ox enzymes, it appears that at least one “bridging propionate” is necessary for optimal binding and catalytic activity, whereas two are preferred. All other configurations studied were substrates for human biliverdin-IXα reductase, albeit poor ones. In the case of mesobiliverdin-XIIIα, extending the propionate side chains to hexanolate resulted in a significant loss of activity, whereas the butyrate derivative retained high activity. For human biliverdin-IXα reductase, we suggest that a pair of positively charged side chains “moving” from a bridging position across the central methene bridge (α isomers) to a “γ-conformation” reveals characteristic behavior that allows us to propose distinct models for the two active sites. For human biliverdin-IXα reductase, as previously discussed for the rat and ox enzymes, it appears that at least one “bridging propionate” is necessary for optimal binding and catalytic activity, whereas two are preferred. All other configurations studied were substrates for human biliverdin-IXα reductase, albeit poor ones. In the case of mesobiliverdin-XIIIα, extending the propionate side chains to hexanolate resulted in a significant loss of activity, whereas the butyrate derivative retained high activity. For human biliverdin-IXα reductase, we suggest that a pair of positively charged side chains play a key role in optimally binding the IXα isomers. In the case of human biliverdin-IXβ reductase, the enzyme cannot tolerate even one propionate in the bridging position, suggesting that two negatively charged residues on the enzyme surface may preclude productive binding in this case. The flavin reductase activity of biliverdin-IXβ reductase is potently inhibited by mesobiliverdin-XIIIα and protohemin, which is consistent with the hypothesis that the tetrapyrrole and flavin substrate bind at a common site.

The formation of linear tetrapyrroles by heme catabolism in mammals has, until recently, been discussed in terms of the IXα isomers of biliverdin and bilirubin as both heme oxygenases I and II (HO-1 and HO-2), and biliverdin-IXα reductase (BVR-A) are reported to exhibit such specificity (1, 2). However, 87% of the bilirubin in human fetal bile has been reported to be the IXβ isomer (3). Yamaguchi et al. (4) purified a novel enzyme from human liver that catalyzes the reduction of the IXβ, IXγ, and IXδ isomers of biliverdin to the corresponding rubin (4). We have shown that this enzyme, biliverdin-IXβ reductase (BVR-B), is identical to NAD(P)H-linked flavin reductase (5). The source of fetal biliverdin-IXβ has not yet been determined; however, this appears to be a pathway that is only operative at any significant level in the fetus. The physiological relevance of the apparent switch in heme degradation from a IXβ pathway in utero to a IXα pathway at birth is unclear, although it may be coupled to the switch from fetal to adult hemoglobin. O’Carra and Collaran (6) have shown that nonenzymic ascorbate-mediated coupled oxidation of “free” heme (pyridine-heme complexes) produces all four isomers of biliverdin-IX (Scheme 1), in approximately equimolar amounts, whereas coupled oxidation of adult hemoglobin produces a mixture of IXα (65%) and IXβ (35%) isomers of biliverdin. The nature of the protein binding the heme is important because ascorbate-mediated coupled oxidation of myoglobin produces 95% biliverdin-IXα (6). It is not known which isomers of biliverdin-IX are produced by ascorbate-mediated coupled oxidation of fetal hemoglobin, although, in preliminary experiments, we cannot support BVR-B-dependent NADPH oxidation using the products of this reaction.

Whereas nonenzymic coupled oxidation is demonstrable in vitro, it is clear that at least two forms of heme oxygenase (HO-1 and HO-2) function in vivo. Both of these enzymes produce the IXα isomer of biliverdin exclusively; however, the nature of the isomer produced by the recently described HO-3 (7) is not known. Quantitative flux through these three HO pathways in mammals has not been studied, although there is considerable interest that there is a requirement for a functioning HO-1 for effective reutilisation of iron in mammals (8).

The discovery that the IXα isomer of bilirubin is a ligand for the aryl hydrocarbon (Ah) receptor (9, 10) may explain the transcriptional up-regulation of the rat GST A5 gene in congenital hyperbilirubinaemia (11) and allows us to suggest that a function for the IXβ isomer (which appears to be a uniquely fetal metabolite) may be related to fetal suppression of the maternal immune system. The Ah receptor is known to be involved in immunosuppression, and, during pregnancy, there is an increased susceptibility to certain types of infection (12–14). Both biliverdin-IXα and bilirubin-IXα have been implicated as modulators of the immune system (15, 16). Intriguingly, the recent suggestion that indoleamine dioxygenase (EC 1.13.11.42) may be involved in fetal suppression of the maternal immune response (17) could also support Ah receptor involvement as tryptophan metabolites are also known to bind to and activate this transcription factor (18).

High levels of the IXα isomer of bilirubin are generally seen in:

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‡ These authors contributed equally to this work.

¶ Supported by the Health Research Board, Ireland.

§ Supported by the Irish National Pharmaceutical Biotechnology Center. To whom correspondence should be addressed. Tel.: 353-1-6083410; Fax: 353-1-6772400; E-mail: aidunn@tcd.ie.

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1 The abbreviations used are: HO, heme oxygenase; BVR-A, biliverdin-IXα reductase; BVR-B, biliverdin-IXβ reductase; GST, glutathione S-transferase; BSA, bovine serum albumin; FR, flavin reductase; PAGE, polyacrylamide gel electrophoresis.

O. Cunningham and T. J. Mantle, unpublished observations.
at birth (so-called physiological jaundice of the newborn) and are potentially cytotoxic if the protective binding capacity of serum albumin (19, 20) is exceeded. However, this is a period when the infant lung experiences a massive increase in the partial pressure of oxygen (21), and the transient increase in serum bilirubin levels seen at this time may represent a temporary boost in the levels of a physiologically significant antioxidant. Indeed, several workers have presented evidence that bilirubin-IXα functions as an antioxidant, both in its free form (22) and bound to albumin (23), and low serum bilirubin-IXα has been shown to correlate with increased risk of coronary artery disease in two independent studies (24, 25).

It is conceivable that linear tetrapyrroles may play significant biological roles in mammals, both as antioxidants and as anti-inflammatory agents, and it is therefore important to extend our knowledge of the substrate specificity of the two human enzymes currently known to catalyze the formation of bilirubin isomers. Further study will need to identify the origin of biliverdin-IXβ in utero and also to define any other linear tetrapyrroles that may occur in the adult and/or the fetus.

The chemical synthesis of the four isomers of biliverdin, cleaved at the α, β, γ, and δ positions using ascorbate-mediated coupled oxidation of pyridine-heme is not amenable to large scale production. Early work by Colleran and O’Carra (26) demonstrated that replacement of the vinyl side chains by propionic acid groups at varying sites along the pigment backbone (Group I verdins, Refs. 27–29; Group II verdins, Refs. 30–32; Group III verdins, Refs. 33 and 34) and report here the initial characterization of these synthetic biliverdin isomers. Further study will need to identify the origin of biliverdin-IXβ in utero and also to define any other linear tetrapyrroles that may occur in the adult and/or the fetus.

EXPERIMENTAL PROCEDURES

Cloning and Overexpression of Human Biliverdin-IXα Reductase and Human Biliverdin-IXβ Reductase

Oligonucleotide primers used to amplify the cDNA encoding human BVR-A were designed based on the cDNA sequence reported by Maines et al. (35). The forward (5′-GCGACATCCAAAGTGAGAACAG-3′) and reverse (5′-AACCAATGTCTGCGCATTGGA-3′) primers contained BamHI and NcoI restriction sites, respectively (underlined). A cDNA library derived from the U937 monocyte cell line was used as target in subsequent amplification reactions. The resulting 960-base pair fragment was digested and ligated into the pGEX-KG expression vector to produce the pGEX-BVR-A plasmid. Escherichia coli strain TG1 was transformed according to procedures described in Sambrook et al. (36).

E. coli cells for large scale purification of the recombinant GST fusion protein were cultured as follows. 10-ml cultures were grown in Luria-Bertani medium overnight at 30 °C in the presence of 2% glucose. After 18 h the cells were harvested by centrifugation (7,700 g for 10 min) and lysed by sonication in the presence of lysozyme (200 g/ml). Following centrifugation at 12,000 g for 45 min, the cell supernatant was passed through a glutathione-Sepharose affinity column, and the fusion protein was eluted with 10 mg/ml glutathione in 50 mM Tris-HCl, pH 8.0. Excess glutathione was removed by gel filtration, and the fusion protein was cleaved overnight at 4 °C with 20 units of thrombin (1 unit/μl). The liberated BVR-A was separated from the GST tag by passage through the glutathione affinity column. The approximate yield of purified BVR-A from a 2-liter culture is 30 mg. Recombinant human biliverdin-IXβ reductase was prepared as described previously by thrombin cleavage of a GST-BVR-B fusion protein (37).

Mass Spectroscopic Analysis of Recombinant Biliverdin-IXα Reductase and Biliverdin-IXβ Reductase

On SDS-PAGE, recombinant human BVR-A migrates with a mobility corresponding to a molecular mass of 40 kDa. Similar behavior has been reported for the native human enzyme (38, 39). This is in contrast to the behavior of the rat, mouse and ox enzymes which exhibit a molecular mass of 34 kDa on electrophoresis (38). Given that the rat enzyme contains 295 amino acids and the human enzyme contains 296 amino acids, mass spectroscopy was carried out to determine the relative molecular mass of human BVR-A. Similar analysis was conducted for BVR-B.

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**Table I**

| Biliverdin isomer | Extinction coefficient \( \text{liters mol}^{-1} \text{cm}^{-1} \) |
|-------------------|-----------------------------------------------|
| Mesobiliverdin IXα| 1.2 × 10^4                                      |
| Mesobiliverdin-XIIIα| 1 × 10^4                                      |
| Mesobiliverdin-IVα| 2.2 × 10^3                                      |
| 12-Ethyl mesobiliverdin-XIIIα| 9.9 × 10^3                                      |
| 12-Ethyl-13-methyl mesobiliverdin-IVα| 1.4 × 10^4                                   |
| Mesobiliverdin-XIIIγ| 1.64 × 10^4                                    |
| Mesobiliverdin-XIIγ| 1.16 × 10^4                                    |
| 8,12-Dimethyl mesobiliverdin-XIIγ| 1.31 × 10^4                                   |
| α,α,α′,α′-Tetramethyl-mesobiliverdin-XIIIα| 7.78 × 10^4                                |
| α,α′-Dimethoxy-mesobiliverdin-XIIa| 1.48 × 10^4                         |
| Mesobiliverdin-XIIIα \((n = 3)\)| 1.36 × 10^4                                    |
| Mesobiliverdin-XIIIα \((n = 4)\)| 1.78 × 10^4                                |

**Table II**

| Verdin isomer | BVR-A | BVR-B |
|---------------|-------|-------|
|               | \( K_{cat} \) | \( K_{m} \) | \( K_{cat} \) | \( K_{m} \) |
| Biliverdin-IXα| 7     | 44    | 0.7     |
| Mesobiliverdin-XIIIα| 11 | 114   | 0.4     |
| Mesobiliverdin-IVα| 76   | 34    | 169     |
| 12-Ethyl mesobiliverdin-XIIIα| 200| 45   | 10      |
| 12-Ethyl-13-methyl mesobiliverdin-IVα| 31 | 6     | 0.3     |
| Mesobiliverdin-XIIIγ| 79   | 0.9   | 213     |
| 8,12-Dimethyl mesobiliverdin-XIIγ| 26  | 0.5   | 30      |
| α,α,α′,α′-Tetramethyl mesobiliverdin-XIIIα| 37 | 1     | 3       |
| mesobiliverdin-IVα| 1.78 × 10^4 | 1.26 × 10^4 | 1.78 × 10^4 | 1.48 × 10^4 |

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**Scheme 1. Biliverdin isomers resulting from haem cleavage.**

Four possible isomers of biliverdin can result from the cleavage of haem because of the nonequivalence of the four methene bridge positions α, β, γ, and δ (P = -CH₂CH₂COOH).

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**Table I**

Calculated extinction coefficients for synthetic biliverdin isomers at maximum absorbance

| Biliverdin isomer | Extinction coefficient \( \text{liters mol}^{-1} \text{cm}^{-1} \) |
|-------------------|-----------------------------------------------|
| Mesobiliverdin IXα| 1.2 × 10^4                                      |
| Mesobiliverdin-XIIIα| 1 × 10^4                                      |
| Mesobiliverdin-IVα| 2.2 × 10^3                                      |
| 12-Ethyl mesobiliverdin-XIIIα| 9.9 × 10^3                                      |
| 12-Ethyl-13-methyl mesobiliverdin-IVα| 1.4 × 10^4                                   |
| Mesobiliverdin-XIIIγ| 1.64 × 10^4                                    |
| Mesobiliverdin-XIIγ| 1.16 × 10^4                                    |
| 8,12-Dimethyl mesobiliverdin-XIIγ| 1.31 × 10^4                                   |
| α,α,α′,α′-Tetramethyl-mesobiliverdin-XIIIα| 7.78 × 10^4                                |
| α,α′-Dimethoxy-mesobiliverdin-XIIa| 1.48 × 10^4                         |
| Mesobiliverdin-XIIIα \((n = 3)\)| 1.36 × 10^4                                    |
| Mesobiliverdin-XIIIα \((n = 4)\)| 1.78 × 10^4                                |
using 5 M HCl. The suspension was centrifuged at 10,000 rpm for 1 h and the supernatant was removed. The erythrocytes were gently resuspended PD-10 column. Samples containing BVR-A and BVR-B were eluted with a 10–350 mM gradient (2 M NaCl) of sodium phosphate, pH 7.2, overnight and then loaded onto a DEAE-cellulose column (5 × 30 cm) equilibrated in 10 mM sodium phosphate, pH 7.2. The column was washed until the conductivity (1 nS to 1 μS), lumichrome (5 to 75 μM), and protoporphin (1 mM to 10 mM). Stock solutions of both proteins were diluted in 200 μl of distilled water to give a final concentration of 0.6 mg/ml and buffer was exchanged into 50 mM ammonium acetate, pH 6.0, through a pre-equilibrated PD-10 column. Samples containing BVR-A and BVR-B were pooled and adjusted to 0.4 and 0.6 mg/ml, respectively. The buffer composition was finally adjusted to 50% (v/v) acetonitrile and 1% (v/v) formic acid. Mass analysis was carried out using a Micromass triple quadrupole electrospray mass spectrometer. A 10-μl sample was introduced into the electrospray mass spectrometer via a Rheodyne injector valve fitted with a 10-μl injection loop. Analysis was carried out in positive ion mode. Raw data were collected between a mass-to-charge ratio of 600–1700 m/z, at a cone voltage of 30 V, HV lens of 0.22 kV, and a capillary voltage of 3.60 kV. The raw data were subjected to maximum entropy analysis according to the Micromass schedule.

**Preparation of Partially Pure Native Biliverdin-IXα Reductase and Biliverdin-IXβ Reductase**

Human erythrocytes were centrifuged at 4,000 × g for 10 min at 4 °C, and the supernatant was removed. The erythrocytes were gently resuspended in three volumes of ice-cold 0.9% (w/v) sodium chloride. Centrifugation was carried out as described above. This washing step was repeated twice. The packed erythrocytes were lysed by the addition of three volumes of ice-cold distilled water. The pH was adjusted to 6.4 using 5 M HCl. The suspension was centrifuged at 10,000 × g for 1 h at 4 °C. The pH was finally adjusted to 7.2 with 5 M KOH. The lystate was dialyzed against 20 liters of 10 mM sodium phosphate, pH 7.2, overnight and then loaded onto a DEAE-cellulose column (5 × 30 cm) equilibrated in 10 mM sodium phosphate, pH 7.2. The column was washed until the A260 was below 0.1, and the two forms of biliverdin reductase were then eluted with a 10–350 mM gradient (2 × 250 ml) of sodium phosphate, pH 7.2. The fractions were assayed for biliverdin-IXα reductase and flavin reductase activity as described below, and the peak fractions were subjected to immunoblotting with antiserum raised against the recombinant BVR-A and BVR-B enzymes.

**Enzyme Assays**

**Biliverdin Reductase Assays**—The biliverdin isomers used in this study were synthesized from mono- and dipyrrole components as described previously (27–34). Dried preparations were dissolved in MeSO to give a final stock concentration of 1 μM. The final concentration of MeSO in the assay was shown to have no effect on enzyme activity.

Preliminary plate assays were conducted at 30 °C in 100 mM potassium phosphate buffer, pH 7.5, containing 50 μM NADPH and the respective biliverdin isomers at a concentration of 20 μM. Initial rate measurements with biliverdin as the variable substrate were made by monitoring biliverdin consumption at 660 nm in the presence and absence of BSA (1 mg/ml) using the extinction coefficients given in Table I. No co-solvents or detergents were added to the assay mixture (Beer Lambert’s law was obeyed over the concentration range used). All assays were conducted in 100 mM potassium phosphate buffer, pH 7.5, and contained NADPH at a saturating concentration of 50 μM (the K_m for BVR-A and BVR-B is 1 μM with biliverdin-IXα and FMN, respectively).

**Flavin Reductase Assay**—The flavin reductase activity of BVR-B was measured using the method described by Yubisui et al. (40). Initial rate studies were carried out under saturating concentrations of FMN (150 μM) and NADPH (50 μM) in 100 mM potassium phosphate, pH 7.5. Activity was monitored by following the decrease in absorbance of NADPH at 340 nm. Inhibition of flavin reductase (FR) activity was determined under the same conditions using mesobiliverdin-IXα (1 mM to 1 μM), lumichrome (5 to 75 μM), and protoporphin (1 mM to 10 mM).

**Treatment of Data**

The initial rate data were fitted to equations for simple hyperbolic kinetics and total and partial substrate inhibition (41). Most data sets showed potent substrate inhibition, where few data points were on the upward limb, and fitting (to either total or partial substrate inhibition) produced negative coefficients or large errors. For this reason estimates of the kinetic parameters (Table II) were obtained by manually generating saturation curves for total substrate inhibition (using, where possible, initial estimates from the curve fitting routines) and visually checking the theoretical line against the data set. For several of the substrates with BVR-B, the substrate inhibition is so potent that the only kinetic parameter obtainable was the substrate inhibitory K_i value, obtained by plotting the reciprocal of the initial rate against the concentration of the tetrapyrrole. For those substrates exhibiting partial substrate inhibition only the linear part of the V_max versus [biliverdin] curve were used to obtain the substrate inhibitory K_i value. All experiments conducted in this study used 50 μM NADPH. More extensive kinetic studies, for example varying NADPH concentration, require the development of an assay that will allow initial rates to be determined at low concentrations of the tetrapyrrole substrate to allow curve fitting routines to be used with confidence.
RESULTS

The cloning of human biliverdin-IX\(^\beta\) reductase into the pGEX-KG expression vector to produce a GST-BVR-B fusion protein has been reported previously (37). We have constructed a similar vector for human biliverdin-IX\(^\alpha\) reductase that allows the production of 40 mg of GST-BVR-A fusion protein/liter of culture. The recombinant protein has been affinity purified on glutathione-Sepharose, and the GST moiety was removed following cleavage with thrombin, yielding a homogenous preparation of BVR-A of the predicted mobility on SDS-PAGE, as shown in Fig. 1. The partial purification of native BVR-A and BVR-B from human erythrocytes was achieved using DEAE-
cellulose chromatography. The elution profile is shown in Fig. 2a and reveals complete separation of the two activities that was subsequently confirmed by immunoblotting (Fig. 2b).

Both the native and recombinant form of human BVR-A migrate on SDS-PAGE with an apparent molecular mass of 40 kDa. Mass spectroscopic analysis of recombinant human BVR-A gave a molecular mass of 34,330 Da (data not shown), suggesting that the protein runs anomalously on SDS-PAGE.

The molecular mass estimated for BVR-B by SDS-PAGE was confirmed using mass spectroscopy as 22,531 Da.

The structures of the verdins used to assess the substrate specificity of BVR-A and BVR-B are shown in Fig. 3. The
natural IXα isomer (1) has vinyl side chains at positions 3 and 18. Fig. 4 shows the result of an overnight incubation of all of the verdin isomers with recombinant human BVR-A (row C) and BVR-B (row E) and the native forms of human BVR-A (row B) and BVR-B (row D) isolated from human red blood cells. The incubations were carried out in the presence of NADPH (50 μM), BSA (1 mg/ml), and 100 mM potassium phosphate buffer, pH 7.5. Rows A and F do not contain enzyme. Rows B and C contain native and recombinant BVR-A, respectively. Rows D and E contain native and recombinant BVR-B, respectively. Numbers 1–16 represent the various verdin structures illustrated in Fig. 3.

The verdins substituted at C10 (13–16) showed no change in the visible spectrum, which is not surprising because they are not reducible at C10. Unfortunately, none of these compounds are particularly effective inhibitors of BVR-A or BVR-B (which might have been a starting point for anti-hyperbilirubinaemia therapy). The methyl derivative (16) was the most potent, exhibiting modest inhibition at 25 μM.

Although the overnight plate incubations allow a crude definition of whether or not the various compounds behave as substrates for the two enzyme forms, it yields little information about the relative rates of reaction. The initial rate kinetics for compounds 1–6, 8, and 9 in the presence and absence of BSA (1 mg/ml) with recombinant BVR-A are shown in Fig. 5. It is clear that the addition of BSA has a pronounced effect on the activity with mesobiliverdin-IVα (3); however, for biliverdin-IXα (1) and 12-ethyl mesobiliverdin-XIIIα (4) the effect is mainly on sequestration of substrate. In previous work with ox kidney BVR-A (42) and rat kidney BVR-A (43), we have attempted to define the effect of BSA as simple sequestration of the verdin substrate; however, detailed work in our laboratory suggests that this is not the only function (41).3 The extinction coefficient for bilirubin-IXα at 460 nm is increased on binding to albumin, and the free concentration of biliverdin IXα is also reduced by binding to BSA; however, it is clear that other factor(s) are also operative (44). By monitoring the ΔA660, as opposed to ΔA460, some of these complications are overcome, albeit at the expense of sensitivity.

As the kinetics of BVR-A involve pronounced substrate inhibi-
bition, because the effect of albumin on the initial rate is not clear and, as the present work shows, these effects vary depending on the substrates used, for the present discussion, we define “good” and “poor” substrates for BVR-A in the following way: good substrates are those that, when assayed in the presence of BSA, exhibit an apparent \( K_m \) biliverdin of less than 10 \( \mu M \) and that exhibit a maximal initial rate of greater than 5 \( \mu mol/min/mg \). We define a poor BVR-A substrate as one with an apparent \( K_m \) biliverdin greater than 20 \( \mu M \) and exhibiting a maximal initial rate no greater than 1 \( \mu mol/min/mg \). It is clear that the compounds with a bridging propionate are all good substrates (compounds 1, 2, and 4) with two propionates being preferred (i.e., biliverdin-IX\( \alpha \) (1) and mesobiliverdin-XIII\( \alpha \) (2)) exhibit lower apparent \( K_m \) values than 12-ethyl-mesobiliverdin-XIII\( \alpha \) (4)). Although all of the other verdins tested were substrates for human BVR-A, the rates were very low compared with those for 1, 2, 4, and 11 (see below).

A quite distinct pattern of substrate specificity is shown by BVR-B. This enzyme cannot tolerate even one bridging propionate side chain in a verdin, so that compounds 1, 2, and 4 do not function as substrates (Fig. 6). The plate shown in Fig. 4 has been allowed to go to completion (i.e. it was left overnight at room temperature) for compounds 1–16. Using the spectrophotometric assay, it was not possible to measure BVR-B activity with compounds 1, 2, and 4. In contrast, several of the compounds that are poor substrates for BVR-A are good substrates for BVR-B (Fig. 6). Propionates in any of the nonbridging positions seem to promote activity, with substitutions at positions 3, 5, 6, 7, and 8 being particularly active. It should be noted that the substrate inhibition with BVR-B is far more potent than that observed with BVR-A, having substrate inhibitory \( K_i \) values in the submicromolar range. This precluded the determination of any standard kinetic parameters for BVR-B using the spectrophotometric assay.

In a separate experiment, we analyzed the effect of the chain length of the carboxylic acid on activity with both BVR-A and BVR-B by using mesobiliverdin-XIII\( \alpha \) where the propionate side chains at positions 8 and 12 have been substituted to produce a dimethoxy (\( \square \)), butyrate (\( \circ \)), and hexanoate (\( \blacklozenge \)) derivative (verdins 10, 11, and 12, respectively) as shown in Fig. 3. The reduction of biliverdin IX\( \alpha \) is also included in this figure (\( \bullet \)).
particularly potent inhibitor of the FR reaction (Ki = 73 μM), and it may be that the marked substrate inhibition seen with BVR-B masks any relatively modest inhibition by lumichrome by shifting the saturation curve to the right. Such an effect may extend the plateau of maximal activity by alleviating substrate inhibition. Intriguingly, protohemin behaves as an activator when BVR-B activity is measured, and this too may reflect an alleviation of substrate inhibition.

**DISCUSSION**

Both BVR-A and BVR-B exhibit a fairly broad specificity in terms of the tetrapyrrole substrate, with human BVR-A able to reduce all of the structures tested. Early work on partially purified preparations of guinea pig BVR-A (that may have been contaminated with BVR-B) also suggested that, although the IXα isomer was preferred, the β, γ, and δ isomers were also substrates for the enzyme (26), which suggests that significant binding energy may be associated with an interaction between the carboxylate side chains and a residue(s) on the enzyme (presumably lysine or arginine). The observation that those compounds with two bridging propionates (1 and 2) have lower apparent Km values than the monopropionate verdin substrates (12-ethyl-mesobiliverdin XIIIα; 4) is consistent with the hypothesis that BVR-A may utilize two basic residues to stabilize tetrapyrrole binding (Fig. 8). BVR-B is most distinct in that the bridging propionate rule for BVR-A is the antithesis in this case. This leads us to suggest that, in contrast to BVR-A, there may be a pair of negatively charged residues in BVR-B that do not permit the IXα isomer to bind productively. If we argue that the reduced pyridine nucleotide (H) binds in a similar position (Fig. 9) for both BVR-A and BVR-B, then we suggest that a ring of positively charged residues may surround the tetrapyrrole pocket of BVR-B to facilitate the “non-alpha” isomers in binding productively as substrates. The IX-α isomers (those with bridging propionates) may bind in a nonproductive mode by rotating through 90°, as illustrated (Fig. 10). This would be in agreement with the competitive kinetics observed for mesobiliverdin-XIIIα against FMN. It should be noted that open chain tetrapyrroles are forced to adopt a slightly helical structure (47) so that the simple rotation of 90° must be accompanied by some degree of “flexibility.” Further work is clearly required to test this hypothesis. We have crys-
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tals of human BVR-B that diffract to 1.6 Å,4 and more recently, have obtained crystals of BVR-A5 that should allow the accuracy of these models to be tested. The number of compounds known to interact with BVR-B/FR now includes a wide range of biliverdin isomers in addition to pyrroloquinoline quinone (48), various hemes, fatty acids, and porphyrins (46).

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REFERENCES

1. Maines, M. D. (1988) FASEB J. 2, 2557–2568
2. Elhirt, K. R., and Bonkovsky, H. L. (1999) Proc. Assoc. Am. Physicians 111, 433–447
3. Yamaguchi, T., and Nakajima, H. (1995) Eur. J. Biochem. 233, 467–472
4. Yamaguchi, T., Komoda, Y., and Nakajima, H. (1994) J. Biol. Chem. 269, 24343–24348
5. Shallowe, F., Elliott, G., Ennis, O., and Mantle, T. J. (1996) Biochem. J. 316, 385–387
6. O’Carra, P., and Colleran, E. (1970) Biochem. J. 119, 42–43
7. McCoubrey, W. K., Jr., Huang, T. J., and Maines, M. D. (1997) Eur. J. Biochem. 247, 725–732
8. Pass, K. D., and Tonegawa, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10919–10924
9. Sinal, C. J., and Bend, J. R. (1997) Mol. Pharmacol. 52, 590–599
10. Phelan, D., Winter, G. M., Rogers, W. J., Lam, J. C., and Denison, M. S. (1998) Arch. Biochem. Biophys. 357, 155–163
11. Igarashi, T., Tsuchiy, T., and Satoh, T. (1991) J. Biochem. (Tokyo) 109, 3–5
12. Salentie, C. E., Halsapple, M. P., and Kaminski, N. E. (1998) Mol. Pharmacol. 53, 623–629
13. Krishnan, L., Guilbert, L. J., Wegmann, T. G., Belosevic, M., and Mosmann, T. R. (1996) J. Immunol. 156, 653–662
14. Krishnan, L., Guilbert, L. J., Russell, A. S., Wegmann, T. G., Mosmann, T. R., and Belosevic, M. (1996) J. Immunol. 156, 644–652
15. Nakagami, T., Toyomura, K., Kinoshita, T., and Morisawa, S. (1998) Biochem. Biophys. Acta 1158, 189–193
16. Mori, H., Otake, T., Morimoto, M., Ueba, N., Kunita, N., Nakagami, T., Yamasaki, N., and Tagi, S. (1991) Jpn. J. Cancer Res. 82, 755–757
17. Munn, D. H., Zhou, M., Atwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., Brown, C., and Mellor, A. L. (1998) Science 281, 1191–1193
18. Health-Paglinso, S., Rogers, W. J., Tullis, K., Seidel, J. D., Brouwer, A., and Denison, M. S. (1998) Biochemistry 37, 11508–11515
19. Water, P. C. (1997) Clin. Pharmacokin. 35, 26–50
20. Chuniand, L., Desant, M., Chantaux, F., Blondeau, J. P., Francon, J., and Trivin, F. (1996) Clin. Chim. Acta 256, 103–114
21. Pitkanen, O. P., and O’Brodovich, H. M. (1998) Ann. Med. 30, 14–24
22. Stoker, R., Yamamoto, Y., Feddagh, A. F., Glazer, A. N., and Ames, B. N. (1987) Science 235, 1043–1046
23. Stoker, R., Glazer, A. N., and Ames, B. N. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5918–5922
24. Schwertner, H. A., Jackson, W. G., and Tolan, G. (1994) Clin. Chem. 40, 18–23
25. Hunt, S. C., Wu, L. L., Hopkins, P. N., and Williams, R. R. (1996) Arterioscler. Thromb. Vasc. Biol. 16, 912–917
26. Colleran, E., and O’Carra, P. (1997) in Chemistry and Physiology of Bile Pigments (Ilerk, P. D., and Berlin, N. I., eds) Department of Health, Education and Welfare Publication 77–1100, National Institutes of Health, Washington, D.C.
27. Trull, F. R., Franklin, R. W., and Lightner, D. A. (1987) J. Heterocyclic Chem. 24, 1573–1579
28. Holmes, D. L., and Lightner, D. A. (1995) J. Heterocycl. Chem. 32, 131–121
29. Sabido, P. M. (1997) Synthesis, Conformational Analysis and Binding Studies of Mesobilirubins-XIII and Their Verdins. Ph.D. Dissertation, University of Nevada, Reno, NV
30. Boiadjiev, S. E., Holmes, D. L., Anstine, D. T., and Lightner, D. A. (1995) Tetrahedron 51, 10663–10678
31. Boiadjiev, S. E., and Lightner, D. A. (1998) J. Org. Chem. 63, 6220–6228
32. Shrot, D. P., Puzicha, G., and Lightner, D. A. (1992) Synthesis, 328–332
33. Kar, A. K., and Lightner, D. A. (1998) Tetrahedron: Asymmetry 9, 3863–3880
34. Kar, A. K. (1998) Synthesis, Conformational Analysis and Properties of C(10)-Substituted Bilirubin and biliverdin Analogues. Ph.D. Dissertation, University of Nevada, Reno, NV
35. Maines, M. D., Polevoda, B. V., Huang, T. J., and McCoubrey, W. K., Jr. (1996) Eur. J. Biochem. 235, 372–381
36. Samuel, J., Pritt, F. R., and Maniatis, T. (1988) in Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 1.82–1.84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Cunningham, O., and Mante, T. J. (1997) Biochem. Soc. Trans. 25, Suppl. 613
38. Rigney, E. M., Phillips, O., and Mante T. J. (1988) Biochem. J. 255, 431–435
39. Maines, M. D., and Trakshel, G. M. (1993) Arch. Biochem. Biophys. 306, 320–326
40. Yubiusi, T., Matsuki, T., Takeshita, M., and Yoneyama, Y. (1979) J. Biochem. (Tokyo) 85, 719–728
41. Phillips, O., and Mante, T. J. (1981) Biochem. Soc. Trans. 9, 275–278
42. Rigney, E. M., and Mante, T. J. (1998) Biochem. Biophys. Acta 957, 237–242
43. Ennis, O., Mayr, M., and Mante, T. J. (1997) Biochem. J. 328, 33–36
44. Phillips, O. (1981) Studies on Biliverdin Reductase and its Role in Haem Catabolism. Ph.D. Dissertation, University of Dublin, Trinity College, Ireland
45. Cunningham, O., Gore, G. M., and Mante, T. J. (2000) Biochem. J. 345, 393–399
46. Xu, F., Quandt, K. S., and Hultquist, D. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2130–2134
47. Boiadjiev, S. E., Pfeiffer, W. P., and Lightner, D. A. (1997) Tetrahedron 53, 14547–14564
48. Xu, F., Mack, C. P., Quandt, K. S., Shafer, M., Massey, V., and Hultquist, D. E. (1993) Biochem. Biophys. Res. Commun. 193, 434–439

4 A. Parraga, O. Cunningham, T. J. Mante, and M. Coll, unpublished observations.
5 A. Dunne, D. Thompson, and T. J. Mante, unpublished observations.
Studies on the Specificity of the Tetrapyrrole Substrate for Human Biliverdin-IXα Reductase and Biliverdin-IX β Reductase: STRUCTURE-ACTIVITY RELATIONSHIPS DEFINE MODELS FOR BOTH ACTIVE SITES
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