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THE ENZYMATIC AND CHEMICAL REDUCTION OF EXTENDED BILIVERDINS

Rosalía B. Frydman, Sara Bari, María L. Tomaro and Benjamín Frydman

Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires
Junín 956, Buenos Aires, Argentina

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SUMMARY: The substrate specificity of rat liver biliverdin reductase was probed using helical and extended biliverdins. The former were the ZZZ-all-syn biliverdins IX α and IX γ, and the latter were the 5Z-syn,10Z-syn,15Z-anti; 5Z-anti, 10Z-syn,15Z-anti; 5Z-syn,10E-anti,15Z-syn; 5Z-syn,10E-anti,15Z-anti and 5Z-anti, 10E-anti,15E-anti biliverdins. It was found that the reduction rates of the biliverdins increased with the progressive stretching of their conformations. The most extended biliverdin was reduced at a higher rate than biliverdin IX α. The chemical reduction rates to bilirubins followed a similar pattern. Nucleophilic addition of 2-mercaptoethanol to the C10 methine was also favored in the extended biliverdins.

Heme catabolism in mammals starts with the enzymatic oxidation of heme to biliverdin IX α which is then reduced by a cytosolic biliverdin reductase to bilirubin IX α (1,2). Biliverdin IX α is the main isomer present in higher animals and plants, while biliverdin IX γ 1 (Fig. 1) is a natural product which was isolated from lepidopters (3), where it was also found to be the prosthetic group of a biliverdin binding protein (4). Free biliverdins have the energetically favored all-syn conformation (as in 1, 5Z,10Z,15Z-all-syn; Fig.1). In solution partially extended biliverdin forms could only be detected when dissolved in solvents which provide strong hydrogen bonds (5), or when the biliverdins were incorporated into liposomes (6). We have recently developed a versatile synthesis by which extended biliverdins (such as 2-6; Fig.1) could be obtained that are stabilized by intramolecular N-ethyl "clips" (7). Some of these extended biliverdins are natural products (3). These stable extended biliverdins allowed us to examine a still unknown feature of the reduction mechanism of biliverdin reductase; namely, if the reduction of the biliverdin requires a helical conformation, or if an extended form can also fit into the catalytic site of the enzyme.

The substrate specificity of biliverdin reductase has been explored using a large number of synthetic helical biliverdins, and it was found that they are reduced to bilirubins as long as they are substituted with two propionate residues (8). Biliverdin reductase is a NADPH-dependent enzyme, and a recent study has established that the catalytic site of the enzyme is similar to that of some nicotinamide-dependent dehydrogenases (9). The analogy between the reductase and the

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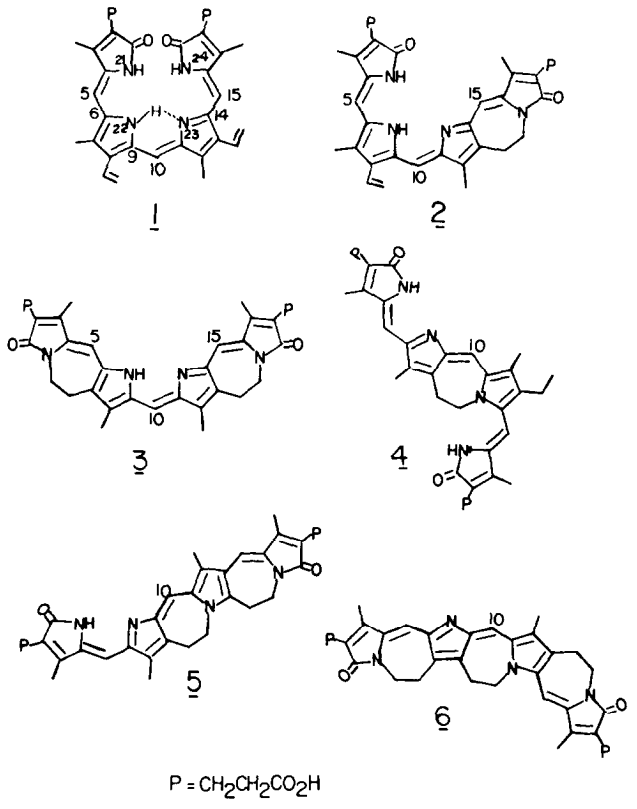

Figure 1. The structures of helical and extended biliverdins of type IX γ .

dehydrogenases break down however, when the nature of their respective substrates is compared. Biliverdins are relatively large molecules where the conformational factor is a crucial structural feature which has to be reckoned with.

Rat liver biliverdin reductase was found to exist in three molecular forms with different reduction rates for the isomers of biliverdin IX (10). Biliverdin IX δ 1 was reduced at a much lower rate than the IX α isomer. The main substitution pattern of 1 (two propionates α to the amide groups; Fig.1) was therefore chosen to prepare the extended analogs 2-6 (Fig.1), where the helix of 1 was progressively stretched in order to detect a possible increase in the enzymatic reduction rates. It was found that the more extended the biliverdin backbone, the more readily is it reduced both enzymatically and chemically to bilirubins.

MATERIALS AND METHODS

Materials. NADPH, NADH, 3-acetyl NADPH, deamino-NADPH, and 2-mercaptoethanol were from Sigma Chem.Co. Sodium borohydride ($NaBH_4$) and all other chemicals and solvents used were of the purest analytical grade from Merck.

Synthetic biliverdins. Biliverdins IX α and IX γ 1 were obtained by the coupled oxidation of hemin IX and were separated as described elsewhere (11). The extended biliverdins were obtained as their methyl esters using the published synthetic procedure (7). The spectra of biliverdins 2 (5Z-syn,10Z-syn,15Z-anti), 3 (5Z-anti, 10Z-syn,15Z-anti), 4 (5Z-syn, 10E-anti, 15Z-syn), 5 (5Z-syn,10E-anti,15Z-anti) and 6

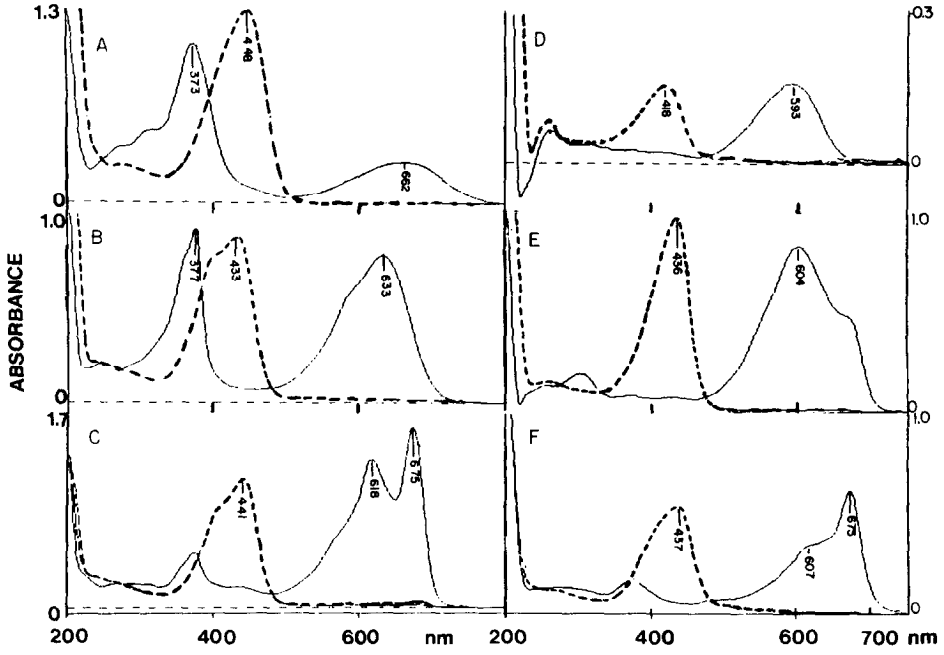

Figure 2. UV-vis spectra in methanol of: (——) biliverdin dimethyl esters, A) biliverdin IX γ 1 (ε 662 nm: 15.2 cm^{-1} mM^{-1}; ε373: 51.0); B) biliverdin 2 (ε633: 35.6; ε377: 42.0); C) biliverdin 3 (ε672: 37.0; ε618: 30.4; ε375: 11.3); D) biliverdin 4 (ε593: 32.2); E) biliverdin 5 (ε604: 39.5); F) biliverdin 6 (ε675: 34.2); and of (---) bilirubin dimethyl esters A) bilirubin IX γ (ε448: 56.0); B) bilirubin of 2 (ε433: 40.7); C) bilirubin of 3 (ε441: 26.5); D) bilirubin of 4 (ε428: 32.0); E) bilirubin of 5 (ε436:44.0) and F) bilirubin of 6 (ε437: 30.0).

(5Z-anti,10E-anti,15E-anti) are shown in Fig.2. The decrease in the near UV peak (B band) and the increase in the vis peak (Q band) indicates the departure from the helical conformation to the more extended one. The COSY and NOESY correlations (500 MHz) showed that the meso bridges which are not kept in the anti conformations by the seven-membered rings retained the favored syn conformations. Reduction of biliverdins 1-6 with $NaBH_4$ afforded the corresponding bilirubins (Fig.2).

Methods. The methyl esters of 1-5 were converted into the acids by saponification with 1N NaOH in methanol at 25°C during 24 h. Biliverdin 6 dimethyl ester was hydrolized with 6N HCl at 25°C during 72 h. The molecular forms 1 and 3 of rat liver biliverdin reductase were 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 (pH 7.4), 100 µM NADPH, the indicated biliverdin at a 13 µM concentration and enzyme, either molecular form 1 or 3 (1-2 µg of protein). The incubations were run at 30°C for the indicated times. NADH, 3-acetyl NADPH and deamino-NADPH were used at a 400 µM final concentration. Blanks were run in which the coenzyme was omitted. After incubation, the volume was completed to 500 ul and the decrease in the vis_{max} of the assayed biliverdins was measured. The ε values (Fig.2) were used to calculate the biliverdin concentrations in each case. Biliverdin dissappearance was further checked by measuring bilirubin formation at their vis_{max} (Fig.2). Adducts of 1-6 with 2-mercaptoethanol were prepared by addition of increasing concentrations of the latter to the biliverdin solutions in 10 mM phosphate buffer, pH 7.4. Biliverdin methyl esters were reduced with $NaBH_4$ in anhydrous solutions.

RESULTS

The enzymatic reduction rates of extended biliverdins. Biliverdin IX γ 1 was reduced by molecular form 1 of biliverdin reductase at a much lower rate that the

isomeric biliverdin IX α (Fig.3A). By stretching the C5-C6 and the C15-C16 bonds (as in 2 and 3, Fig.3B) the reduction rates increased slightly as compared to the helical conformer 1. It should be noted that in both 2 and 3 the C10 methine bridge (which is the one reduced by the enzyme) kept its Z-syn conformation. Biliverdin 4 (dihydrophorcabilin, Fig.1) where the C10 methine is held in an extended conformation (E-anti) was reduced at a much higher rate than 1 (Fig.3B). Further stretching of the bilitriene backbone as in 5 (Fig.1) increased the substrate activity of the biliverdin towards the enzyme (Fig.3B). The fully extended form 6 (Fig.1) was the best substrate of molecular form 1 of biliverdin reductase and its reduction rate was even higher than that of biliverdin IX α (Fig.3C). The visible spectrum of the fully extended biliverdin 6 (Fig.4D) shows that more than one conformer (or an associated form) is present in the solution (see (7)); at least two can be detected by their vis_{max} at 607 and 675 nm. They were not reduced by biliverdin reductase at the same rates (Fig.3C and D), although both were reduced to the same bilirubin derivative as judged by the spectroscopic evidence.

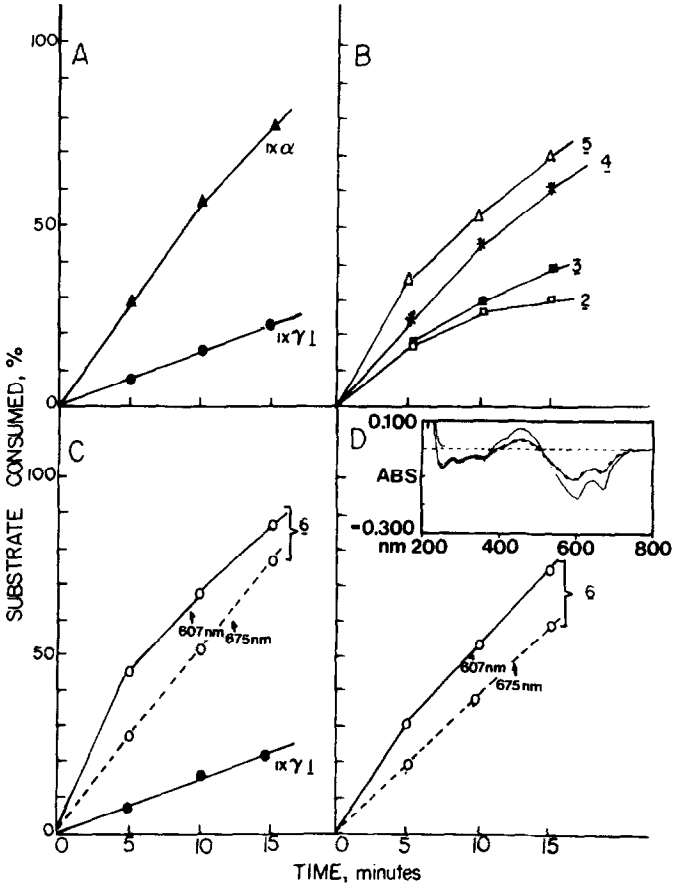

Figure 3. Enzymatic reduction rates of helical and extended biliverdins. A,B,C) molecular form 1 of biliverdin reductase was used. D) molecular form 3 was used. Inset: Differential spectra of 6 when reduced with: (——) form 1; (---) form 3.

Due to the extended and rigid overall structure of 6 it is conceivable that it could face steric problems when interacting with the catalytic site of the reductase, if the latter is buried in the protein structure. We have recently discussed the topological differences between molecular forms 1 and 3 of biliverdin reductase (12), and we have shown by using chemical modifications on the enzyme that in form 3 the catalytic site is less exposed than in form 1. It was therefore no surprise to find out that biliverdin 6 was reduced at a lower rate by form 3 as compared to form 1 (Fig.3D). The more flexible biliverdins 2-5 were reduced at similar rates by both molecular forms of the enzyme.

NADPH was the only nucleotide capable of reducing all the assayed biliverdins. We reported elsewhere (13) that in the case of many synthetic and natural helical biliverdins NADPH could be replaced by NADH, 3-acetyl NADPH and, in certain cases, deamino NADPH. However none of the extended biliverdins could be reduced when the latter nucleotides were used as coenzymes.

The fact that the C10 methine was reduced in the extended biliverdins by biliverdin reductase at higher rates than in the helical conformers could be attributed either to an increase in the nucleophilic character of the C10 methine in the extended forms, or to conformational factors affecting the interaction of substrates and enzyme. The first factor can be experimentally determined by measuring the addition at C10 of nucleophilic reagents as well as by measuring the reduction rates of the biliverdins using chemical reducing agents.

Formation of bilirubinoid adducts of 2-mercaptoethanol and extended biliverdins. 2-Mercaptoethanol adds to the C10 methine of the helical Z-all-syn biliverdins to give -S-C10 bilirubinoid adducts with vis_{max} around 420 nm (14). A similar regioselective necleophilic addition also takes place with biliverdin reductase, where the addition of a thiol residue of the enzyme leads to the tetragonalization of the trigonal C10 and very likely preceeds the reduction of the methine by the hy-

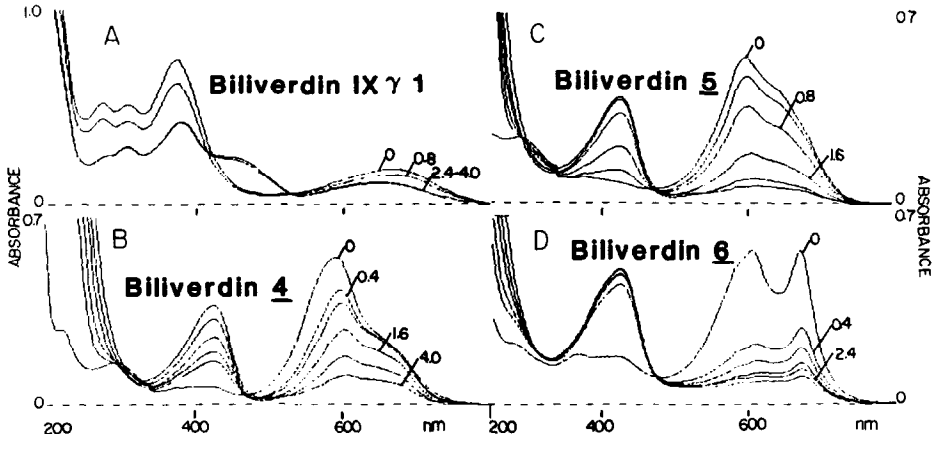

Figure 4. Spectra of the thiobilirubinoid adducts formed by addition of 2-mercaptoethanol to biliverdins. Thiol concentrations are mM. Adduct formation is measured by the increase of the 420-430 vis_{max}.

dride transfered from the dihydropyridine nucleotide (9). Similar reaction mechanisms are common to many nicotinamide-dependent dehydrogenases (15). When 2-mercaptoethanol was added in aqueous solution to the biliverdins 1-6, the formation of the thiobilirubinoid adducts could be detected by the increase in their vis_{max} around 420 nm (Fig.4). The decrease in the verdinoid absorptions was simultaneous with the increase in the bilirubinoid maxima. By measuring the formation of the adducts at increasing 2-mercaptoethanol concentrations, it was evident that the fully extended biliverdin 6 was the most prone to add to the nucleophile (Fig.5A). The ^1H NMR data showed that the addition took place at the C10 position. It is noteworthy that the nucleophilic addition took place more readily with the form absorbing at 606 nm than with the one absorbing at 675 nm (Fig.4D and 5A) thus paralleling their reduction rates with biliverdin reductase. The extended biliverdins 4 and 5 where the C10 bridge is held in an extended conformation were more readily converted into their thiobilirubinoid adducts than biliverdins 1, 2 and 3 where the C10 bridge is still in a Z-syn conformation (Fig.5B). It is evident that there is a good correlation between the enzymatic reduction rates of the extended biliverdins and their readiness to undergo nucleophilic addition by a thiol residue.

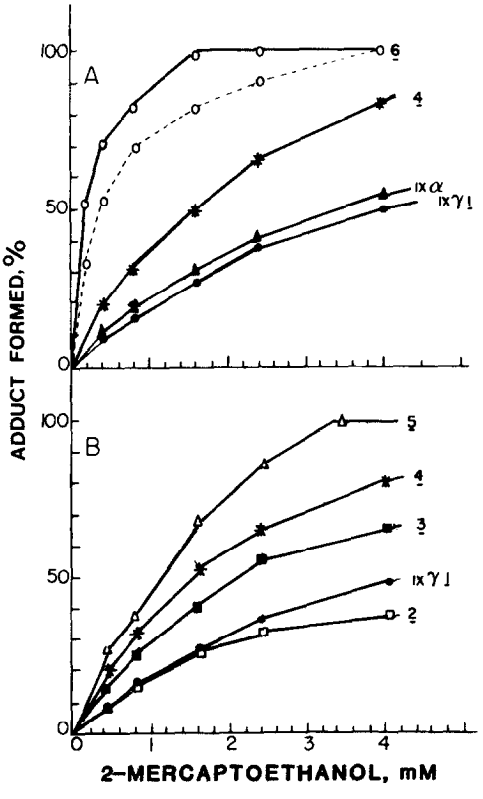

Figure 5. Bilirubinoid adduct formation as a function of 2-mercaptoethanol concentration. The additions were carried out in buffer solutions of biliverdins (18 μM).

TABLE I

Comparative reductions of the helical and extended biliverdins by sodium borohydride

Biliverdin	$NaBH_4$	% of biliverdin reduced		
	μM	0.5 min	2 min	10 min
Biliverdins IX α or IX γ 1	600	5	10	10
	2,000	6	15	20
Biliverin 2	600	10	20	20
	2,000	10	25	30
Biliverdin 3	600	30	32	32
	2,000	45	45	45
Biliverdin 5	100	75	100	100
Biliverdin 6	30	80	100	100

The methyl esters of the indicated biliverdins (18 μM concentrations) were dissolved and reduced in anhydrous methanol. The spectra were recorded at the indicated times after addition of $NaBH_4$. The data are the mean of three independent determinations which differed in less than 5%.

The chemical reduction with sodium borohydride of helical and extended biliverdins. The reduction of biliverdins to bilirubins with borohydrides is a chemical reaction which mimicks the enzymatic reduction since both involve a nucleophilic attack by a hydride on the C10 methine. It could therefore serve as a good model to find out if the extended biliverdins are also more readily reduced to bilirubins by the chemical reductant than their helical analogs. Table I shows that indeed this is the case. The fully extended biliverdin 6 was completely reduced to its bilirubin in 2 min using a 30 μM $NaBH_4$ concentration, while a 600 μM $NaBH_4$ concentration only reduced 10% of the helical biliverdin IX γ 1 under identical reaction conditions. Under the same reduction conditions it was evident that the more extended the biliverdin, the fastest was its reduction by $NaBH_4$. Again, as was the case with the addition of an electron donor, the extended conformation of the bilitriene facilitated its attack by a nucleophilic agent.

DISCUSSION

Reduction rates and nucleophilic additions were faster in extended biliverdins as compared to their helical conformers (Fig.3 and 5, and Table I). These experimental results suggest that an extended conformation could be a transient enzyme-substrate intermediate on which fast reduction takes place. There are still no X-ray diffraction clues to understand the stereochemical interaction of biliverdin reductase with its substrates. We already mentioned that the reductase resembles several nicotinamide-dependent dehydrogenases both in its structural (9)

and kinetic features (16). These dehydrogenases are among the more stereospecific enzymes in their interaction with substrates (15). A helical ZZZ-all-syn biliverdin is a mixture of two enantiomers of opposite helical sense which are present in equal proportion in solution (and is therefore devoid of optical activity). Of the few examples known where a protein binds non-covalently to a biliverdin, it was found to bind only one of the enantiomers which retains the ZZZ-configuration although it adopts a more extended conformation (4). If biliverdin reductase also binds to one of the enantiomers of the helical conformation and reduces it to bilirubin, a rapid interconversion of the helical enantiomers in solution should take place to account for the high reduction yields of biliverdin IX α to bilirubin IX α. If the enzyme-bound biliverdin adopts an extended geometry (and is therefore in a more strained state), it could be expected that extended biliverdins which are devoid of enantiomeric forms will have higher initial reduction rates, as is apparently the case with 5 and 6 (Fig.3B and C). It should be mentioned that in the case of the dehydrogenases the pyridine nucleotides which exist in solution in folded (or stacked) forms, adopt extended forms when binding to the active sites of the enzymes (15). These steric factors could also favour the enzymatic reduction of the extended biliverdins, in addition to their enhanced susceptibility towards nucleophilic attack.

Since biliverdins IX α and IX γ are reduced with $NaBH_4$ at the same rates (Table I) and also behave in a similar manner towards electron donors, the difference in their enzymatic reduction rates (Fig.2A) could be due to their differential steric interaction with the enzyme; either in the interconversion of the helical enantiomers or in the geometry which they adopt on interaction with the enzyme.

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