Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 752-758

MOLECULAR FORMS OF BILIVERDIN REDUCTASE FROM RAT LIVER WITH DIFFERENT REDUCTION RATES FOR BILIVERDIN-IX

Rosalía B.Frydman, María L.Tomaro,
Josefina Awruch and Benjamín Frydman

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

Received January 26, 1982

SUMMARY: Biliverdin reductase from normal rat liver was separated into two forms by DEAE-cellulose. The major component (peak 1) had the properties ascribed to purified biliverdin reductase of rat liver. It reduced biliverdin-IXα at the highest rate, while it had a lower affinity for the other three biliverdin isomers. The minor component (peak 2) reduced biliverdin-IXβ at a rate similar to that of biliverdin-IXα, and its molecular and kinetic properties are different from those of peak 1. When the rats were pretreated with $CoCl_2$, peak 2 remained unchanged, while the major peak 1 dissappeared and was replaced by a new major form (peak 3) which reduced biliverdin-IXβ at the highest rate and biliverdin-IXα at a slightly lower rate. The molecular form 3 of biliverdin reductase differed from the other two forms of the enzyme in its molecular and enzymatic properties.

Heme IX is the prosthetic group of hemoglobin and of a large number of hemoproteins. While the chemical oxidation of heme IX is a random process which takes place by an oxidative attack at any of the four meso-bridges and results in a mixture of the four biliverdin isomers (-IXα, -IXβ, -IXγ and -IXδ), heme IX enzymatic break down in nature is a strict α-selective oxidation reaction which forms biliverdin-IXα (1-4). The reduction of biliverdin-IXα to bilirubin -IXβ by biliverdin reductase (5) is the next step in the metabolic sequence of heme degradation in mammals. It was shown however, that the biles of adult human, rhesus monkey, and dog contain 2-5% of bilirubin-IXβ (6). It is also known that the chemical oxidation of hemoproteins (hemoglobin, catalase) results in the formation of only biliverdin-IXα and -IXβ (7). We found that a crude biliverdin reductase preparation from livers of cobaltous chloride treated rats reduced a mixture of the four biliverdin isomers to the corresponding bilirubins (2). The reduction rates of biliverdin-IXα and -IXβ were similar and much higher that the reduction rates of the other two isomers. A crude biliverdin reductase preparation of normal rats, reduced the -IXα isomer at a much higher rate. These results led us to the search of different molecular forms of the reductase in rat liver preparations.

0006-291X/82/060752-07$01.00/0

MATERIALS AND METHODS

Biliverdins-IX α, -IX β, -IX γ, and -IX δ were obtained by the oxidation of hemin IX in aqueous pyridine (8). The mixture of the four biliverdin isomers was separated as their methyl esters by tlc procedures. A first run on silica-gel plates (Merck, 20 x 20 cm, 0.25 mm layer thickness) at 22°C using 5% of acetone in chloroform as the developing system, allowed the separation of the methyl esters of biliverdin IX-β (the fastest band), biliverdin IX-δ (the slowest band), and of a mixture of the IX-α and IX-γ isomers (the middle band). Each band was eluted and run again at 22°C on similar plates using hexane: acetone: propionic acid, 48:12:11 as the developing system. In this solvent the isomers were completely separated and identified by their decreasing Rf as biliverdins-IX β, -IX δ, -IX α, and -IX γ. Each isomer band was eluted, dissolved in 2 ml of methanol and 2 ml of 1N KOH, the mixture was kept at 37°C during 2 hr, then adjusted to pH 4 with glacial acetic acid, and the biliverdin acids were extracted with chloroform. Bilirubins IX α-δ were prepared from the corresponding biliverdin methyl esters by reduction with sodium borohydride followed by a saponification step (9). NADPH, NADH, and DEAE-cellulose were purchased from Sigma Chemical Company. DEAE-cellulose was pretreated according to the procedure of Petersen and Sober (10).

Preparation of biliverdin reductase from rat liver. Biliverdin reductase was obtained from either normal (control) or cobaltous chloride injected (treated) animals. Wistar albino female rats (150-180 g) were used. The treated rats were injected subcutaneously with a single dose of $CoCl_2$ (200 mg/Kg) and were later fasted for 18 hr. They were then anesthetized with ether and the excised livers were repeatedly washed with an ice-cold saline solution. All the further operations were performed between 0-4°C. The livers (4 g) were homogenized in 3 volumes of an ice-cold 0.25 M sucrose solution and 0.05 M phosphate buffer (pH 7.4) in a Potter-Elvehejm homogenizer. The homogenate was centrifuged at 20,000 g for 15 min and the supernatant was further centrifuged at 105,000 g for 60 min. The 105,000 g supernatant was used as the crude biliverdin reductase source for the further fractionation steps. The livers from the control rats were extracted following the same procedure after the animals were fasted for 18 hr.

Assay of biliverdin reductase. When the crude enzymes of both control and treated animals were assayed, the incubations were carried out at 37°C during 10 min. The incubation mixture contained in a final volume of 100 µl: 12.5 µmol of pottasium buffer (pH 7.4), 60 nmol of NADPH, 13 nmol of biliverdin, and 25 µl of enzyme (0.3 mg of protein). When NADPH was substituted by NADH, 240 nmol of the latter were used. Biliverdin reductase activity was measured by diluting the incubation mixture to 1 ml with water, and by estimating the formed bilirubin by its ΔOD 455-520 nm (3). This difference was proportional to the bilirubin concentration under the used assay conditions, as determined with authentic bilirubin samples. The absorbance values at 455 nm (peak of bilirubin absortion) and 520 nm were the same for all the four bilirubin isomers. An ε=50 mM^{-1} cm^{-1} was found by using standard bilirubin solutions assayed under the conditions of the incubation mixture. When the purified bilirubin reductase was assayed, the above indicated incubation mixture was incubated in a final volume of 200 µl during 20 min and 100 µl of enzyme (5 µg of protein) was then used.

RESULTS

Separation of different molecular forms of biliverdin reductase. Crude biliverdin reductase obtained from the liver of normal rats was purified by a DEAE-cellulose column equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. The enzyme was eluted with a linear potas

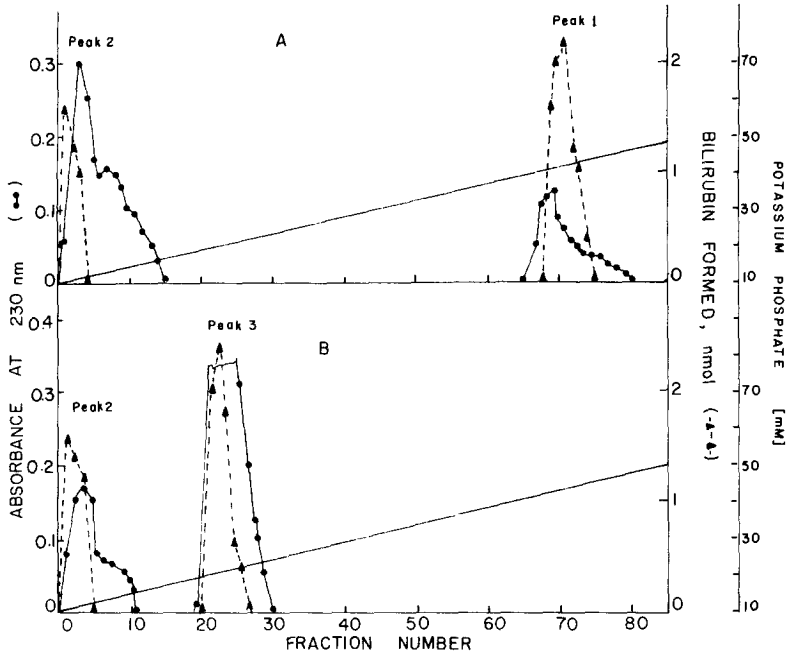

Figure 1. Separation of the different molecular forms of liver biliverdin reductase from control (A), and $CoCl_2$-treated (B) rats. The crude extract (1 ml, 14 mg protein, 12 nmol of bilirubin formed/mg protein/5 min) was applied to a DEAE-cellulose column (1,5 x 18 cm) equilibrated and eluted as described in Materials and Methods. Only the gradient elution pattern is depicted. Fractions of 1 ml were collected and assayed as described. Identical elution patterns were obtained when the rat livers were homogenized in the presence of 1 mM of phenylmethylsulfonylfluoride. (-●-) Absorbance at 230 nm. (-▲-) Biliverdin reductase activity.

sium phosphate buffer (pH 7.4) gradient between 10 and 100 mM containing 0.25 M sucrose. Two biliverdin reductase forms were thus eluted; peak 2 (Fig.1A) was eluted between 11-13 mM of the phosphate buffer, while peak 1 was eluted between 30-42 mM of the same buffer. Peak 1 was the major form and carried about 2/3 of the biliverdin reductase activity. It reduced biliverdin-IXα to bilirubin-IXα at the highest rate (Fig.3A), while biliverdin-IX δ was a better substrate than biliverdin-IXβ and biliverdin-IX γ was the poorest substrate. These reduction rates are similar to those reported for biliverdin reductase from rat spleen and liver (11). Since the molecular properties of this form are also similar to those reported for the reductase (see below), it can be conluded that the biliverdin reductase described elsewhere (11, 12) is the form eluted as peak 1. Peak 2 was the minor component of the system and it reduced biliverdins-IXβ and -IXα at a similar rate (Fig.2B).

When crude biliverdin reductase from rat liver of $CoCl_2$-treated rats was purified by DEAE-cellulose following the above mentioned procedure, it was found that peak 2 was also present in this preparation (Fig.1B). Peak 1 however was absent, and a new form (peak 3) was eluted at 20-23 mM phosphate buffer. It

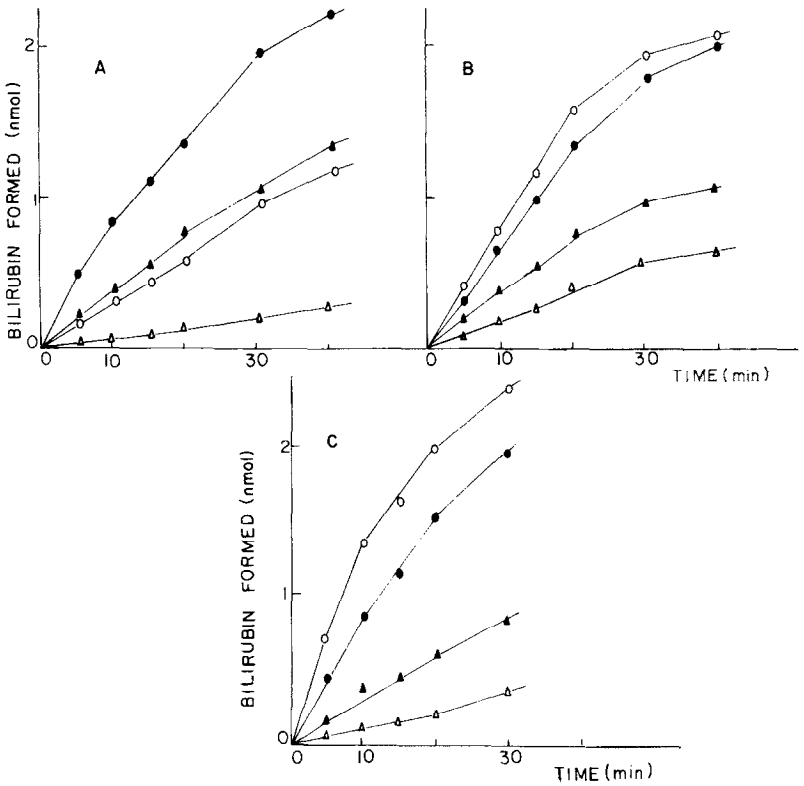

Figure 2. Reaction rates of the three forms of biliverdin reductase with the four biliverdin isomers. The biliverdin reductase forms separated by DEAE-cellulose as described in Results were used. Peak 1 (A), peak 2 (B), and peak 3 (C). Biliverdin-IX α (●); -IX β (○); -IX γ (△) and -IX δ (▲).

was the major component of the enzyme of treated rats. This new form reduced biliverdin-IX β at a slightly higher rate than the α-isomer (Fig.2C). The different forms of the reductase present in the preparations of the normal and $CoCl_2$-treated rats, explain the difference in the reduction rates found for the biliverdin isomers in the crude extracts.

Properties of the different biliverdin reductase forms. The molecular forms 1 and 3, the major components of biliverdin reductase from normal and treated rats, differed not only in the reduction rates of biliverdin-IX β but also in a number of important molecular properties. Their molecular weights were determined by the method of Andrews (13) using 100 mM phosphate buffer (pH 7.4), 2 M KCl, and as mass markers, bovine serum albumin (68,000 daltons), pepsin (34,500 daltons), chymotrypsinogen A (25,000 daltons), and cytochrome C (12,500 daltons). Peak 3 was found to have a molecular weight of about 66,000 daltons, while peak 1 contained a major form with a molecular weight of 34,000 daltons and a minor dimeric form with a molecular weight of 68,000 daltons. When KCL was omitted from the buffer (i.e., under conditions of lower ionic strenght), only the dimeric form of peak 1 (68,000 daltons)

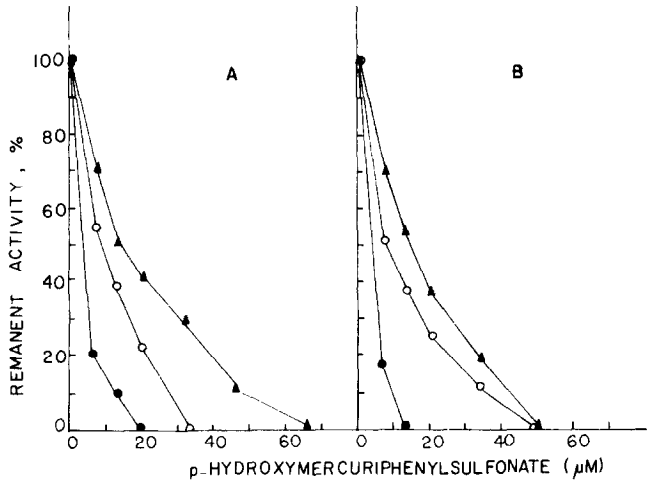

Figure 3. The effect of p-hydroxymercuriphenylsulfonate concentration on the activity of the different forms of biliverdin reductase. The assay conditions were the indicated in Materials and Methods except for the addition of the inhibitor. The DEAE-cellulose separated peak 1 (●), peak 2 (○), and peak 3 (▲), were used. Biliverdin-IXα (A) and -IXβ (B) were used as substrates.

was present. Therefore, under physiological conditions this form has very likely the dimeric structure, although the monomer has the full catalytic activity. A molecular weight of 34,000 daltons was reported for biliverdin reductase from rat liver (11, 12), lending further support to our former suggestion that the described reductase is form 1. The fact that peak 3 has the molecular weight of a dimer under the conditions where peak 1 is mainly a monomer, can be linked to the different oxidation states of the sulfhydryl groups of the enzyme derived from the $CoCl_2$ treatment. It was found that of the three reductase forms, peak 1 was the most sensitive to sulfhydryl reagents while peak 3 was the least sensitive. This property is exemplified by their relative sensitivities toward p-hydroxymercuriphenylsulfonate (Fig.3). Peaks 1 and 3 also differed in their inactivation rates at 60°C (Fig.4). Their activities were assayed using biliverdin-IXα as substrate.

Peak 2 of both the control and treated rat livers had the same molecular properties. A molecular weight of about 56,000 daltons was determined for the peaks of both sources using Andrews' method. They also had similar optimum pH's, identical inactivation rates at 60°C (Fig.4), as well as the same sensitivity toward sulfhydryl reagents (Fig.3). The full details of the molecular and enzymatic properties of the three biliverdin reductase forms will be given elsewhere.

DISCUSSION

The existence of the hitherto undetected forms of biliverdin reductase (peaks 2 and 3) with their high affinity for biliverdin-IXβ suggest that this

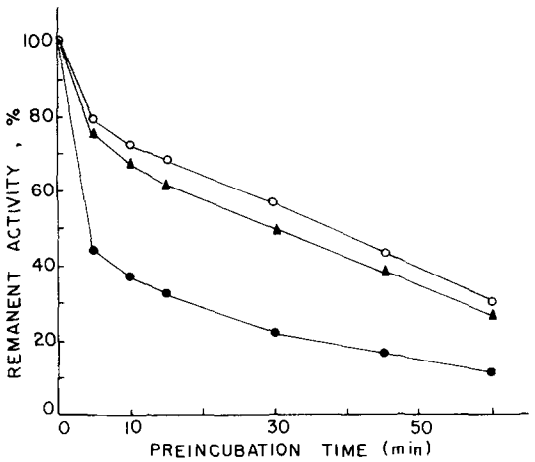

Figure 4. Inactivation rate of the three forms of biliverdin reductase. DEAE-cellulose separated biliverdin reductase forms were preincubated at 60°C during the indicated times, and then biliverdin-IXα and NADPH were added and the incubation was carried out at 37°C for 30 min. Peak 1 (●), peak 2 (○), peak 3 (▲).

isomer if formed under normal conditions during hepatic hemoprotein break down processes, and that its formation is enhanced when hemoprotein oxidations were accelerated. While the different biliverdin reductase forms also reduce isomers-IXγ and -IXδ, there is clearly no metabolic regulation involved in the reduction of these isomers. The fact that all the forms of biliverdin reductase have high reduction rates for biliverdin-IXα is clearly the result of the α-specific oxidation of heme by heme oxygenase (1-4). The high reduction rate of biliverdin IXβ by the novel form 2 suggest that this isomer is formed even under normal conditions, very likely by the chemical (non-enzymatic) oxidation of hemoproteins (7). It was shown (14) that hepatic catalase contains biliverdin in a 1:3 ratio with respect to heme, and that the former is an equimolar mixture of the α and β isomers; a fitting example of chemical in situ heme cleavage. The administration of $CoCl_2$ which leads to an extensive oxidation of liver hemoproteins, induces the appearance of peak 3 with its high reduction rates for both the α- and β - isomers. While the induction of heme oxygenase by the $CoCl_2$ (2) will form biliverdin-IXα from free heme, non-enzymatic oxidation of hemoproteins by Co^{2+}-O_2 adducts (15) will simultaneously form the isomer IXβ (7).

ACKNOWLEDGMENT

This work was made possible by grant GM -11973 from the NIH (PHS).

REFERENCES

1. Tenhunen, R., Marver, H.S., and Schmid, R. (1969) J.Biol.Chem. 244, 6388-6394.

2. Frydman, R.B., Awruch, J., Tomaro, M.L., and Frydman, B. (1979) Biochem. Biophys.Res.Commun. 87, 928-935.
3. Frydman, R.B., Tomaro, M.L., Buldain, G., Awruch, J., Díaz, L., and Frydman, B. (1981) Biochemistry 20, 5177-5182.
4. Awruch, J., Lemberg, A., Frydman, R.B., and Frydman, B. (1981) Biochim. Biophys.Acta 714, 209-216.
5. Tenhunen, R., Ross, M.E., Marver, H.S., and Schmid, R. (1970) Biochemistry 9, 298-303.
6. Blumenthal, S.G., Taggart, D.B., Ikeda, R.M., Ruebner, B.H., and Bergstrom, D.E. (1977) Biochem.J. 167, 535-548.
7. O'Carra, P. (1975) in Porphyrins and Metalloporphyrins, pp. 123-153, Elsevier Sci.Pub.Co., Amsterdam-New York-Oxford.
8. Bonnett, R., and McDonagh, A.F. (1973) J.Chem.Soc.Perkin I, 881-888.
9. Blanckaert, N., Heirwegh, K.P.M., and Compernolle, F. (1976) Biochem.J. 155, 405-417.
10. Peterson, E.A., and Sober, H.A. (1962) in Methods in Enzymology 5, pp.3-6, Academic Press, New York.
11. Noguchi, M., Yoshida, T., and Kikuchi, G. (1979) J.Biochem. (Tokyo) 86, 833-848.
12. Kutty, R.K., and Maines, M.D. (1981) J.Biol.Chem. 256, 3956-3962.
13. Andrews, P. (1964) Biochem.J. 91, 222-233.
14. Morell, D.B., and O'Carra, P. (1974) Ir.J.Med.Sci. 143, 181 (Abstr.).
15. Wilkins, R.G. (1971) in Bioinorganic Chemistry (Gould, R.F.Editor) pp.111-134, American Chemical Society Editions, Washington D.C.