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ISOLATION FROM RAT LIVER OF A PEROXISOMAL ENZYME WHICH CONVERTS MOLECULAR FORM 1 OF BILIVERDIN REDUCTASE INTO MOLECULAR FORM 3

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SUMMARY: Cobaltous chloride induced in rat liver an enzyme which converted biliverdin reductase molecular form 1 into the molecular form 3. This conversion involves the oxidation of two sulfhydryl groups of form 1 giving rise to a disulfide bond in form 3. The converting enzyme was isolated from the liver peroxisomal fraction (which was devoid of biliverdin reductase activity), and was absent in liver peroxisomes of control rats. The enzyme was solubilized by treatment of the peroxisomes with 0.1% sodium deoxycholate, and partially purified by DEAE-cellulose and Sephadex G-100 filtration. It is a NAD^+ dependent enzyme which was inactivated by trypsin and heat treatments. It did not oxidize either reduced glutathione or cysteine. The converting enzyme had a molecular weight of about 54,000 daltons. The oxidation of biliverdin reductase molecular form 1 mediated by the converting enzyme did not affect the latter's molecular weight or activity.

Biliverdin reductase exists in three different molecular forms in rat liver (1). Under normal conditions molecular form 1 is the major molecular form of the enzyme and its outstanding feature is the high reduction rate for biliverdin IX α, while the reduction rates for the other three biliverdin IX isomers (β, γ, δ) are much lower (1). Molecular form 2 is the minor form of biliverdin reductase of normal rat liver and it has similar reduction rates for both biliverdins IX α and IX β. The third molecular form of biliverdin reductase is form 3 which is induced in liver of rats by treatment with $CoCl_2$ or phenylhydrazine, and posesses high reduction rates for biliverdins IX α and IX β. A similar substrate specificity of the three molecular forms of biliverdin reductase was found when biliverdins XIII α, XIII β and XIII γ were used as substrates (2). Biliverdin reductase isolated from the livers of $CoCl_2$ treated rats although devoid of form 1 still has the minor molecular form 2 (1). We have shown that the appearance of form 3 was due to a gradual transformation of form 1 of the enzyme into form 3 which is produced in the liver by the treatment of the animals with the aforementioned chemicals (3). This transformation involved a *de novo* mRNA and protein synthesis and it was therefore proposed that it is very likely an enzyme mediated process (3). Since molecular form 3 could be transformed back into molecular form 1 by an *in vitro* treatment with reduced thioredoxin (3), it was conceivable that the *in vivo* transformation of form 1 into form 3 was mediated

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by an enzymatic oxidation reaction which converted two sulfhydryl groups into a disulfide bond. Support for this suggestion was derived from the fact that molecular form 1 had three sulfhydryl groups per molecular weight of 34,000 daltons, while molecular form 3 had four sulfhydryl groups per molecular weight of 68,000 daltons (1,3). We now report the isolation, characterization, and partial purification of a NAD^+ dependent enzyme which converts biliverdin reductase form 1 into form 3. It was isolated from rat liver peroxisomes of $CoCl_2$ treated animals, and it was absent in liver peroxisomes isolated from normal animals.

MATERIALS AND METHODS

Biliverdins IXα and IXβ were obtained and separated as previously described (1,4). NAD^+, $NADP^+$, FAD, NADPH, reduced and oxidized glutathione, dithiothreitol, cysteine, phenylmethylsulphonylfluoride (PMSF), and DEAE-cellulose were purchased from Sigma Chemical Co. The latter was pretreated according to the procedure of Peterson and Sober (5). Protein was estimated by the method of Lowry et al.(6). The reactive sulfhydryl groups were measured by titration with 5,5'-dithiobis(2-nitrobenzoic acid) according to Ellman (7). All other chemicals were of analytical reagent grade.

Biliverdin reductase was obtained from livers of normal rats and from rats injected with $CoCl_2$ as described elsewhere (1,3). The three molecular forms of the enzyme were separated on DEAE-cellulose following the described procedure (1,3).

Preparation of the liver fractions from $CoCl_2$ treated rats. Wistar albino female rats (180-200 gr) were used. The rats were injected subcutaneously with a single dose of $CoCl_2$ (170 mg/kg) and were later fasted during the course of the experiment. The animals were sacrificed 2.25 h after the $CoCl_2$ treatment. They were anesthesized with ether and the excised livers were perfused with an ice-cold saline solution before homogenization. The livers were minced and homogenized in 3 vol of an ice-cold solution of 0.33 M sucrose, 0.2 mM EDTA and 2 mM PMSF in 10 mM potassium phosphate buffer (pH 7.4) using a Potter Elvehejm homogenizer. The homogenate was then centrifuged at 700 g for 10 min, the pellet was discarded and the supernatant was centrifuged at 6,000 g for 10 min. The resulting pellet consisted mainly of heavy mitochondria (8), and is therefore called the mitochondrial fraction. The supernatant fraction was centrifuged for 10 min at 20,000 g and the pellet was washed with the buffer used for liver homogenation. This pellet was then dissolved in 20 mM phosphate buffer (pH 7.4) and was considered to be the peroxisomal fraction (8). The 20,000 g supernatant carried the biliverdin reductase activity while the peroxisomal fraction was entirely devoid of this activity. The 105,000 g precipitate was the microsomal fraction where heme oxygenase activity was located.

Assay of the enzyme converting form 1 of biliverdin reductase into form 3. The converting enzyme was solubilized and purified from the peroxisomal fraction as described in Results. The assay was performed in a final volume of 1 ml by incubation of 0.5 ml of biliverdin reductase form 1 (total activity, 50-70 nmol of bilirubin formed in 10 min) with either the peroxisomal suspension (200 μl, 3 mg of protein), or the solubilized enzyme (200 μl, 30-300 μg of protein depending of the purification stage), and 1 μmol of NAD^+. The incubation was carried out in the presence of 50 mM phosphate buffer (pH 7.4) for 30 min at 37°C. The incubation mixture was then applied on a DEAE-cellulose column (1 x 18 cm) previously equilibrated with 10 mM phosphate buffer (pH 7.4) and 0.25 M sucrose. Molecular forms 1 and 3 of biliverdin reductase were eluted and their activity was measured as described elsewhere (1,3). The activity of the converting enzyme was expresed as the percentage of the activity of molecular form 1 which was transformed into molecular form 3. Controls in which the NAD^+ or the preincubation step were omitted were simultaneously performed and analyzed as described above.

RESULTS

Isolation and partial purification of the biliverdin reductase converting enzyme. Molecular form 3 started to appear in rat liver 2.5 h after the treatment of

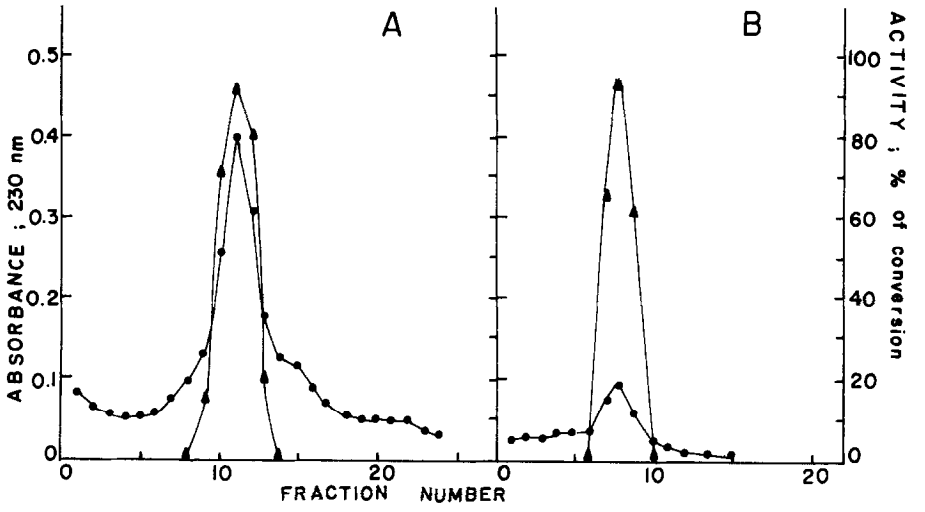

Figure 1. Elution profiles of the chromatographic steps in the purification of the converting enzyme. (A) DEAE-cellulose chromatography. The 0.1% deoxycholate solubilized enzyme (15 ml, 37 mg of protein) was applied on a DEAE-cellulose column (3 x 30 cm) previously equilibrated with 50 mM phosphate buffer (pH 7.4). The column was washed with 500 ml of the same buffer and the enzyme was eluted with 70 mM phosphate buffer. Fifteen ml fractions were collected. Only the portion eluted with 70 mM phosphate buffer was depicted. (B) Sephadex G-100 elution profile. The enzyme eluted from DEAE-cellulose was concentrated (3.5 ml, 4.5 mg of protein) and applied on a Sephadex G-100 column (3 x 40 cm). Fractions of 5 ml were collected. Activity was measured as described in Materials and Methods. (●) Absorbance at 230 nm; (▲) Converting enzyme activity.

the rats with $CoCl_2$ (3). It was therefore conceivable that any enzyme converting molecular form 1 into molecular form 3 had been already induced by this time. Hence the presence of the enzyme was investigated in livers isolated 2.25 h after the $CoCl_2$ administration. The enzyme was looked for in the different liver fractions (see Materials and Methods) and it was found to be present in the peroxisomal fraction while it was absent in the other liver fractions (mitochondria, microsomes, and cytosol). The peroxisomal fraction was devoid of any biliverdin reductase activity. The enzyme was solubilized by treatment of the peroxisomes with 0.1% sodium deoxycholate and was further purified by DEAE-cellulose chromatography and Sephadex G-100 filtration (Fig. 1A and B). A one hundred fold purification was achieved by these treatments. The conversion of molecular form 1 into molecular form 3 catalyzed by this enzyme required the addition of NAD^+ when the purified enzyme (after the DEAE-cellulose stage) was assayed. At the former purification stages the addition of NAD^+ was not always essential, very likely due to the presence of the cofactor in the crude preparations. The requirement for NAD^+ was essential for the activity of the converting enzyme and could not be replaced either by $NADP^+$ or FAD.

Properties of the biliverdin reductase converting enzyme. The conversion of biliverdin reductase form 1 into the molecular form 3 catalyzed by the converting enzyme increased with time of preincubation and enzyme concentration (Fig.2A and B).

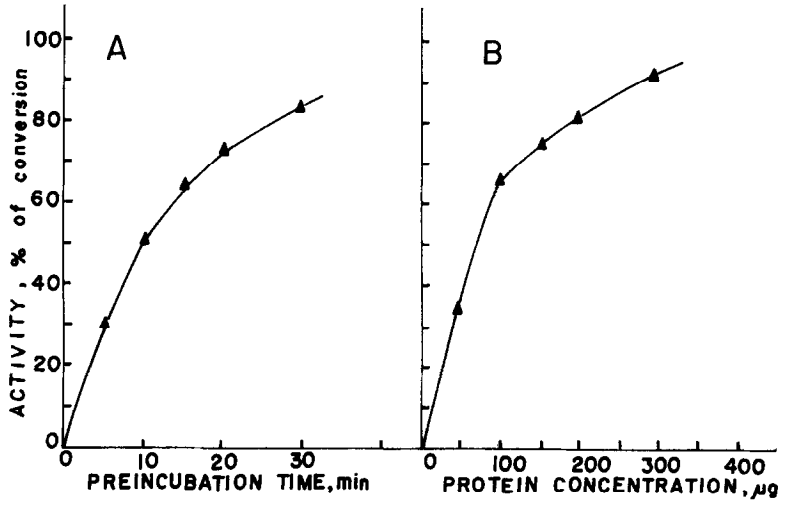

Figure 2. Effect of preincubation time (A) and enzyme concentration (B) on the conversion of biliverdin reductase form 1 into form 3. The DEAE-cellulose purified enzyme was used. The incubation conditions and the activity assay were the described in Materials and Methods. In (A) 260 µg of protein were used.

The enzymatic activity was completely abolished by treatment of the enzyme with trypsin for 30 min. When the Sephadex G-100 purified enzyme was heated 10 min at 65° about 70% of its activity was lost.

The molecular weight of the enzyme was determined by the method of Andrews (9) using 100 mM phosphate buffer (pH 7.4), 2M KCl, and as mass markers bovine albumin (68,000), ovoalbumin (43,000 daltons), and chymotrypsinogen A (25,000 daltons). The converting enzyme was found to have a molecular weight of about 54,000 daltons.

The enzyme did not oxidize reduced glutathione or cysteine as measured by sulfhydryl content (7). It did not oxidize biliverdin reductase molecular form 2 either.

We have previously shown (1,3) that the conversion of form 1 into form 3 of biliverdin reductase involves the oxidation of two sulfhydryl groups to form a disulfide bond. This oxidation is therefore mediated by the NAD^+ dependent converting enzyme. In the course of this reaction neither the charge nor the activity of the converting enzyme were affected (Fig.3). The enzymatic reaction triggered by the addition of NAD^+ converted form 1 into form 3 (Fig.3B) but did not affect the elution pattern from DEAE-cellulose of the converting enzyme (compare Fig.3A and B with C). No change was either detected in the molecular weight of the converting enzyme after its enzymatic action on the biliverdin reductase form 1.

DISCUSSION

It has been known for long that $CoCl_2$ induces hemoprotein degradation in liver (10), and it has been established that the major contribution to this degradation is the induction of heme oxygenase by the Co^{2+} which results in the α-selective oxidation of heme to biliverdin IX α (11)(4)(12). The data presented in this paper show

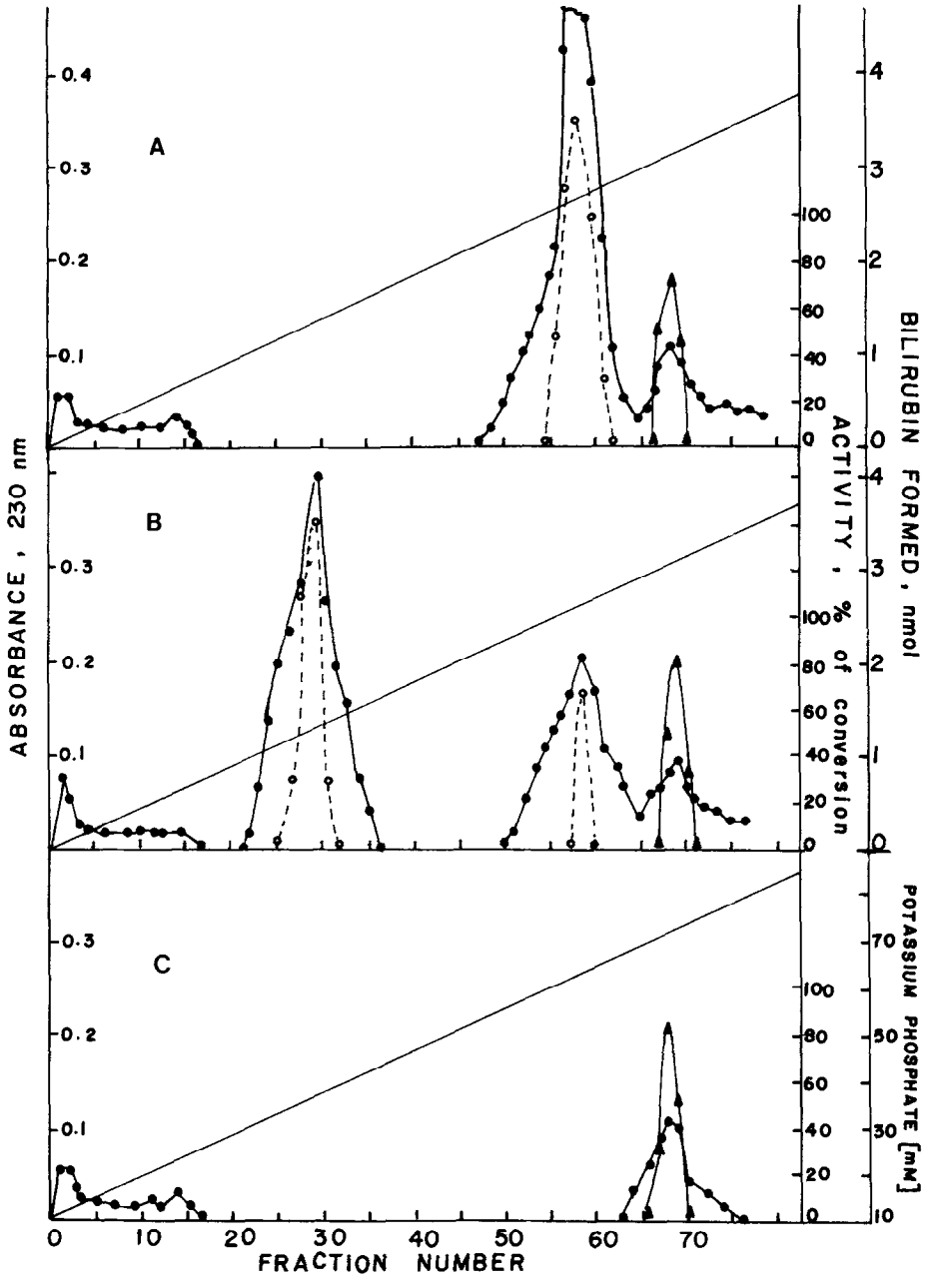

Figure 3. Enzymatic interconversion of liver biliverdin reductase form 1 into form 3. DEAE-cellulose elution profiles of: (A), Biliverdin reductase form 1 preincubated with the DEAE-cellulose purified converting enzyme for 30 min at 37°C in the absence of NAD^+. The incubation mixture (1.5 ml) was applied to a DEAE-cellulose column (1 x 18 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose. The column was washed with the same buffer (100 ml) and then eluted with a linear potassium phosphate buffer (pH 7.4) gradient between 10 and 100 mM containing 0.25 M sucrose. Fractions of 1 ml were collected. The reductase and the converting enzyme were assayed as described in Materials and Methods using a 100 µl aliquot; (B) As in (A) but NAD^+ was added to the preincubation mixture; (C), Converting enzyme without addition of biliverdin reductase. (●) Absorbance at 230 nm; (○) Biliverdin reductase activity; (▲) Converting enzyme activity.

that Co^{2+} also induces a peroxisomal dehydrogenase which modifies biliverdin reductase by converting its major molecular form 1 into another major molecular form 3. The net result of this conversion is the increase in the rate of biliverdin IX β reduction. This could reflect a situation of increased biliverdin IX β formation as a result of a chemical hemoprotein degradation due to the administration of the Co^{2+}, a situation which we have already discussed elsewhere (1,3). There is also the possibility that the presence of oxidizing cobalt species (13) is countered by the decrease in the number of sulfhydryl groups of biliverdin reductase and by the formation of a new form of the latter which is less sensitive to sulfhydryl reagents (1). In any case the induction of the converting enzyme by $CoCl_2$ is undoubtely an hitherto unrecognized new metabolic regulation step in the process of heme degradation.

The converting enzyme appears to be different, in its coenzyme requirements and subcellular localization, from the known thiol-protein disulfide oxidoreductases (14).

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