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A NOVEL 34 KDA GLUTATHIONE-BINDING PROTEIN IN MATURE RAT OVARY

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A novel protein that binds to a glutathione-Sepharose affinity column has been detected in mature, but not immature, rat ovary. This protein could be resolved from all identifiable components when the affinity-purified material, containing primarily glutathione transferases, was analyzed on reversed phase-HPLC. The unidentified protein migrated with an apparent molecular weight of 34 kDa on SDS-PAGE. After purification by affinity chromatography and subsequent preparative electrophoresis, the protein was subjected to N-terminal amino acid sequence analysis. The sequence obtained demonstrated a high degree of homology with an internal amino acid sequence in human carbonyl reductase (EC 1.1.1.184).

Glutathione-linked enzymes are in most cases involved in protection against reactive, electrophilic compounds and oxidative processes (1). The most extensively studied substrates for glutathione transferases are xenobiotics and/or their metabolites, but steroids have also been found to be substrates for these enzymes (2, 3). Previous reports have shown an increase in the level of hepatic glutathione transferases in male and female rats after puberty (4). It has also been reported that treatment of immature rats with pregnant mare's serum gonadotropin significantly stimulates hepatic glutathione transferase activity (5, 6). Those results indicate that at least certain glutathione transferases are regulated by gonadotropic hormones and/or steroids. Carbonyl reductase (EC 1.1.1.184) reduces a great variety of carbonyl compounds, utilizing NADPH as the electron donor. Carbonyl reductase from human brain has been cloned and sequenced (7). This enzyme has also been purified from the rat ovary (8) and previous reports indicate that it is involved in ovulatory processes (9).

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MATERIALS AND METHODS

Animals. Cytosol was prepared by homogenizing the ovaries of female Sprague-Dawley rats, either immature (26 days old) or sexually mature (230 g), in 0.25 M sucrose, 20 mM Tris-Cl, pH 7.4, and subsequent centrifugation at 100,000 x g for 1 hr.
For purification of the 34 kDa protein we used immature rats (26 days old) treated with a single dose of 10 IU pregnant mare's serum gonadotropin, which induces follicular maturation. The rats were decapitated 48 hours after administration of pregnant mare's serum gonadotropin, i. e., just prior to ovulation (10). The 34 kDa protein was found to be most abundant at this time (not shown).

Enzyme purification. The cytosols were first passed through a Sephadex G-25 column (Pharmacia LKB Biotechnology) in order to remove endogenous glutathione. The protein fraction was then applied to a glutathione-Sepharose 4B column (Pharmacia LKB Biotechnology) (11), which had been pre-equilibrated with 10 mM potassium phosphate buffer containing 0.15 M KCl, 1 mM EDTA and 2 mM dithiothreitol, pH 7.0 (buffer A), and, after sample application, was washed again with the same buffer. Elution was performed with a buffer containing 0.1 M Tris-Cl, pH 9.1, 5 mM S-hexylglutathione and 5 mM glutathione. In order to separate the 34 kDa protein from the other glutathione transferases, a linear gradient obtained by mixing buffer A and 7.5 mM each of S-hexylglutathione and glutathione in 0.1 M Tris-Cl, pH 9.1, was used.

HPLC analysis was performed using a reversed-phase column (4.6 x 250 mm) (Dynamax 300 A, C4-83-503-C5; Rainin Instruments, Woburn, MA, USA) on a Gilson HPLC system. The glutathione transferase subunits were separated by gradient elution with acetonitrile and aqueous 0.1 % trifluoroacetic acid, with a flow rate 1 ml/min. The gradient employed was a modified version of that described by Ostlund Farrants *et al.* (12).

SDS-PAGE was performed according to the method of Laemmli (13) with a 15 % (w/v) acrylamide concentration. In order to visualize the proteins after electrophoresis, the silver staining method of Blum *et al.* (14) was employed.

Preparative electrophoresis. The 34 kDa protein was purified from the affinity-purified material by continuous-elution electrophoresis using a Model 491 Prep Cell (Bio-Rad Laboratories). The acrylamide concentration was 12 % and the run was performed at 40 mA constant current. The fractions were analyzed by SDS-PAGE and silver staining.

N-terminal amino acid sequencing. The solution containing the purified 34 kDa protein was run through a pair of ProSpin Sample Preparation Cartridges (Applied Biosystems, Foster City, California) to eliminate SDS from the sample. Sequence analysis was carried out on an Applied Biosystems (Foster City, California) 477A Pulsed Liquid Phase sequencer with an online PTH 120A Analyzer. Sequencing was performed with cycle programs adapted to our reaction cartridges using chemicals from the manufacturer.

Enzyme assays. Glutathione transferase activity towards 1-chloro-2,4-dinitrobenzene was determined spectrophotometrically (15).
Reduction of menadione (carbonyl reductase activity) was measured in a reaction mixture consisting of 60 mM sodium phosphate, pH 6.5, 0.08 mM NADPH, 0.25 mM menadione and enzyme (16).
Protein concentration was determined by the modified Lowry procedure described by Peterson (17). The purified 34 kDa protein was quantitated on the basis of the N-terminal sequencing, due to the very small quantities present.

RESULTS AND DISCUSSION

The original purpose of this investigation was to characterize the subunit compositions of the cytosolic glutathione transferases in the ovaries of immature and sexually mature rats. After purification by affinity chromatography on a glutathione-Sepharose column, the different

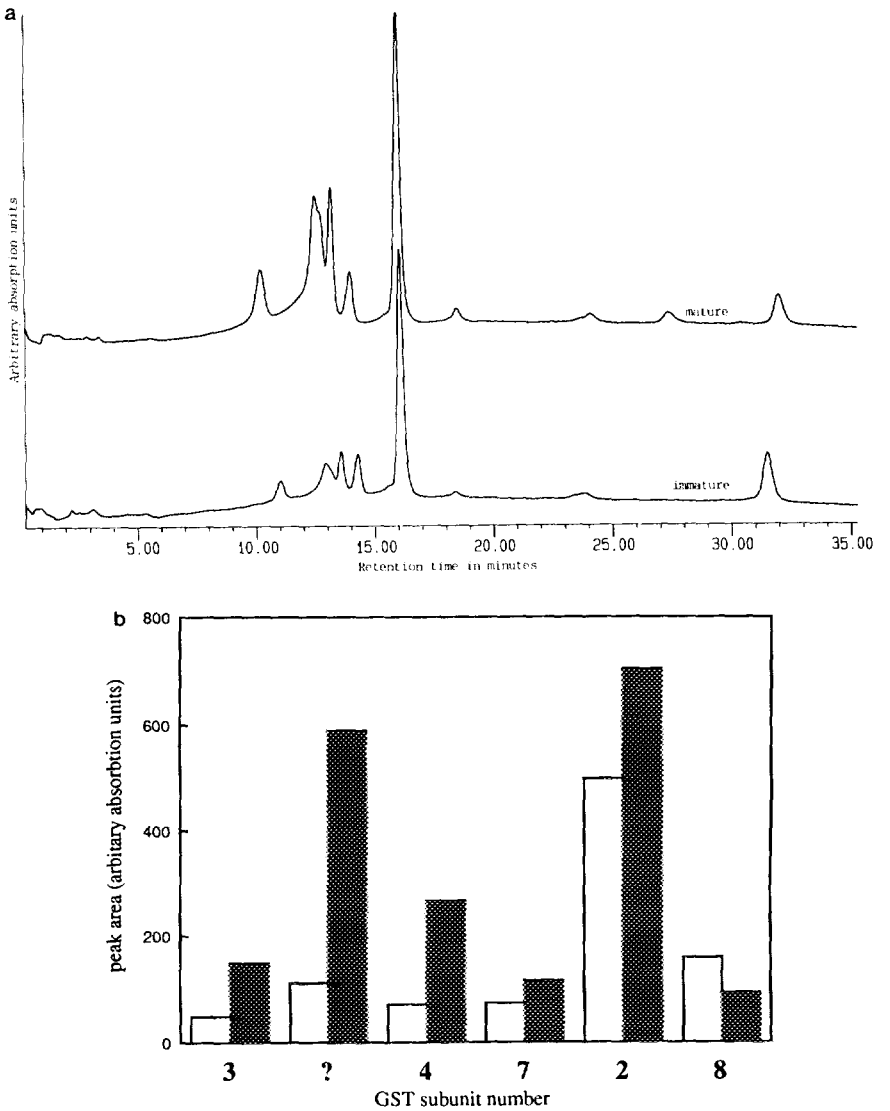

Figure 1. **HPLC chromatogram of glutathione transferase subunits, comparing the affinity purified (glutathione-Sepharose column) cytosolic fraction from immature and sexually mature rat ovaries.** Each chromatogram in Figure 1a represents one of three experiments with equal amounts of cytosolic protein from ovaries pooled from an experimental group of 10 mature or 20 immature rats. The subunits are eluted from the HPLC column in the same order as they are given in Figure 1b, which represents quantitation of the areas under the peaks in the HPLC chromatogram (open bars = immature rats, filled bars = mature rats).

subunits were analyzed by HPLC using a reversed-phase column and identified by comparing their retention times with those of isoenzymes purified from rat liver. Subunits 2, 3, 4, 6, 7 and 8 could be identified (Fig. 1 a and b). The chromatograms show that the mature rat ovary contains considerably more of all the glutathione transferase subunits than does the immature organ. The largest difference, five-fold, was observed with an unidentified peak eluting just after subunit 4.

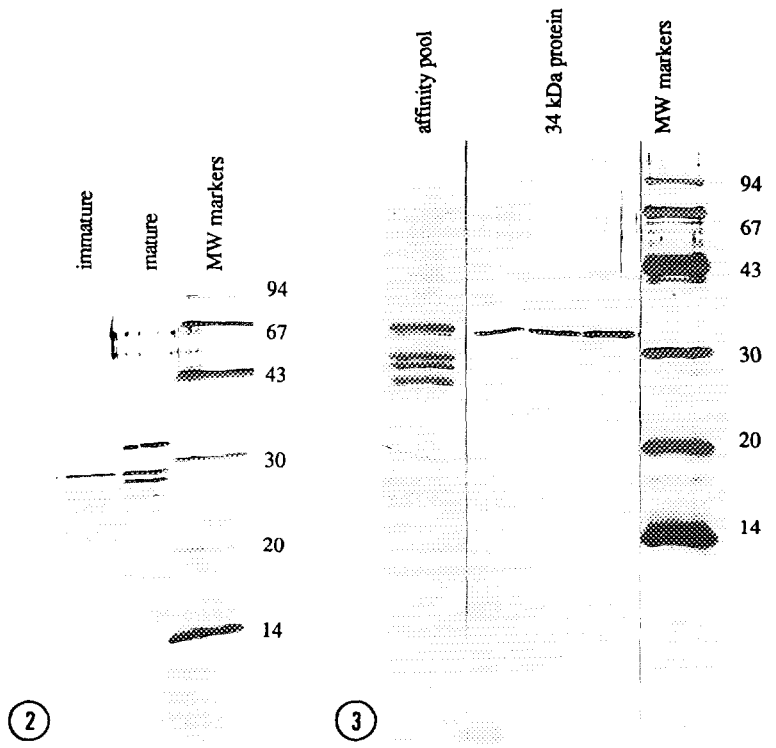

Figure 2. **SDS-PAGE and silver staining of ovarian cytosolic proteins purified on a glutathione-Sepharose affinity column.** The samples analyzed were obtained by applying equal amounts of cytosolic protein from ovaries pooled from an experimental group of 10 mature or 20 immature rats to the affinity column. Equal amounts of protein were applied to each slot.

Figure 3. **SDS-PAGE and silver staining of the 34 kDa protein purified by preparative electrophoresis.** Cytosolic protein from ovaries pooled from immature rats treated with PMSG was affinity purified on a glutathione-Sepharose column. The 34 kDa protein was purified from the affinity pool by continuous-elution electrophoresis using a Model 491 Prep Cell (Bio-Rad) with an acrylamide concentration of 12 % (w/v). The fractions were analyzed by SDS-PAGE (15 % w/v acrylamide) and silver staining. Three fractions from the preparative electrophoresis contained the purified 34 kDa protein.

Upon running the samples on SDS-PAGE, a band with an apparent molecular weight of 34 kDa was seen, in addition to the bands corresponding to subunits 2 at 28 kDa, 3 and 4 at 26.5 kDa and 7 at 24 kDa (Fig. 2). The difference in the intensity of the 34 kDa band between immature and mature rats was similar to the difference seen in the HPLC chromatogram. This 34 kDa protein could not be detected in male or female rat liver (not shown).

In order to further investigate the 34 kDa component, this protein was purified using Bio-Rads system for preparative SDS-PAGE, continuous-elution electrophoresis. SDS-PAGE and silver staining (Fig. 3) revealed that the unidentified protein was pure after this procedure. Subsequently, automated N-terminal amino acid sequencing was performed (Fig. 4). When the 34 amino acid-long N-terminus of the 34 kDa protein was compared with known amino sequences of cytosolic glutathione transferase subunits, no homology was found. Subsequently, this sequence was compared with other protein sequences by a FASTA search in the SWISS.

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                                  10                  20                  30
34 kDa protein      H I Q A E V T M K T N F F G T Q D V X K E L L P I I K P Q G R D V N H

human carbonyl      _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ R _ _ C T _ _ _ _ L _ _ _ _ _ _ V _ _ V
reductase                      110                 120                 130
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Figure 4. **N-terminal amino acid sequence of the 34 kDa protein compared to an internal sequence of human carbonyl reductase (EC 1.1.1.184).** The residues which differ are indicated.

PROT. database (031293). There was no homology found with any other N-terminal sequence, but we found an 85 % identity with a 34 amino acid-long sequence starting 105 amino acids from the N-terminus of human carbonyl reductase (EC 1.1.1.184)(6).

The 34 kDa protein was also purified in native form. To this end, the glutathione-Sepharose 4B column was eluted with a linear gradient formed by mixing washing buffer A and 10 mM Tris-Cl, pH 9.1, containing 7.5 mM of both S-hexylglutathione and glutathione. The 34 kDa protein was more tightly bound to the glutathione-affinity column than the other glutathione transferases, eluting after these (Fig. 5). The other glutathione transferases were also partly separated from one another by this procedure.

The enzyme activity of the isolated native 34 kDa protein was investigated. Glutathione conjugation with CDNB was catalyzed at a rate of 0.16 μmol/mg min. The 34 kDa protein was also found to significantly catalyze the reduction of menadione by NADPH (0.33 μmol/mg min), a catalytic activity normally described for carbonyl reductase.

The fact that this protein has the ability to conjugate glutathione with CDNB indicates that it is a new form of cytosolic glutathione transferase. Since it demonstrates no sequence homology

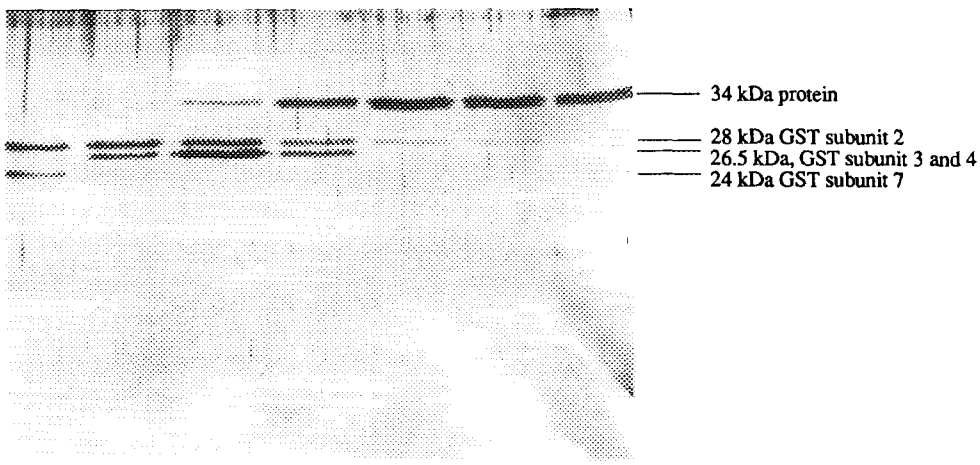

Figure 5. **SDS-PAGE and silver staining of the 34 kDa protein purified by gradient elution from an glutathione-Sepharose column.** Cytosolic protein from ovaries pooled from immature rats treated with PMSG was affinity purified on a glutathione-Sepharose column. The 34 kDa protein was eluted and separated from the other glutathione transferases with a linear gradient formed by mixing washing buffer A and 10 mM Tris-Cl, pH 9.1, containing 7.5 mM of both S-hexylglutathione and glutathione. Fractions 1-7 (left to right) were analyzed by SDS-PAGE (15 % w/v acrylamide) and silver staining.

with earlier described glutathione transferases, this protein does not seem to belong to any of the known classes of glutathione transferases (alpha, mu, pi, theta or microsomal). However, the determination of the complete amino acid sequence will provide the definitive answer to this question. The high degree of homology with a region in human carbonyl reductase indicates that these two enzymes contain a common domain. This may explain the ability of the 34 kDa protein to reduce menadione.

Further studies will focus on characterization of the entire amino acid sequence and of the catalytic activity of this protein; its relationship to human carbonyl reductase; and its hormonal regulation, both in terms of the underlying mechanism and physiological significance.

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