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Mouse-Liver Glutathione Reductase: Inactivation by NADPH of Two Allelic Variants

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Mouse-liver glutathione reductase has been purified to homogeneity from strain SWR/J by ammonium sulfate precipitation (40-80 %) and two additional steps of affinity chromatography in ATPR-Sepharose and 2′, 5′-ADP-Sepharose from which it was specifically eluted by using $NADP^+$ gradients. After 2032-fold purification the pure enzyme has a specific activity of 146 U/mg. The SWR/J protein is slightly more basic than the other allelic variant from strain DBA/2J, with PI 7.0 and 6.5 respectively. Both pure proteins are immunologically identical, either by immunodiffusion or by quantitative immunoprecipitation. They can however be distinguished by their rate of inactivation in the presence of NADPH, their reduced cofactor. The SWR/J protein is much more resistant to that inactivation ($t\frac{1}{2} = 14$ min) than the DBA/2J enzyme ($t\frac{1}{2} = 5$ min).

Glutathione (γ-glutamyl-cysteinil-glycine) is a tripeptide which in its reduced state participates, by means of its thiol group, in several reactions essential for the integrity of the cell (1). The enzyme glutathione reductase (EC 1.4.6.2) catalyzes the production of reduced glutathione (GSH) from its oxidized form (GSSG). The enzyme is a flavoprotein which utilizes NADPH as electron donor according to the reaction:

$$GSSG + NADPH + H^+ \rightleftharpoons 2\,GSH + NADP^+$$

Mouse-liver glutathione reductase has been recently purified to homogeneity by a fast and efficient procedure based on a new gel for use in affinity chromatography named ATPR-Sepharose (3). The pure protein has been studied in detail from a physicochemical, spectral and kinetic point of view. The pure enzyme oscillates between two states, active and inactive,

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according to the levels of its reduced cofactor (NADPH) and oxidized substrate (GSSG). Incubation with NADPH promotes a fast and complete inactivation of the enzyme in the absence of GSSG. Oxidized glutathione protects the enzyme from its inactivation by reduction and also promotes its fast reactivation (3). It has been suggested that the redox interconversion of glutathione reductase could play an important role in the *in vivo* regulation of this enzyme (3). Yeast glutathione reductase behaves in a similar way (6), and a similar process has been suggested for the rabbit enzyme (7).

In the mouse, mapping has located the gene coding for glutathione reductase in chromosome 8, and the gene shows two electrophoretic alleles (4). The enzyme was first purified from the mouse inbred strain DBA/2J, which has the GR-1A allele (3). This protein is more acidic than that encoded by the GR-1B allele present in other strains (4) and in particular the strain SWR/J. These two electrophoretic variants form an excellent model for investigating the biochemical differences among two very similar proteins, and could provide alternative procedures for the detection of genetic variants.

The present work shows the purification to homogeneity of the second allelic variant of mouse-liver glutathione reductase from strain SWR/J. The two pure protein variants have been compared using electrophoretic, immunological and biochemical criteria in order to detect structural and functional differences between them.

Materials and Methods

The origin of the different reagents used has been described previously (3). SWR/J male mice, eight weeks old, were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA). The animals were sacrificed by cervical dislocation and the livers removed and frozen at —70° C for use later in enzyme purification.

Enzyme assays, protein determination, polycrilamide gel electrophoresis and glutathione reductase purification were performed as in the procedures previously described (3). One unit of enzyme activity is defined as the amount of enzyme which reduces 1 μmole of oxidized glutathione per minute under the conditions of the standard assay (3).

Isoelectric focusing was carried out with either crude liver extracts or pure glutathione reductase from strains DBA/2J and SWR/J. A preparative column LKB 8101 (110 ml) with 2% ampholine pH 5-8 was used. A constant voltage of 800 V was maintained for 64 hours. The column was then fractionated in 1 ml samples for enzyme assays and pH measurements.

Antibodies against pure DBA/2J glutathione reductase were prepared in rabbits by the method previously described (3). The method of OUCHTERLONY (5) was used to perform gel immunoprecipitation in 0.046 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 0.01% sodium azide and 1% agar noble. Liquid immunoprecipitation was carried out by diluting 1 μg of each pure protein to a final volume of 0.12 ml, with control serum and increasing amounts of immune serum. The mixtures were incubated at 37° C for 1 hour and kept overnight at 4° C. They were then centrifuged for 4 min at $3.000 \times g$ and the supernatants assayed for residual enzyme activity.

Inactivation of glutathione reductase was studied as previously described (3), incubating each pure protein at 37° C aerobically, in 0.05 M Tris-HCl buffer, pH 8.0. NADPH was added at a final concentration of 0.3 mM.

Results

Purification of glutathione reductase from SWR/J mice. The enzyme has been

Table I. *Purification of mouse-liver glutathione reductase from strain SWR/J.*

Purification step	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification -fold	Yield (%)
Crude extract	1549	21601	0.072	1	100
40-80 % $(NH_4)_2SO_4$	1774	9020	0.197	2.7	115
ATPR-Sepharose	1252	93	13.462	187	85
2′,5′-ADP-Sepharose	629	4.3	146.279	2032	41

purified to homogeneity using ammonium sulfate fractionation between 40 and 80% saturation, and affinity chromatography. Two different gels were used, ATPR-Sepharose [8-(6-aminohexyl)-amino-2′-phosphoadenosine diphosphoribose], and 2′,5′-ADP-Sepharose [N^6-(6-aminohexyl) adenosine 2′,5′-biphosphate]. Specific elution was carried out using 0-0.5 mM and 0-10 mM $NADP^+$ gradients respectively. After this last step the enzyme was pure, as was shown by electrophoresis in both denaturing and non-denaturing acrylamide gels. Table I summarizes the purification procedure. Starting with 230 g of liver, 4 mg of pure glutathione reductase were obtained in only 5 days after a purification of two thousand fold with a 41% recovery. The maximum specific activity obtained was 146 units of enzyme per mg of protein.

Isoelectric focusing. The first description of mouse glutathione reductase polymorphism was based on starch gel electrophoresis (4). In order to obtain quantitative information about the electrophoretic behaviour, isoelectric focusing was performed with both allelic variants. When crude extracts of DBA/2J and SWR/J livers were mixed in a 1:3 ratio (fig. 1A) three glutathione reductase peaks were detected. The SWR/J protein is more abundant in the mixture, so the higher peak with the more basic PI (7.0) must correspond to it. The more acidic peak (PI = 6.5) corresponds to the DBA/2J strain as has been previously described (3). The third peak had intermediate hight

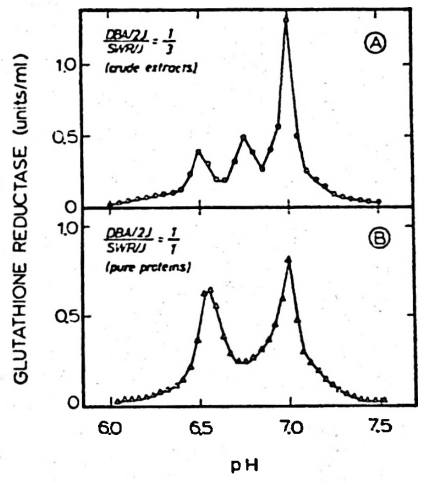

Fig. 1. *Isoelectric focusing of SWR/J and DBA/2J glutathione reductases.* *A:* an SWR/J crude liver extract containing 8 units of enzyme (110 mg of protein in 1 ml) was mixed with a DBA/2J extract containing 2.6 units of glutathione reductase (33 mg of protein in 0.3 ml). *B:* 30 μg of pure SWR/J enzyme (4.5 units) were mixed with 28 μg of DBA/2J pure protein (4.5 units).

and PI. When the two pure glutathione reductases were mixed in a 1:1 ratio (fig. 1B) only two peaks of enzymatic activity could be detected with nearly equal size, one around pH 6.5 and the second at pH 7.0.

Immunological studies. The process of immunization of a rabbit against pure DBA/2J glutathione reductase is shown in figure 2A. The antibody titre was studied in control serum and in sera obtained

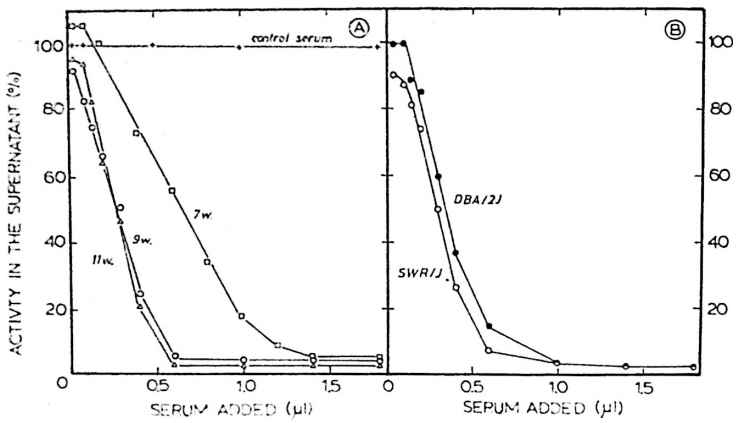

Fig. 2. *Liquid immunoprecipitation of the two allelic enzymes by rabbit anti-DBA/2J antibodies.*

A: antibody titre of control serum (+——+) and sera obtained after 7 (□——□), 9 (○——○) and 11 (△——△) weeks of immunization. *B:* Quantitative immunoprecipitation of 1 µg of SWR/J (○——○) or DBA/2J (●——●) pure proteins by 11 weeks antibodies.

after 7, 9 and 11 weeks of immunization. 1.15 µl of the first immune serum precipitated 1 µg of pure DBA/2J enzyme. In contrast only 0.56 µl of the second and 0.49 of the third immune sera were required to produce a similar immunoprecipitation.

The antibodies produced against the enzyme from DBA/2J mice were tested against both pure allelic variants by immunodiffusion (fig. 3). A continuous immunoprecipitation line, lacking any spurs, could be observed from the DBA/2J well to the SWR/J well, thus suggesting the identity of the antigenic determinants on both proteins. The immunological identity of both allelic variants was further confirmed by liquid immunoprecipitation (figure 2B), in which 1 µg of both pure proteins was allowed to interact with increasing amounts of the same immune serum. The anti-DBA/2J antibodies precipitate with the same, or even slightly better efficiency, the SWR/J enzyme.

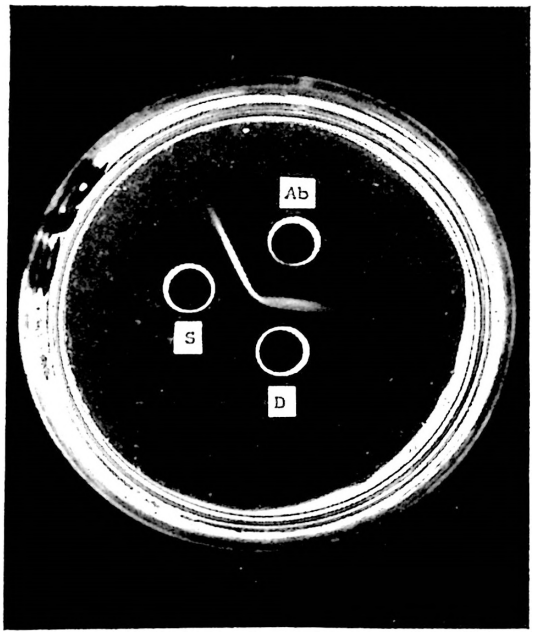

Fig. 3. *Gel immunoprecipitation of SWR/J and DBA/2J glutathione reductase by anti-DBA/2J rabbit antibodies.*

Ab: antibodies; *S:* 5 µg of pure SWR/J protein; *D:* 5 µg of pure DBA/2J protein.

Inactivation by NADPH. The effect of the reduced cofactor NADPH on the

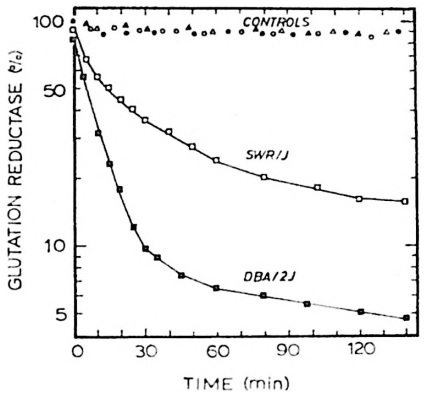

Fig. 4. *Differential inactivation by NADPH of the two allelic glutathione reductases.* Ten μg of each pure protein were incubated at 37° C without additions (△—△, ▲—▲), in the presence of $NADP^+$ (○—○, ●—●) or NADPH (□—□, ■—■) at a concentration 0.3 mM. Open symbols, SWR/J enzyme; solid symbols, DBA/2J enzyme.

activity of both glutathione reductases is shown in figure 4. In the absence of NADPH or in the presence of $NADP^+$, both enzymes maintain the initial activity throughout the incubation. In contrast, in the presence of NADPH both enzymes undergo an inactivation. The SWR/J enzyme is, however, much more resistant to this inactivation by reduction ($t_{1/2} = 14$ min) than the DBA/J enzyme which shows a half life of just 5 min under the same conditions.

Discussion

The present paper shows that the purification procedure devised for the glutathione reductase of DBA/2J mice can be successfully applied to the purification of the other allelic variant of this enzyme from SWR/J mice. In contrast with the 66 % yield published for the purification of the DBA/2J protein, only 41 % was obtained with the SWR/J protein. This difference might be due to the fact that the purification procedure had been optimized specifically for the DBA/2J protein, and the second allelic variant could have a slightly different behaviour.

The similarity between the maximum specific activity of pure SWR/J enzyme, 146 U/mg, and the DBA/2J enzyme, 156 U/mg, corroborates the existence of a close relationship between both proteins. The purification-fold is only 2032 for the SWR/J protein, in contrast with the almost five thousand times necessary to obtain a pure DBA/2J enzyme (3). This can be attributed to the higher initial specific activity of the first protein, probably due to a much more complete saturation with FAD, its prosthetic group (2, 3).

Isoelectric focusing experiments with crude extracts and pure enzymes confirm the existence of two protein species, one with PI = 6.5 produced by strain DBA/2J (GR-1A allele), and the other with PI = 7.0 produced by strain SWR/J (GR-1B allele). The third peak, with intermediate PI, appearing after isoelectric focusing of crude extracts could be due to the formation of hybrid dimers (3) with one subunit coming from each strain.

No differences were detected in the immunological behaviour of both DBA/2J and SWR/J glutathione reductases, either by immunodiffusion or by quantitative immunoprecipitation, thus indicating a complete identity of antigenic determinants. This is in agreement with the rather small PI differences between the two allelic proteins, and in strong contrast to the total lack of cross-reactivity between the mouse and yeast glutathione reductases (3).

Important differences between the two allelic glutathione reductases were observed in their sensitivity to inactivation under reducing conditions. A decreased affinity of the SWR/J enzyme towards NADPH could explain that behaviour, although further experiments are needed to clarify the molecular basis of that differential inactivation. We are now planning to obtain, by mutagenesis, molecules of gluta-

thione reductase with different sensitivities to inactivation by reduction, although totally functional from all other points of view.

Resumen

La enzima glutatión reductasa de hígado de ratón ha sido purificada hasta homogeneidad a partir de la estirpe SWR/J mediante su precipitación fraccionada con sulfato amónico (40-80 %) y dos pasos adicionales de cromatografía de afinidad en geles de ATPR-Sefarosa y 2',5'-ADP-Sefarosa de los que fue eluida específicamente mediante sendos gradientes de $NADP^+$. Tras una purificación de 2.032 veces la enzima pura muestra una actividad específica de 146 μ/mg. La proteína de la estirpe SWR/J es ligeramente más básica que la procedente de la otra variante alélica DBA/2J, con PI 7,0 y 6,5, respectivamente. Ambas proteínas puras son inmunológicamente idénticas, sea en inmunodifusión o en inmunoprecipitación cuantitativa. Se distinguen, sin embargo, por su diferente velocidad de inactivación con NADPH, su cofactor reducido. La proteína de SWR/J es mucho más resistente a tal inactivación ($t_{1/2}$ = 14 min) que la enzima procedente de la estirpe DBA/2J ($t_{1/2}$ = 5 min).

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