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Separation of the Isoenzymes of Glyoxalase I from Human Red Blood Cells by Electrophoresis and Isoelectric Focusing on Polyacrylamide Gel and by Ion Exchange Chromatography

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Methods have been devised for the separation of the isoenzymes of glyoxalase I(*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) from human red blood cells by electrophoresis and electrofocusing on polyacrylamide gel slabs. Three different staining methods were used for the location of the enzyme. Three electrophoretic phenotypes of the enzyme were resolved, the fast and slow types with one band and the intermediate type with three glyoxalase I activity bands. In gel electrofocusing (pH gradient 3.5–9.5) two glyoxalase I activity bands were found for all electrophoretic types. In electrofocusing on gel with a narrow pH gradient, at least four separate enzyme components were resolved for the fast and slow electrophoretic types and at least six components for the intermediate type. The phenotypes could be distinguished correspondingly to the electrophoretic results. Preparative separation of the isoenzymes was achieved by ion exchange chromatography on DEAE-Sephacel but gel chromatography on Sephadex G-100 gave the same elution volume for all enzyme phenotypes. This corresponds to an apparent molecular weight of about 47 000.

Glyoxalase I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5) and glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6) form the glyoxalase activity ubiquitously present in living cells.[1] Both glyoxalase I[2–5] and glyoxalase II[6] have been obtained in purified form from animal sources. The occurrence of genetically determined polymorphism of glyoxalase I in human red blood cells was first found by Kömpf *et al.* by starch electrophoresis.[7] Considerable interest in the isoenzymes of glyoxalase I has arisen from the finding that the gene for glyoxalase I is located on chromosome 6 in man in close linkage to the major histocompatibility complex[8,9] and that the enzyme is useful in population genetics and paternity studies.

We have devised methods for the separation of glyoxalase I isoenzymes by polyacrylamide gel electrophoresis and supplemented these experiments with investigations by isoelectric focusing on polyacrylamide gel and ion exchange column chromatography. Three different staining methods, based on the disappearance of the reaction of glutathione at sites of the enzyme with 2-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), iodine and phthalaldehyde, respectively, have been used. The different staining and separation techniques have been compared. A preliminary report of the work has appeared earlier.[10]

MATERIALS AND METHODS

Chemicals. Acrylamide (specially purified) was obtained from BDH, *N*,*N*′-methylenebisacrylamide and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine from Eastman and ammonium peroxodisulfate from E. Merck. DEAE-Sephacel was a product of Pharmacia. Ampholytes for isoelectric focusing (Ampholine, pH ranges 3.5–10, 3–5, 4–6 and 5–8) and thin-layer focusing gels (Ampholine PAG plates No. 1804-101, pH 3.5–9.5) were purchased from LKB. MTT, 2,6-dichlorophenolindophenol and phthalaldehyde were obtained from Sigma.

Preparation of hemolysates. The blood samples were obtained in 0.31 % (w/v) sodium citrate. Distilled water (1.5 vol) was added to the washed

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red cells and mixing continued for 45 min at 4 °C. The preparation was centrifuged at 23 000 *g* for 20 min. The particle-free supernatant (hemolysate) was used in part of the studies. Because in some of our electrophoretic systems hemoglobin interfered in the visualization of glyoxalase I in gels, a further treatment was done for the samples.[5] Similar volumes of the hemolysate and of a mixture of chloroform, ethanol and water (5:4:6, v/v/v) were combined and the mixture slowly stirred for 45 min at 4 °C. The solution was then centrifuged at 23 000 *g* for 20 min. The supernatant was virtually free from hemoglobin but contained most of the original glyoxalase I activity. The preparations were either used immediately or stored at −70 °C where they could be kept for at least two weeks with unchanged results.

Electrophoretic methods. Method A. Polyacrylamide slab gels (139 × 82 × 2.7 mm) were prepared with the gel slab casting apparatus of Pharmacia (GSC-8) according to the gel system 1 of Maurer[11] for the small-pore gel. The electrophoretic run was performed at 10 °C with the Pharmacia gel electrophoresis apparatus (GE-4) at a current of 30 mA/gel. The samples (130 µl) were the hemoglobin-free supernatants containing 20 % glycerol (v/v) and 0.05 % bromophenol blue (w/v). The bromophenol blue front was run to the lower edge of the gels which took approximately 3 h.

Method B. Polyacrylamide slab gels (113 × 248 × 1.5 mm) for the Multiphor 2117 apparatus (LKB) were prepared by the technique described by the manufacturer (see LKB application note No. 306). The gel mixture contained 22.2 ml acrylamide solution (containing 22.2 g acrylamide and 0.6 g *N,N'*-methylenebisacrylamide per 100 ml), 22.0 ml citrate−phosphate buffer pH 7.3 (2.94 g citric acid.H_2O and 30.6 g $Na_2HPO_4.2H_2O$/l), 18.5 ml 1 % (w/v) soluble starch (Merck), 0.03 ml *N,N,N',N'*-tetramethylethylenediamine and 3.2 ml ammonium peroxodisulfate (10 mg/ml). The gel was allowed to polymerize overnight. The samples were pipetted into 10 µl slots made during polymerization by a slot former. The sample contained 10 parts hemolysate, 2 parts glycerol and one part 0.25 % bromophenol blue. The electrode buffer was a 1:3 dilution of citrate−phosphate pH 6.4 (6.51 g citric acid.H_2O and 24.4 g $NaHPO_4.2H_2O$/l). The gel was cooled by circulating tap water and was run at 150 mA, 150 V for 8 to 9 h. The bromophenol blue front moved under these conditions to the anodal edge of the gel in 5 to 6 h.

Isoelectric focusing on gels. Some electrofocusing experiments were done on slab gels (pH 3.5−10) commercially available from LKB. For other experiments gels with a narrow pH gradient were prepared. The gel mixture contained 10 ml 29.1 % (w/v) acrylamide, 10 ml 0.9 % (w/v) *N,N'*-methylenebisacrylamide, 23 ml of a solution of 7.5 g sucrose in water, 14 ml 1 % (w/v) soluble starch, 0.4 ml Ampholine pH 3−5, 2.4 ml Ampholine pH 4−6, 0.2 ml Ampholine pH 5−8 and 0.6 ml 0.004 % riboflavin. Photopolymerization of the gel took 4 to 5 h at room temperature. Anode and cathode solutions were 0.2 % H_3PO_4 and 0.4 % ethylenediamine, respectively. The samples (up to 24 in one gel) were applied by filter papers near the cathode. Focusing was done with the Multiphor 2117 apparatus for 4 h with the following maximum settings on the power supply: power 25 W, voltage 900 V and current 50 mA. In some experiments the starch solution was replaced by water or the riboflavin solution was replaced by ammonium peroxodisulfate (1.6 ml of a solution containing 10 mg ammonium peroxodisulfate per ml in the above gel mixture).

Localization of glyoxalase I on gels. Method Ia. This was used for alkaline gels (Method A). The slab gels were preincubated three times for 5 min in a large volume of 0.2 M phosphate buffer pH 6.8 and then kept in darkness at 37 °C in the following mixture: 80 mM imidazole-HCl buffer pH 6.8, 10 mM $MgSO_4$, 70 mM methylglyoxal, 10 mM GSH, NaOH to bring the pH to 6.8 (about 20 mM) and 0.4 mg/ml MTT. During about 2 h the gel turned a blue colour at the sites not containing enzyme. The principle was first described by Kömpf *et al.*[7]

Method Ib. The gel was treated with a solution similar to that of method Ia but without MTT. The solution was applied on and below the gel with Whatman 17C-papers of the size of the gel. After incubation (20 min at 37 °C) one of the papers was removed. A second solution was prepared just before use by combining 14 ml of a solution of MTT (1 mg/ml) in 0.1 M Tris, 0.5 ml 2,6-dichlorophenolindophenol (2 mg/ml in H_2O) and 240 mg agar (Bacto-Agar, Difco) in 16 ml 0.1 M Tris. The agar solution was at 50 °C. The combined solution was rapidly poured on the gel and the result inspected after 30 min.

Method II. The gel was treated with the same first solution as in method Ib. Then an iodine solution was applied on the gel.[12] The stock iodine solution contained 10 mg I_2 and 40 mg KI per ml; this was diluted 1:12 just before use. Positive blue bands appeared at the sites of the enzyme almost immediately. The bands were unstable but could be brought back several times by reapplications of the iodine solution.

Method III. The gel was treated with the first solution as in method Ib. Then the following two solutions were combined and the mixture immediately applied on the gel: Ten milligrams of phthalaldehyde in 2 ml methanol and 240 mg agar in 28 ml of 50 mM potassium phosphate buffer

pH 8.0 (kept at 50 °C). After gelling of the staining solution the result was inspected under long-wave ultraviolet light. The sites of the enzyme were seen as non-fluorescent bands against the fluorescent background.

Electrofocusing gels. The gels were preincubated three times for 5 min in 0.1 M phosphate buffer pH 8.0 to increase the pH of the area where the enzyme is located (see Results). Staining was then performed as for the electrophoresis gels.

Treatment with neuraminidase. Hemolysates were incubated at 4 °C for 24 h in the following mixture: 20 mM sodium phosphate buffer, pH 6.8, 1 mM Ca^{2+} and 50 units/ml neuraminidase (Calbiochem, (Calbiochem, from *Vibrio cholerae*, EC 3.2.1.18). Controls without neutraminidase were included. The samples were then studied by electrophoresis and electrofocusing by the usual procedures.

Assay of glyoxalase I activity. This was done as described previously.[2]

RESULTS

Electrophoretic separation of glyoxalase I isoenzymes. Polyacrylamide gel electrophoresis resolved three different phenotypes of glyoxalase I in human red cells, the fast and slow types which gave one enzyme band and the intermediate type which gave three bands (Fig. 1). This result is in accord with the original report[7] obtained by starch electrophoresis. With the alkaline gel system (Method A) only the staining method Ia was successful. A preincubation was required to lower the pH of the gel and thus lower the rate of the base-catalyzed nonenzymic staining of the gel at sites containing GSH. Optimal resolution was, however, achieved by the alkaline system with suitable preincubation (Fig. 1). For the neutral electrophoresis system (Method B) staining methods Ib, II and III were useable.

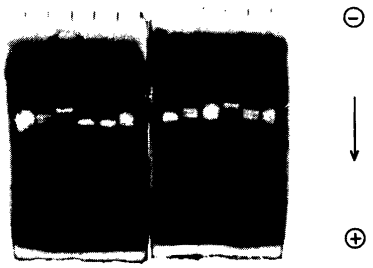

Fig. 1. Electrophoresis of human red cell glyoxalase I on polyacrylamide gel slabs (Method A). Phenotypes (from left to right): Intermediate, intermediate, slow, fast, fast, intermediate, fast, intermediate, intermediate, slow, intermediate, intermediate. Staining by the MTT method (Ia).

Gel electrofocusing. When hemolysates were electrofocused on slab gels with a broad pH gradient purchased from LKB (pH 3.5–9.5) or made in the laboratory (pH 3.5–10) two glyoxalase I activity bands were constantly found for every sample tested. Both bands given by samples from persons with the fast electrophoretic type were located slightly more anodically than those from persons with the slow electrophoretic enzyme type. The bands from persons with the intermediate type were slightly broader than those of the others. These differences were not, however, sufficient for reliable identification of the types because small inhomogeneities in the pH gradient of the gel also produced differences of this degree.

In the narrow pH gradient system (see Methods) the three phenotypes were reliably distinguished from each other but the number of enzyme components resolved was increased. The more anodic activity band found by the broad pH gradient

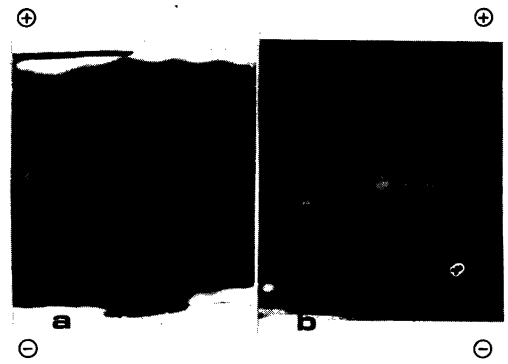

Fig. 2. (a). Isoelectric focusing of human red cell glyoxalase I on polyacrylamide gel slab in a narrow pH gradient (see Methods). The hemolysates from left to right had the following electrophoretic phenotypes: Fast, intermediate, fast, intermediate, fast, slow, intermediate, slow, fast. (b). A similar experiment but the photograph was taken two days after staining. Electrophoretic phenotypes (from left to right): Fast, slow, intermediate, slow, fast, intermediate, fast, intermediate, fast. Staining by method Ib.

method was by this method resolved into two separate components for all samples (Fig. 2). The bands of samples with the fast electrophoretic phenotype were somewhat more acidic (pI about 4.40 and 4.45) than those given by the slow electrophoretic types (pI about 4.47 and 4.52) and the intermediate types gave bands between these. The less anodic activity band found in the broad pH gradient method was by this technique resolved into at least two components for the homozygous electrophoretic types and to several (at least four components for the heterozygous type (Fig. 2). Fig. 2b in which some fading of the bands had occurred between staining and photographing shows that only one major (less anodic) band was found for the homozygous types and three major bands for the heterozygous type. These bands had clearly separate locations for the fast electrophoretic type (pI about 4.90) and the slow type (pI about 5.05).

Some interference was caused by an apparent reaction between methylglyoxal and ampholytes around pH 5.0 producing a dark continuous band. In freshly stained gels this band was seen between the main components given by the fast and slow electrophoretic types (Fig. 2a). The band faded away within 2 days after staining. Fig. 2b shows the gel, stained by the MTT method, after this period. Some fading of the enzyme bands, especially the minor ones, also occurred but the main bands were seen more clearly.

To find out whether or not the two separate glyoxalase I forms, found for all electrophoretic phenotypes even in the broad pH gradient system, are inconvertible during the focusing procedure, a refocusing experiment was conducted for each of the enzyme froms separated by the first focusing after cutting the corresponding areas out from the first slab. Both forms gave only one band at the original pI. This showed that the two forms are separate rather than arising in the focusing.

When the electrofocusing was performed on gels in which starch in the gel solution had been replaced by water the focusing result did not change in any way. Also replacing of riboflavin with ammonium peroxodisulfate in the gel polymerization mixture produced no difference in the results. Attempts were made to influence the number of glyoxalase activity bands found by pretreatment of the hemolysates with thiols. However, neither reduced glutathione (up to 130 mM) nor dithiothreitol (50 mM) (30 min incubation with the thiol before the focusing) affected the focusing result in either broad or narrow pH gradient. Neither had pretreatment of the hemolysates with neuraminidase any effect.

Ion exchange chromatography. When a hemolysate containing homozygous (fast or slow) electrophoretic glyoxalase I phenotype was chromatographed on a DEAE-Sephacel column, a single narrow enzyme activity peak was obtained. When the same volumes of hemolysates with fast and slow electrophoretic phenotypes were mixed and chromatographed on the DEAE-Sephacel column, two activity peaks far from each other were found in the eluate. The peaks were pooled separately, concentrated by ultrafiltration and dialysed. Electrophoresis showed that the first and second peak of the column had the same mobilities as the slow and fast hemolysate types, respectively. These fractions also gave typical results for the slow and the fast phenotypes in isoelectric focusing. Chromatography of a hemolysate with intermediate glyoxalase I phenotype on DEAE-Sephacel yielded three separate peaks of activity (Fig. 3). The peaks A, B and C, as marked in Fig. 3, were shown to represent, respectively, the slow, the intermediate (only the

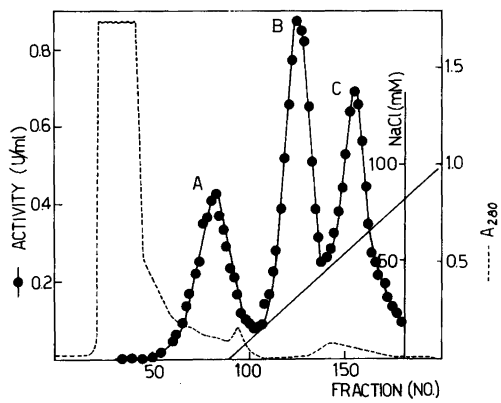

Fig. 3. Separation of the isoenzymes of glyoxalase I by ion exchange chromatography. A hemolysate containing the intermediate glyoxalase I phenotype was applied to a DEAE-Sephacel column (2.0 × 32 cm, load 35 ml, hemolysate prepared by adding 5 vol water to washed red cells instead of the usual 1.5 vol) equilibrated with 50 mM potassium phosphate pH 7.0. After washing of the column with equilibration buffer a linear NaCl gradient (2 × 250 ml, from 0 to 0.15 M NaCl) was used for elution. Fraction volume was 3 ml.

middle band) and the fast electrophoretic form of the enzyme.

Gel chromatography on Sephadex G-100. Hemolysates with all three electrophoretic phenotypes gave a single symmetrical peak with the same elution volume. From the elution volumes of the five standard proteins run in the same column a linear curve was obtained in a log M_r *versus* V_e/V_o plot.[13] From this plot the apparent molecular weight of human red cell glyoxalase I is 46 800.

DISCUSSION

Purified glyoxalase I from sheep liver[2] and pig erythrocytes[5] have been reported to be dimers with the apparent molecular weights of 45 900 and 48 000, respectively. The human red cell glyoxalase I isoenzymes studied in the present work all had the same molecular weight which corresponds to that found from the other animal sources. The finding of the three electrophoretic phenotypes as shown in Fig. 1 is compatible with the dimeric structure for the enzyme. During our purification studies of glyoxalase I from liver[2] a tendency of the enzyme to form artificial electrophoretic heterogeneity has been noted. This heterogeneity is reversed by treatment with thiols. However, thiol treatment had no effect on the electrophoretic results given by fresh human hemolysates. The electrophoretic results of this study were highly reproducible and Methods A and B always gave agreeing phenotype for the same sample. Kömpf *et al.*[7] also showed that the glyoxalase I phenotype is hereditary.

We have developed suitable electrophoretic systems for the separation of the isoenzymes of glyoxalase I on polyacrylamide gel because we obtained much better resolution on polyacrylamide than on starch used previously.[7] We have also made successful typings on agarose[14] with the modification that in addition to agarose (1 %) soluble starch (0.4 %) was incorporated in the gel and staining done by the iodine method (essentially method II). Agarose gels have, however, the disadvantages of fragility and low capacity. Of the staining methods described, the iodine method and the phthalaldehyde method have been the most convenient in our hands. The latter staining where fluorescence is measured has a high potential sensitivity. These methods have been used to find out the frequencies of the alleles determining fast and slow glyoxalase I phenotypes in a Finnish population.[15]

The electrophoretic study of glyoxalase I isoenzymes was in this work supplemented with electrofocusing experiments and ion exchange chromatography. A narrow pH gradient system was required in the gel electrofocusing to distinguish clearly the three phenotypes of glyoxalase I. Two groups of enzyme bands were resolved for each phenotype. The less anodic enzyme bands (which represented the main components) gave a pI difference of about 0.15 pH unit for the slow and fast electrophoretic phenotypes. For the more anodic enzyme bands of the slow and fast electrophoretic phenotypes the pI difference was 0.05 to 0.1 pH unit. The less anodic band was resolved in the narrow pH gradient system into at least one major and one minor component for the homozygous types and to three major and a number of minor components for the heterozygous type. In addition the more anodic band was in the narrow gradient system resolved into two components for all types. These many bands may represent true heterogeneity or may be formed artificially (*e.g.* as a result of enzyme-ampholyte complexes[16]). Neither riboflavin nor ammonium peroxodisulfate is a prerequisite for the multiplicity of the bands because gels in which one of them was lacking gave similar results. Refocusing of the enzyme forms after first focusing and cutting the separated enzyme bands, suggested that the two components which are resolved also by the broad pH gradient system are separate forms of the enzyme. Aronsson and Mannervik have reported[5] that glyoxalase I from pig erythrocytes gave two activity peaks on column electrofocusing. According to these authors the more acidic glyoxalase I component represented a complex of glyoxalase I with glutathione, as evidenced by its disappearance by the incubation of the enzyme with high concentration of reduced glutathione (resulting in the formation of free enzyme and oxidized glutathione).[5] We have been unable to reduce the number of glyoxalase I bands found for human hemolysates in gel electrofocusing by preincubation of the samples in high concentrations of reduced glutathione or dithiothreitol. The possibility that these agents cannot influence effectively in the crude hemolysate cannot be excluded but seems remote.

Glyoxalase I isoenzymes can also be separated in preparative scale by ion exchange chromatography. With suitable care this succeeds directly from the hemolysate as shown in Fig. 3. After the completion of this manuscript a report on the purification of the

three glyoxalase I isoenzymes from pooled human red blood cells has appeared.[17]

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