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CHARACTERIZATION OF HUMAN GLUCOSE-6-PHOSPHATE
ISOMERASE OF DIFFERENT SIZES

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Glucose phosphate isomerase (GPI) was purified from human placenta utilizing cross-linked spherical particle phosphocellulose. In three steps, GPI could be purified approximately 5500 fold with greater than 50% recovery. The purified enzyme exhibited four bands upon non-denaturing PAGE and isoelectric focusing (IEF) when stained with GPI specific activity stain. The four isozymes were isolated by preparative IEF. The isoelectric points of the isozymes were determined. Sodium dodecyl sulfate (SDS) gel electrophoresis showed two types of subunits with different molecular weights. Structural analyses showed both types of subunits had blocked amino termini. Other properties of the isozymes and subunits, including immunological reactivity, pH stability, peptide mapping and amino acid composition, were also established.

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LIST OF ABBREVIATIONS

<u>Abbreviations</u>	<u>Full Name</u>
BME	2-mercaptoethanol
EDTA(H)	Ethylenediamine-tetraacetic acid, acid form
GPI	Glucose 6-phosphate isomerase
IEF	Isoelectric focusing
MTT	Dimethylthiazolyl diphenyl tetrazolium
NADP	β -nicotinamide adenine dinucleotide 2'-phosphate
PAGE	Polyacrylamide gel electrophoresis
PMS	Phenazine methosulfate
SDS	Sodium dodecyl sulfate
BTP	Bis tris propane
TEA	Triethanolamine
TRIS	Tris(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

Occurrence and Metabolic Function of Glucose 6-Phosphate Isomerase

Glucose 6-Phosphate Isomerase (GPI, [EC 5.3.1.9]), also called phosphoglucose isomerase (PGI) or phosphohexose isomerase (PHI), plays a central role in the metabolism of phosphorylated sugars. GPI is an aldose-ketose isomerase, that catalyses the reversible interconversion of *D*-glucose-6-phosphate and *D*-fructose-6-phosphate by promoting the intramolecular transfer of a proton between C-1 and C-2.

The isomerases have been widely studied because of their extremely high rates of catalysis, stereospecificity, and clinical diagnostic value. The catalytic mechanism of isomerization has been studied extensively (Achari, 1981). GPI has been found in all living organisms, and is ubiquitous in all tissues. The isomerase has been isolated from a wide variety of sources. It is present in large excess compared to the rates of metabolic flux and has long been considered to be a bifunctional enzyme of glycolysis and gluconeogenesis. Some genetic variants of tissue-specific or

time-specific temporal genes have also been found that provide information on the genetic switches that occur during development (Duboule and Burki, 1985, Mo et al., 1975, Peterson et al., 1978, West et al., 1987). In bony fishes, two independent structural genes coding for two distinct homodimers (GPI-a and GPI-b) and a hybrid heterodimer have been found (Avisé and Selander, 1972, Avisé and Kitto, 1973, Dando, 1974). In *Astyanax mexicanus*, GPI-b was found to predominate in muscle whereas the GPI-a predominated in the liver and other organs (Avisé and Selander, 1972). In catfish, the distinctly different amino acid compositions, stability, and peptide maps of the isozymes verified that they are the result of two independent genes (Mo et al., 1975).

Time-specific temporal genes have been found in mice. Three electrophoretic variants (GPI-A, GPI-B, and GPI-C), coded by these alleles, have been characterized in mice (De Lorenzo and Ruddle, 1969, Padua et al., 1978, Duboule and Burki, 1985, Gilbert and Solter, 1985). Studies in mouse embryos have shown that GPI-A and B in fertilized embryonic cells were found at the beginning and then were gradually replaced by GPI-C which was first detected on days 3 to 4 (at the morula stage) (Duboule and Burki, 1985).

Basic genetic studies have revealed the chromosomal location of the GPI structural gene in several species. The locus of the human gene was assigned to chromosome 19

(McMorris *et al.*, 1973, Ruddle, 1973, Mckusick and Ruddle, 1977). In mice, the GPI gene is located on chromosome 7 (Hutton and Roderick, 1970, Carter and Parr, 1967, De Lorenzo and Ruddle, 1969). Several other loci of different species have also been assigned (Hutton and Roderick, 1970, Hart, 1979, Maitra and Lobo, 1977). Three different gene loci in tetraploid fish were observed (Ferris and Whitt, 1980, Schmidtke *et al.*, 1975) and attributed to the reduplication of at least one of the original pairs of GPI gene loci.

Multiple Electrophoretic Forms of the Enzyme

As reviewed by Gracy (1982), multiple electrophoretic forms of GPI have been reported in many species. For example, GPI is present in multiple forms in pig muscle (Achari *et al.*, 1981) and bovine tissues (Cini *et al.*, 1988), and the relative distribution of the isozymes varies depending upon the animal species from which the enzyme is isolated (Gracy, 1982, Mo *et al.*, 1975). The molecular basis of the isozymes of GPI has been the subject of many investigations. Multiple forms of GPI can arise from oxidation (Lu and Gracy, 1981, Blackburn *et al.*, 1972, Noltmann, 1964), proteolysis (Baumann and Brand, 1988), deamidation of specific Asn-Gly regions (Cini *et al.*, 1988), as well as from genetic variability (Gibson *et al.*, 1977,

Yuan *et al.*, 1979, Detter *et al.*, 1968). The multiple forms can also be generated by modification of the enzyme during isolation (Lowe and Reithel, 1975). These "artifactual" forms can often be prevented by very mild isolation procedures or by the presence of protecting agents.

Allelic variation of the GPI gene locus has been found very commonly in man and other animals (Gracy, 1982). The GPI allozymes of *Trypanosoma cruzi* (*T. cruzi*) have multiple electrophoretic forms and high activity in tissue extracts, and hence have been consistently used as a genetic marker of *T. cruzi* subgenus (Widmer *et al.*, 1986, Miles, 1983). The stability of *T. cruzi* GPI electrophoretic profiles under different isolation, culture and electrophoretic conditions or in different life cycle stages are well documented (Widmer *et al.*, 1986, Miles, 1983, Miles *et al.*, 1980, 1984). The wealth of data on the geographical distribution of GPI isozyme profiles contrasts with the lack of information on its physical and biochemical properties (Widmer *et al.*, 1986).

An inherited variation of the enzyme in humans was first reported by Detter *et al.* (1968), who described eight different electrophoretic phenotypes in addition to the common phenotype. With the development of rapid screening methods, many genetic variants of the enzyme have been discovered (Gibson *et al.*, 1977, Blume and Beutler, 1972, Smith and Lee, 1974). The frequency of human GPI

electrophoretic variants has been studied in a variety of populations. The results indicate that all the variant phenotypes are rare, some of which have been observed with a frequency of about 1% in Asiatic Indians (Chahal et al., 1987, Detter et al., 1968, Papiha and Chahal, 1984), and Japanese (Shinoda, 1970, Ishimoto and Kuwata, 1974). Some of the variants are associated with genetic deficiencies, most of which are asymptomatic (Gibson et al., 1977, Gracy, 1982, Tilley et al., 1974). In addition, nongenetic factors (Cini et al., 1988) have been postulated to be responsible for the polymorphism of GPI from numerous origins with no clear evolutionary pattern.

Molecular Properties of the Enzyme

Studies on GPI at the molecular level are the focus of vigorous research in a number of laboratories. The reported molecular weights vary between 110 to 140 KDal for the enzyme from rabbit muscle (Pon, 1970), pig muscle (Gee et al., 1980), bovine heart (Cini et al., 1988), and human erythrocytes (Tilley et al., 1974). The apparent isoelectric points vary widely depending on the species: *T. cruzi* = 5.5 to 5.7 (Widmer et al., 1986), catfish isozyme A = 6.2 and isozyme B = 7.0 (Mo et al., 1975); rabbit muscle = 8.5 (Tilley et al., 1973), and human = 9.25 (Tilley et al., 1974). The enzyme, in its native state, exists as a

homodimer and, in the presence of a denaturant, is dissociated into monomers with a subunit molecular weight of 59 to 66 kdal depending on the species (Gracy, 1982, Achari et al., 1981, Green, 1988, Cini et al., 1988).

The amino acid composition of GPI from various species is similar and a comparison is given in Table IV. The greatest differences thus far recognized between the enzymes from different species appears to be the cysteine content. For example, the rabbit enzyme appears to contain six cysteine residues per subunit (Pon, 1970), while the swine GPI contains only three residues per subunit (Chaput et al., 1988). Yeast GPI (Green et al., 1988, Wesolowski-Louvel et al., 1988) is about 60% homologous with the mammalian enzymes, but is devoid of cysteine. The second most apparent difference in the enzyme from different species is the number of arginine residues (Green, 1988, Wesolowski-Louvel et al., 1988).

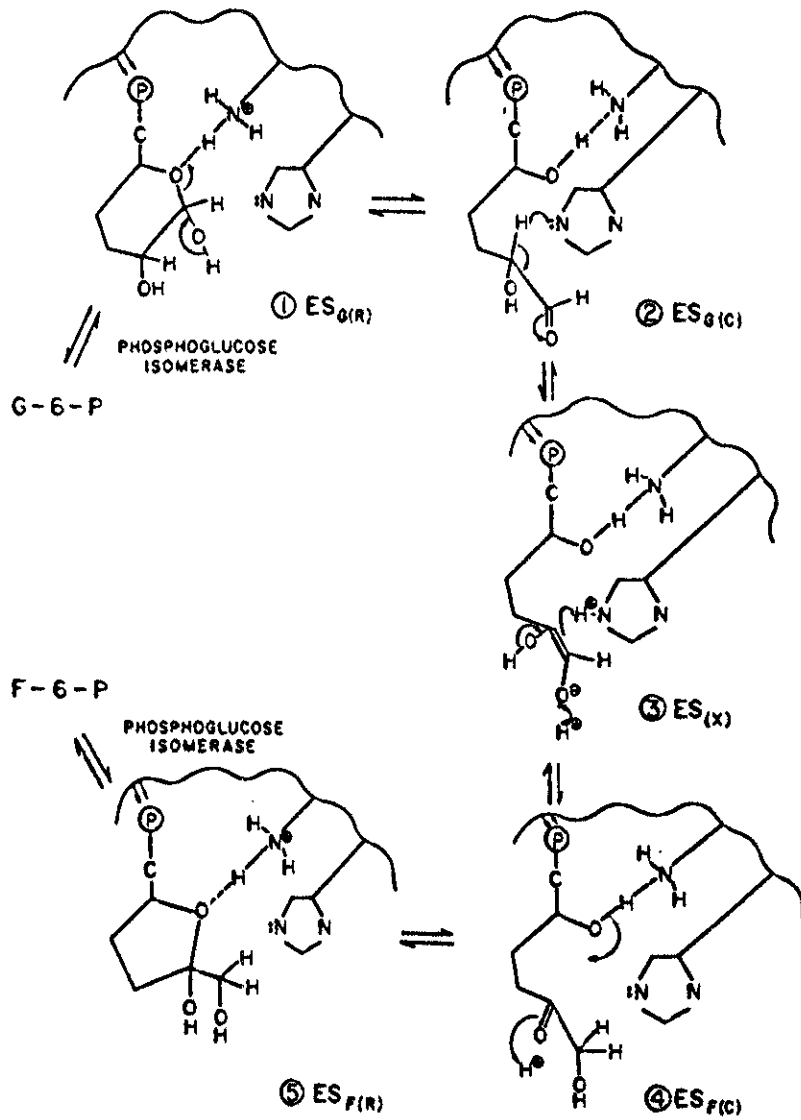
The structural properties of GPI have been reviewed by Gracy, (1982), and Achari et al., (1981). The crystal structure of the enzyme from pig muscle has been determined at a resolution of 2.6 Å. The structure is of the $\alpha\beta$ type. Each subunit consists of two domains (Achari et al., 1981). There are two active sites per GPI dimer. GPI is specific for a single substrate-product pair and has no requirements for cofactors or metal ions (Gracy, 1982, Achari et

al., 1981). It is a highly efficient enzyme and exhibits a turnover number of about 1,000 catalytic events per second (Gracy, 1982, Achari et al., 1981). The actual substrate for the isomerization reaction is the acyclic form in which the ring has been opened (Salas et al., 1965). The evidence for the mechanism for the acid base-catalyzed interconversion of aldoses and ketoses via a *cis*-enediol anion has been reviewed by Rose (1975). Binding of substrate analogues show that inter-subunit interactions are required for GPI activity at both sites (Shaw and Muirhead, 1976, Bruch et al., 1976, 1973, Gracy and Schnackerz, 1977). Two ionizable groups in the enzyme with average pK values of 6.75 and 9.3 are involved in catalysis (Bruch et al., 1976). Based on kinetic studies, Dyson & Noltmann (1968) proposed a mechanism involving a protonated lysine and a non-protonated imidazole in which the enzyme first catalyzes the ring-opening step and then the isomerization via the enediolate intermediate (Fig 1).

The Dyson & Noltmann model has been supported by affinity labeling and chemical modification experiments. The substrate analog N-bromo-acetyethanolamine-phosphate (BAEP) resembles the 5-carbon sugar phosphates which are powerful competitive inhibitors of the enzyme (Gibson et al., 1977, 1980, Chirgwin et al., 1975, Chirgwin and Noltmann, 1975). BAEP causes the rapid, stoichiometric, covalent labeling

Figure 1. The Postulated Mechanism for the Glucose Phosphate Isomerase-catalyzed Reaction.

(G-6-P = glucose 6-phosphate, F-6-P = fructose 6-phosphate, ES = enzyme-substrate complex, G = glucose 6-phosphate, F = fructose 6-phosphate, C = the straight chain form of the substrate, R = the ring form of the substrate, and X = the assumed enediol intermediate)



of a specific histidine residue in the catalytic center. Another critical residue in the active center is a lysine. Pyridoxal-5-phosphate stoichiometrically binds to this lysine, and the resultant Schiff base can be reduced with NaBH_4 (Gibson *et al.*, 1977, Lu *et al.*, 1981, Schnackerz and Noltmann, 1971). The relative position of the lysine residue has been located near the C-terminus (Lu *et al.*, 1981, Lu and Gracy, 1981). Chemical modification studies have also suggested that an essential glutamic acid (O'Connell and Rose, 1973, Schray and Howell, 1979), arginine (Lu *et al.*, 1981), and tryptophan (Lu *et al.*, 1981) may be involved in the catalytic mechanism.

The Diseases Associated with Glucose 6-Phosphate Isomerase and the Relationship to Neuroleukin and gp120

The isomerases have been of interest for clinical reasons. Mathias (1980) has reported the highly diagnostic sensitivity and specificity of GPI in cerebrospinal fluid as an indicator of bacterial and viral meningitis. GPI deficiency has a profound effect on the metabolism of red blood cells. Clinically, the disease is manifested as a non-spherocytic hemolytic anemia, now recognized as the third most common enzyme defect of the red cell (Blume *et al.*, 1972, Paglia and Valentine, 1974). A human allelic GPI variant responsible for this deficiency disease was first

described by Banghan et al. (1968). Since then, with the development and implementation of rapid screening procedures for this disease, a large number of genetic variant forms of the enzyme have been described. Most of the variants have been identified by electrophoretic methods and have usually been observed in the heterozygous state (Gibson et al., 1978, Gracy, 1982, Paglia and Valentine, 1974, Arnold, 1979, Brewer, 1980, Sullivan and Glader, 1980). Clinical syndromes arising from GPI deficiency in tissues other than blood have also been reported. Ultrastructural and enzyme histochemical abnormalities of skeletal muscle in a patient have been found in a case with a variant (type Homburg) of GPI deficiency, associated with congenital nonspherocytic hemolytic anemia and muscle weakness (Bardosi et al., 1985).

Another interesting aspect of this enzyme is that elevated plasma GPI levels appear to be a good marker for neoplastic disease (Cunietti and Greco, 1977, Munjah et al., 1976). The early observations of Bodansky (1954, 1955) suggested that the dramatic increase of GPI level in the serum of patients might be useful in diagnosing cancers. The studies of DeYoung et al. (1980) and Munjal and Goldenberg (1976) have suggested the usefulness of monitoring levels of both GPI and carcinoembryonic antigen. Three GPI variants were recently isolated from human gastrointestinal tumor tissue (Baumann and Brand, 1988). The results obtained

support the hypothesis that those variants were due to a specific intracellular cleavage of the enzyme in the malignant cells (Baumann and Brand, 1988).

The multiple forms of GPI in mammals began attracting particular attention when recent studies (Chaput *et al.*, 1988, Faik *et al.*, 1988) demonstrated amino acid sequence homology of approximately 90% with the neurotrophic factor neuroleukin (NLK). NLK is found in the muscle, brain, heart, and kidney (Chaput *et al.*, 1988, Gurney, 1984, Gurney *et al.*, 1986), and is a neurotrophic factor for spinal and sensory neurons. It also exhibits lymphokine activity on mitogen-stimulated T-cells and induces immunoglobulin secretion by human lymphocytes (Gurney *et al.*, 1986). Since it was shown that the 56 KDa NLK shares at least 90% amino acid sequence homology with GPI, it was suggested that a specific cleavage of GPI might be responsible for the conversion of the enzyme to the trophic factor (Chaput *et al.*, 1988, Baumann and Brand, 1988). It has been suggested that GPI has both glycolytic catalytic activity as well as trophic lymphokine activity, or that the trophic lymphokine activity may be mediated by a peptide fragment or fragments of the native enzyme (Faik *et al.*, 1988). Interest in the relation between GPI and NLK is further increased due to the observations that there is significant sequence homology between NLK, GPI and gp120, the envelope glycoprotein of human immunodeficiency virus 1 (HIV-1) and simian

immunodeficiency virus (SIV) (Muesing *et al.*, 1985, Sanchez-Pescador and Power, 1985). HIV-1 and SIV both inhibit neuron growth in the presence of NLK (Lee *et al.*, 1987). Thus, it has been postulated that NLK may play a role in the pathogenesis of AIDS (acquired immune deficiency syndrome) - related dementia (Faik *et al.*, 1988).

Purpose of This Investigation

Earlier work from this laboratory showed that human GPI could be purified by an affinity elution of the enzyme with its substrate from cation exchangers (Tilley *et al.*, 1974, Gibson *et al.*, 1978, Gracy and Tilley, 1975) and the method has subsequently been developed into a standard procedure for isolation of the enzyme from many other species (Gracy and Tilley, 1975). In view of the potential relation between GPI and NLK it was deemed essential to develop an improved method for isolation of the protein from human tissues which was very rapid, mild, and yielded high recovery, permitting examination of the enzymes for possible heterogeneity.

The second objective of this study was to characterize the physical/chemical properties of the human isoforms of GPI. It was anticipated that this work would be helpful in a further understanding of the molecular properties of GPI and its relation to NLK and pg120.

CHAPTER II

MATERIALS AND METHODS

Materials

Enzymes and Proteins

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and NADP⁺ were obtained from Sigma Chemical Co. (St. Louis, MO) as crystalline suspensions free of GPI activity. The protein kits for molecular weight calibration were obtained from Pharmacia Fine Chemicals (Piscataway, NJ), Sigma, United States Biochemical Corporation (Cleveland, Ohio), and Bio-Rad Laboratories (Richmond, CA). Isoelectric focusing markers were from Pharmacia.

Substrates and Chemicals

Fructose- and glucose-6-phosphate, 2-mercaptoethanol (BME), ethylenediamine-tetraacetic acid, acid, dimethylthiazolyl diphenyl tetrazolium, phenazine methosulfate, bis tris propane, triethanolamine, and tris(hydroxymethyl)aminomethane were all obtained from Sigma,

and were of the highest purity available. Reagents for automated Edman degradation were purchased from Applied Biosystems Inc (Foster City, CA).

Chromatographic and Electrophoretic Supplies

Cellulose phosphate was obtained from Sigma. Cross-linked spherical particle cellulose phosphate was from Serva Fine Biochemicals Inc. (Westbury, NJ). All reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad, and isoelectric focusing reagents were from Pharmacia and LKB Instruments (Bromma, Sweden). The amino acid standards were from Sigma, and the other reagents used in amino acid analysis were from Pickering Laboratory (Mountain View, CA) and Pierce (Rockford, IL). Cellulose acetate sheets for activity staining were from Sartorius (Gottingen, W. Germany).

Immunochemicals

Rabbit anti human GPI antisera was obtained as described by Purdy et al. (1980). Peroxidase conjugated IgG fraction of goat anti-rabbit IgG was obtained from Cooper Biomedical Inc. (Worthington; Freehold, NJ). Hydrogen peroxide (30%) and 4-chloro-1-naphthol were obtained from Sigma. Nitrocellulose membranes, Tween-20, and gelatin were obtained from Bio-Rad.

Methods

Enzyme Activity Assays

Catalytic activity of GPI was measured according to the method of Noltmann (1972). The enzyme was assayed in the direction of fructose 6-phosphate to glucose 6-phosphate by coupling the product to glucose 6-phosphate dehydrogenase and measuring the rate of reduction of NADP⁺. The assay mixture contained: 50 mM triethanolamine (TEA)-HCl, pH 8.3; 4.0 mM fructose-6-phosphate; 0.5 mM NADP⁺; and 1.0 unit of glucose-6-phosphate dehydrogenase in a final volume of 1.0 ml. The reduction of NADP⁺ was followed spectrophotometrically by the increase in absorbance at 340 nm. The temperature was maintained at 30°C.

Protein Assays

Protein content was routinely measured by the method of Bradford (1976). A Bio-Rad Protein Assay Kit was used with bovine serum albumin (BSA) as the calibration standard. Column effluents were monitored by absorbance at 280 nm. An extinction coefficient of $E_{1\text{cm}}^{1\%} = 13.9$ was used to convert

absorbance at 280 nm to protein concentration (Lu et al., 1981).

Isolation of Glucose 6-Phosphate Isomerase from Human Placenta

The method of Tilley et al. (1974) including the modifications of Purdy et al. (1980) was utilized. Three to five human placentas were homogenized in extraction buffer (50 mM TEA-HCl, 5 mM EDTA, 10 mM KCl, 0.1% BME, pH 8.2) at a ratio of 150 g wet tissue per 225 ml of buffer. The homogenate was centrifuged at 9800 x g for 1 h at 4°C, and the supernatant passed through glass wool and dialyzed against two changes of 20 l of buffer A (10 mM TEA-HCl, 0.1% BME, 1 mM EDTA, pH 7.2) for 48 h. Damp cross-linked spherical phosphocellulose, which had been cycled more than three times through 0.5 N HCl/0.5 N NaOH/distilled water washes and preequilibrated in buffer A, was mixed with the dialyzed homogenate (1000 ml:400 g). The mixture was washed with buffer A and buffer B (25 mM TEA-HCl, 0.1% BME, pH 8.2) in a 3000 ml sintered glass funnel, and then poured into a column (4 x 100 cm). The column was washed with buffer B. When the effluent was protein-free (as judged from the absorbance at 280 nm), the enzyme was eluted with buffer B containing 10 mM fructose 6-phosphate (300 to 500 ml).

Preparative Isoelectric Focusing

Preparative isoelectric focusing (IEF) was carried out on an LKB Multiphor system using LKB Ultrodex or Pharmacia Sephadex granules (4 g in 100 ml distilled water) and Ampholines [LKB pH 8-9.5 (3 ml), plus pH 9-11 (0.5 ml), or Pharmalyte pH 8-10.5 (3.5 ml)]. The degassed slurry was poured into the tray and dried to give a stable gel matrix. The samples were applied in a narrow zone, prefocused at 15W (constant) for 25 min at 4°C and then resolved at 30W for 20 h at 4°C.

Preparative SDS-Polyacrylamide Gel Electrophoresis

A BRL 1100 PG Preparative Gel Electrophoresis system (Bethesda Research Laboratories, Inc.) was used to separate the subunits. The tube (1 cm inner diameter) contained a 4 cm high 10% acrylamide resolving gel and a 1 cm high 3% acrylamide stacking gel. The running buffer contained 50 mM Tris, 0.38 M glycine, and 0.1% SDS at pH 8.2. Electrophoresis was carried out at constant current (8 mA) at room temperature. The flow rate was set at 10 ml/h, and fractions of 0.75 ml were collected. Complete separation was achieved in 6 h.

Analytical Electrophoresis and Isoelectric Focusing

Nondenaturing alkaline gel electrophoresis. Nondenaturing gels were cast as described by Maizel (1971). Slab gels (140 x 160 x 1.5 mm) consisted of 3% acrylamide stacking and 7.5% acrylamide resolving (120 mm high) portions. Electrophoresis was conducted at 4°C. Field strength was 20 mA until the tracking dye entered the resolving gel and 30 mA thereafter. The composition of modified 5% nondenaturing gel is shown in Table I.

Analytical SDS-polyacrylamide gel electrophoresis: SDS-PAGE was performed as described by Laemmli (1970). Polyacrylamide slabs (140 x 160 x 0.75 mm; 10% acrylamide resolving gel, 12 cm high; 5% acrylamide stacking gel; 0.1% SDS) were run at 20 mA per slab at room temperature. Subunit molecular weights were estimated using protein standards of known molecular weights. The proteins were stained with Coomassie Brilliant Blue or silver (Merril et al., 1981, Yuksel and Gracy, 1985).

Analytical isoelectric focusing: For the pH range of 3.5-9.5, Ampholine PAGplates (LKB) were used for analytical IEF. The gel was focused at constant power (1 watt/cm) for

Table I. Composition of Modified Nondenaturing Polyacrylamide Gels for Electrophoresis

Reagent	1	2	3	4	5	6	Water
	(ml)	(ml)	(ml)	(ml)	(μ l)	(μ l)	(ml)
Resolving gel (5%)	5	2	1.75	-	15	150	21.1
Stacking gel (3%)	1	-	-	1.25	5	100	7.64

Reagent 1: 30 g of acrylamide and 0.8 g of bis-acrylamide (N,N-bis-methylene acrylamide) dissolved and diluted to 100 ml with distilled water.

Reagent 2: 3M Tris-HCl, pH 9.3.

Reagent 3: 1.5 M Bis tris propane (BTP)-HCl, pH 9.3.

Reagent 4: 0.5 M Tris-HCl, pH 8.2.

Reagent 5: TEMED (N,N,N,N-tetramethylethylenediamine)

Reagent 6: 10% Ammonium persulfate.

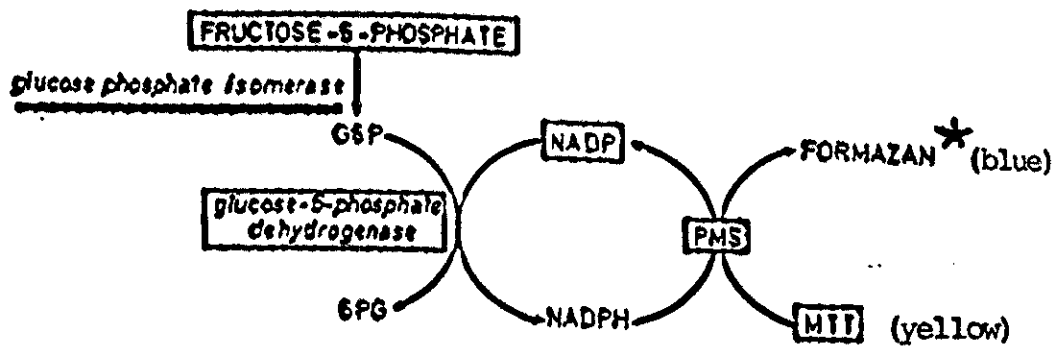
Running buffer: Tris 25mM, glycine 192mM, pH 8.2, for both upper and lower buffer chambers.

30 min, the sample applicators were removed, and electrofocusing continued for an additional hour. Anode-electrode buffer was 1 M phosphoric acid (H_3PO_4), and the cathode-electrode buffer was 1 M NaOH. Apparent isoelectric points were determined against protein standards of known pI values.

For the pH range 8-10.5; Reagent 1 (see Table I, 2.5 ml), sorbitol (1.5 g), Ampholine (LKB, pH 8-9.5 0.7 ml, pH 9-11 0.25 ml) or Pharmalyte (Pharmacia, pH 8-10.5 0.95 ml), and H_2O (10.5 ml) were mixed and degassed. Reagents 5 (15 μ l) and 6 (100 μ l) were then added and mixed. This was sufficient to cast a 0.75 X 140 x 110mm gel. The gel was pre-focused for 15 min at 5 Watts, the samples applied, and power increased to 14 Watts for 30 min. The sample applicators were then removed and focusing continued for an additional hour at 14 Watts. The cathode solution was 1 M NaOH, and the anode solution was 0.25 M HEPES.

Activity staining: The native enzyme was located by the activity staining procedure of Scopes (1964;1968). Freshly run gels were stained for GPI activity by overlaying the gel with a cellulose acetate sheet soaked in the substrate and coupling enzymes. The principle of the assay is shown in Fig.2.

Figure.2. The principle of the GPI activity staining.
(G6P = glucose 6-phosphate; 6PG = 6-phosphate-gluconolactone; NADP = β -nicotinamide adenine dinucleotide phosphate; NADPH = β -nicotinamide adenine dinucleotide phosphate (reduced form); PMS = phenazine methosulfate; MTT = dimethylthiazolyl diphenyl tetrazolium)



Western blotting

GPI from SDS-PAGE was electroblotted onto nitrocellulose at 10V/cm (constant) for 1 h at 4°C in 25 mM Tris-HCl:0.19 M glycine containing 20% methanol, pH 8.3. The unoccupied sites were blocked with 3% gelatin in Tris buffered saline (TBS, 20mM Tris:0.5 M NaCl, pH 7.5) for 30 min, then washed three times for 5 min each with the same solution containing 0.05% Tween-20. Primary antibody (rabbit anti-human GPI antisera) was applied for 1 h at room temperature. After washing with TBS containing 0.05% Tween-20 (3 X 5 min), secondary antibody (peroxidase conjugated goat anti-rabbit IgG) was applied (40 min at room temperature), and the blot rinsed with water. Color developer consisted of 20ml of TBS, 4ml of 4-chloro-1-naphthol stock solution (3 mg in 1 ml methanol) and 8 µl of 30% hydrogen peroxide, all freshly mixed. The blot was developed in the dark for 5-7 minutes and was then rinsed with water.

Amino Acid Analysis

GPI subunits were separated by 10% SDS PAGE. After electrophoresis, the gels were soaked in transfer buffer (10mM CAPS, 10% methanol, pH 11.0) for 5 min. and blotted

onto Polyvinylidene difluoride (PVDF; Immobilon™, Millipore) membrane by method of Matsudaira (1987). The blotted proteins were visualized by staining with 0.1% Coomassie Blue R-250 in 50% methanol for 5 min., and then the background was destained in 50% methanol for 5-10 min. at room temperature. The membrane was finally washed in distilled H₂O for 4 times (each time for 5 min), and air dried. The protein bands were cut and stored at -20°C. The samples (pieces of PVDF membrane containing protein) were hydrolyzed for 24, 48, and 72 h in 6N HCl *in vacuo* at 110°C. The PVDF membrane was resistant to acid hydrolysis. Nonetheless, blanks were analyzed to compensate for the background. Values for Serine and Threonine were extrapolated to zero time of hydrolysis while values for the hydrophobic residues were obtained from the 72 h hydrolysis. Analysis was quantitated by post column fluorescence detection with *o*-phthalaldehyde (Roth, 1971).

N-terminal Analysis

Amino terminal sequence analyses were carried out by automated Edman degradation on an Applied Biosystems model 475-A gas phase sequencer.

Cyanogen Bromide Cleavage and Peptide Mapping

The cleavage at the methionine residues was carried out with CNBr by the modified method of McGowan *et al.* (1987). The protein (about 0.1 mg) was dissolved in 50% (v/v) formic acid and a small crystal of cyanogen bromide was added. Cleavage at methionine residues was allowed to proceed for 20 h at 4°C and the solutions were then dried by lyophilization. The cleaved GPI peptides were dissolved in 25 mM TEA buffer, pH 8.9, and resolved on a 20% SDS PAGE.

pH Stability of the Isozymes

pH stability studies were conducted on the purified isozymes. The samples (1-2 units/ml) were incubated at pH 6 in 10 mM TEA-HCl, containing 5 mM BME and 5 mM EDTA, or at pH 10 in 10 mM CAPS, 5 mM BME, and 5 mM EDTA, at 37°C. Following incubation, the enzyme was immediately assayed for catalytic activity at pH 8.3 as described above.

CHAPTER III

RESULTS

Isolation of the Protein

Some ion exchangers, such as phosphocellulose and CM-Sephadex, have been evaluated to improve the purification of GPI from humans (Gracy and Tilley, 1975, Carter and Yoshida, 1969, Tsuboi et al., 1971). Phosphocellulose has traditionally been used for the ion exchange isolation of many proteins including GPI (Gracy and Tilley, 1975). A limitation of this method is the relatively slow flow rate that prohibits large-scale isolation of proteins. Cross-linked spherical particle phosphocellulose was found to yield better results in terms of purification, speed and recovery. This method routinely resulted in a 5500-fold purification with a recovery of over 50% and provided enzyme with a specific activity of 820 to 1000 units/mg. Comparative data (Table II, Fig. 3 & 4) show that the spherical particle phosphocellulose procedure was substantially superior to the use of conventional phosphocellulose in terms of selectivity (specific activity of 820 vs. 670, respectively), yield (>50% vs. 40%) and speed (4.8 h vs. 30.8 h for a complete

Table II

Comparison of purification of GPI using fibrous and cross-linked spherical particle cellulose phosphates^a

Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)	Time required (hours)
Dialysate	209	1390	0.15	(1)	(100)	-
P-cellulose						
crosslinked	115	0.14	821	5470	55.0	4.8
fibrous	87	0.13	669	4460	41.6	30.8

^a For procedure see Figure 3 and method section.

Figure 3. Flow-chart of the procedure for purification of GPI by using different phosphocelluloses.

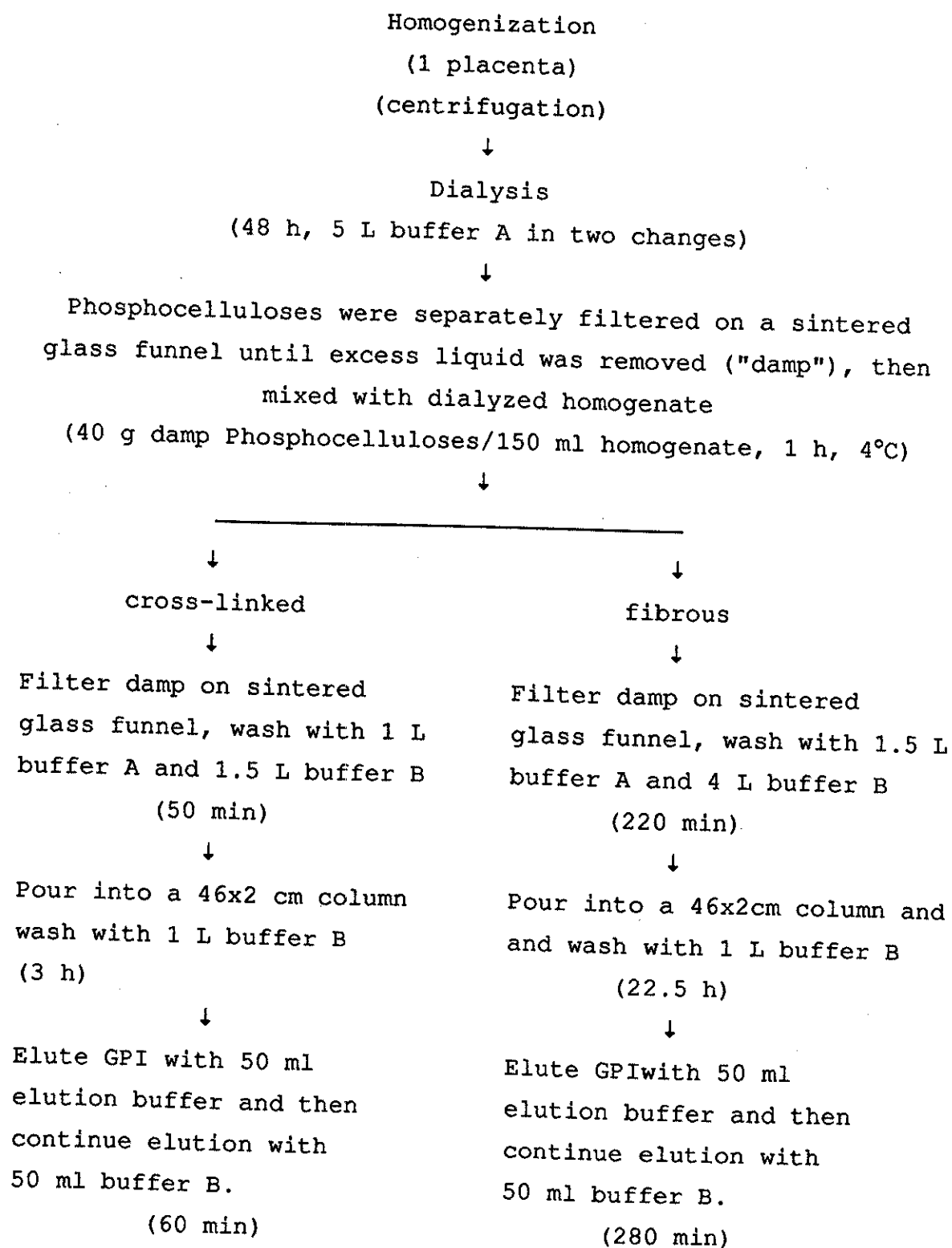
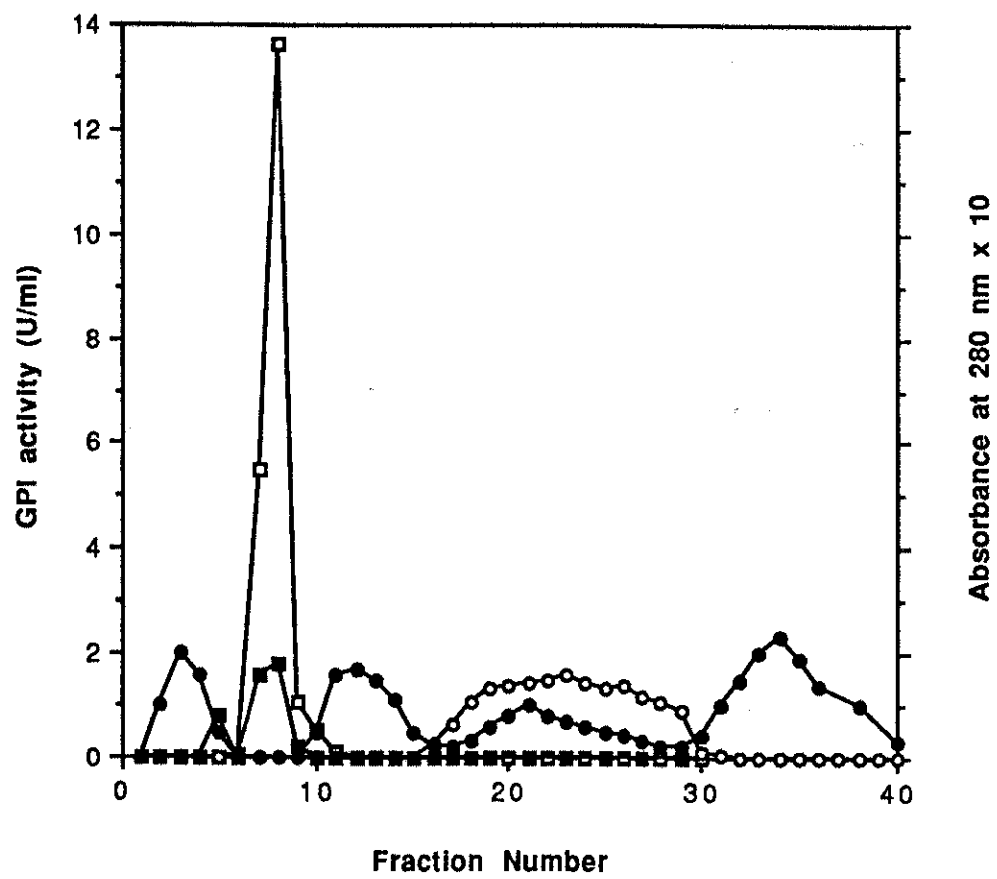


Figure 4. Elution profiles of GPI from different types of phosphocellulose. Open symbols denote GPI activity, closed symbols absorbance at 280 nm. Circles and squares show the elution from fibrous and cross-linked spherical particulate phosphocellulose, respectively. Fractions (150 drops/tube) were collected after the elution buffer was started. The protein concentration was determined by measuring absorbance at 280 nm. Enzyme activity was assayed as described in Methods.



purification). In addition, the contaminated proteins were more easily removed by this procedure, requiring less volume of washing buffers. From 150 g of placenta (wet weight) approximately 150 μ g GPI was obtained.

Electrophoretic Properties of the Native Isozymes

Multiple electrophoretic forms of GPI were observed upon non-denaturing PAGE. Since the human placenta GPI has a relatively high pI(9.2), relatively large molecular weight (132 kdal) and is somewhat unstable, isolation of the isozymes by electrophoresis was laborious.

An attempt was made to optimize the resolution of isozymes in several separation media so that the isozymes could be isolated preparatively by using discontinuos electrophoresis. There are three primary physical effects responsible for the resolving power of discontinuous electrophoresis (Maurer, 1971, Simpson et al., 1983, Osterman, 1984, Andrews, 1986): (a) molecular sieving, (b) electrical charge, and (c) buffer ions. All of these were evaluated to obtain optimum resolution. From Fig. 5, one can see that increasing the pore size of the resolving gel (i.e. decreasing acrylamide concentration from 7.5% to 5%) diminished friction (the sieving effect) and allowed the isozymes to move faster. On the other hand, increasing pH of the gel (changing the stacking gel pH from 6.7 to 8.3 and

resolving gel pH from 8.9 to 9.3) not only increased the mobility but also improved the separation of the isozymes. At high pH, the isozymes were separated into four bands, while at low pH only three bands were observed (Fig. 5).

To study the effects of buffer ions, different bases were evaluated in the resolving gel buffer. One was Tris and the other bis tris propane (BTP). Fig. 6 shows that when Tris was used alone, the isozymes could be separated, but the resolution was not good. BTP alone did not separate the isozymes at all. But, when Tris and BTP were mixed at a molar ratio of 2:0.875, the isozymes were adequately separated and resolved. The difference between Tris and BTP buffers can be attributed to the ionic strength.

Figure 5. Resolution of human placental GPI isozymes:
effects of pH and polyacrylamide concentration.

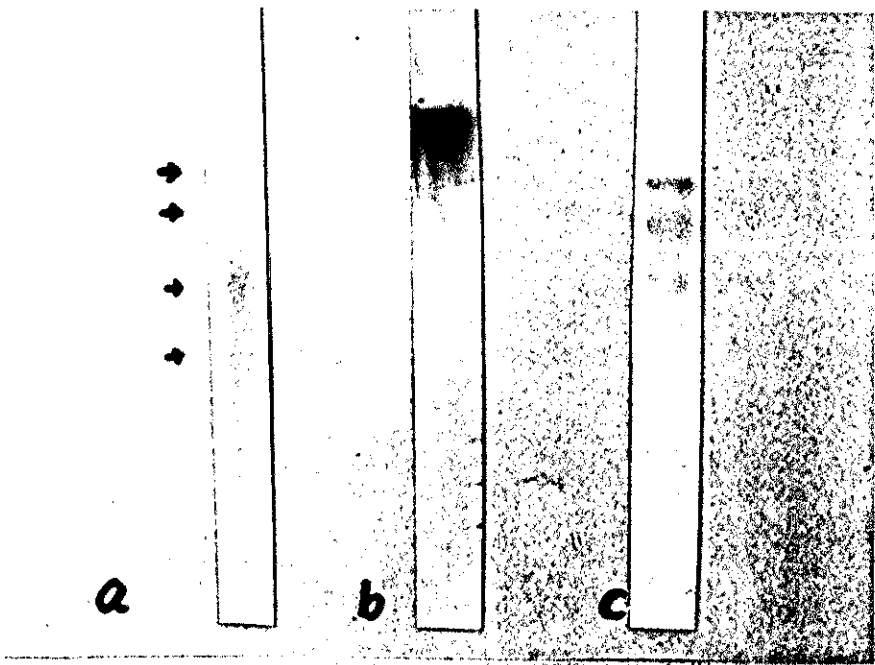
	I	II
stacking gel buffer (pH)	6.7	8.3
resolving gel buffer (pH)	8.9	9.3

Polyacrylamide concentration: a) 7.5%, b) 5%; electrode
buffer: 25 mM Tris : 192 mM glycine, pH 8.3.

	I		II	
	a	b	a	b
- ↓	— — —	— — —	— — — —	— — — —
+				—

Figure 6. Resolution of human placental GPI isozymes: effects of resolving gel buffer ions. left: 0.2 M Tris, center: 0.875 M Bis Tris Propane (BTP), right: Tris:BTP = 2:0.875 M.

Resolving gel 5% polyacrylamide, pH 9.3; stacking gel 3% polyacrylamide, pH 8.3; electrode buffer: 25 mM Tris, 192 mM glycine, pH 8.3.



Preparative Resolution and Characterization of the Isozymes

In order to characterize the different forms of the enzyme, a suitable method for the separation of the isozymes was required. Considering the basic principles of protein separation, IEF would be a useful method for separating the different GPI isozymes since the method does not effect the catalytic activity of the enzyme. Thus, preparative IEF was chosen and resulted in resolution of the isolated GPI into four forms with apparent pI values of 9.13, 9.00, 8.84, and 8.62 (Table III, Fig. 7 & 8). The relative amount of each of the isozymes determined by total protein content were 48%:36%:13%:3%, respectively. Each of the isozymes isolated by preparative IEF was subjected to SDS-PAGE. Two different size subunits were found: A = 63.2 kdal and B = 69.8 kdal (Table III, Fig.7 & 9). The two more basic isozymes designated #1 and #2 (which comprise approximately 85% of the isolated protein) exhibited a single band on SDS-PAGE (subunit A) suggesting that they were dimers of identical size subunits, and that the basis for their difference was charge only. The subunit size of the basic isozymes (63.2 KDal) was essentially identical with those previously observed for human GPI (Tilley et al., 1974) as well as the enzymes from other species (Cini et al., 1984, Chaput et al., 1988, Gee et al., 1980, Pon, 1970).

Figure 7. Molecular properties of the GPI subunits.

GPI isozymes and subunits purified as described in the text were analyzed by isoelectric focusing, SDS-PAGE and immunochemical methods.

Abbreviations: PC = phosphate cellulose, cPC = cross linked phosphate cellulose, prep SDS = preparative SDS-PAGE, IEF = isoelectric focusing.

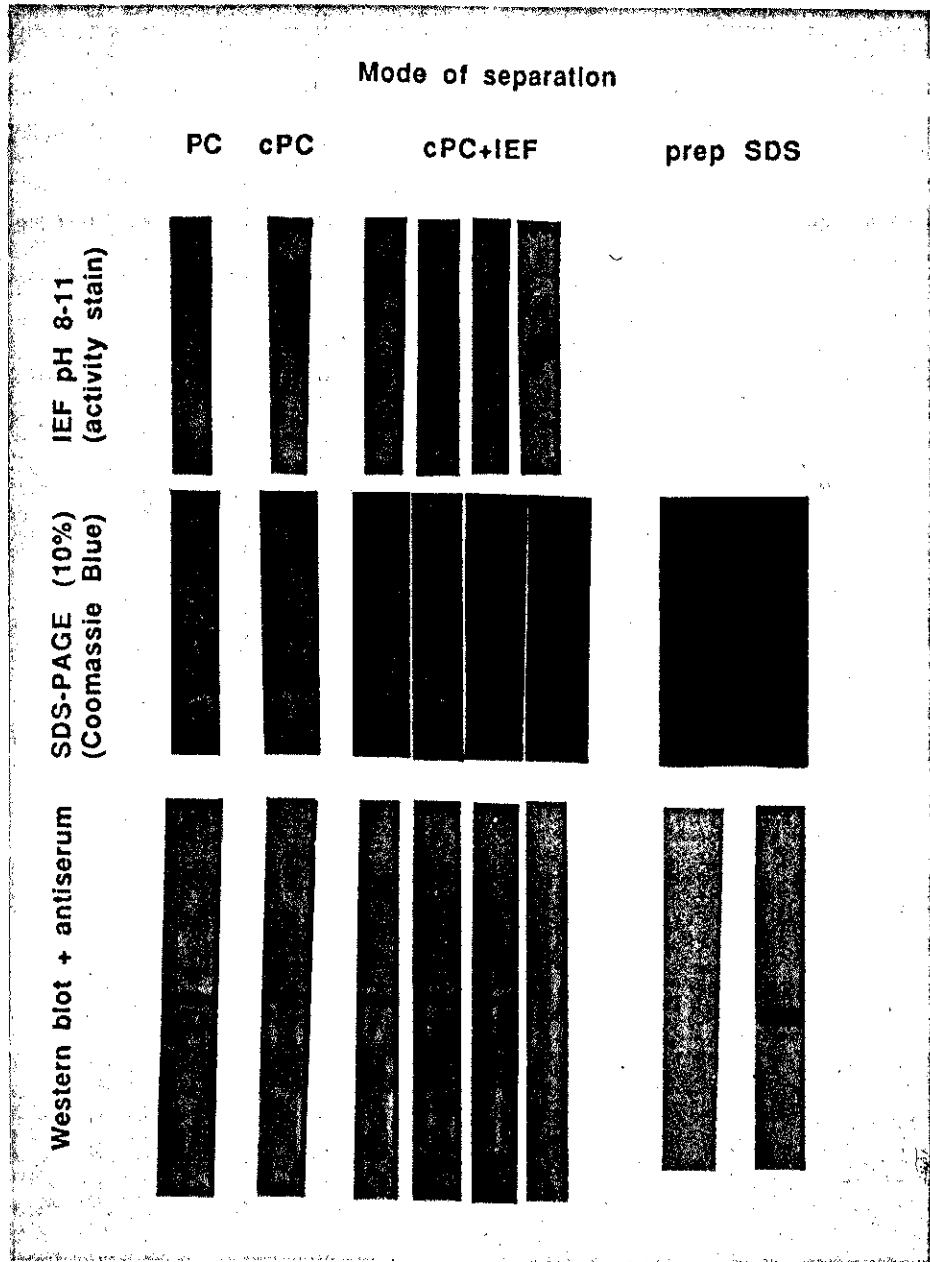


Figure 8 Determination of isoelectric points of the isozymes.

Isoelectric points were determined on PAGplate (pH 3.5-9.5), and calculated against protein standards of known pI values. The equation and correlation coefficient shown are for the best fitting line through the data points. The protein standards were trypsinogen (pI 9.3), lentil lectin-basic band (pI 8.65), lentil lectin-middle band (pI 8.45), lentil lectin-acidic band (pI 8.15), myoglobin-basic band (pI 7.35), myoglobin-acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β -lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.50).

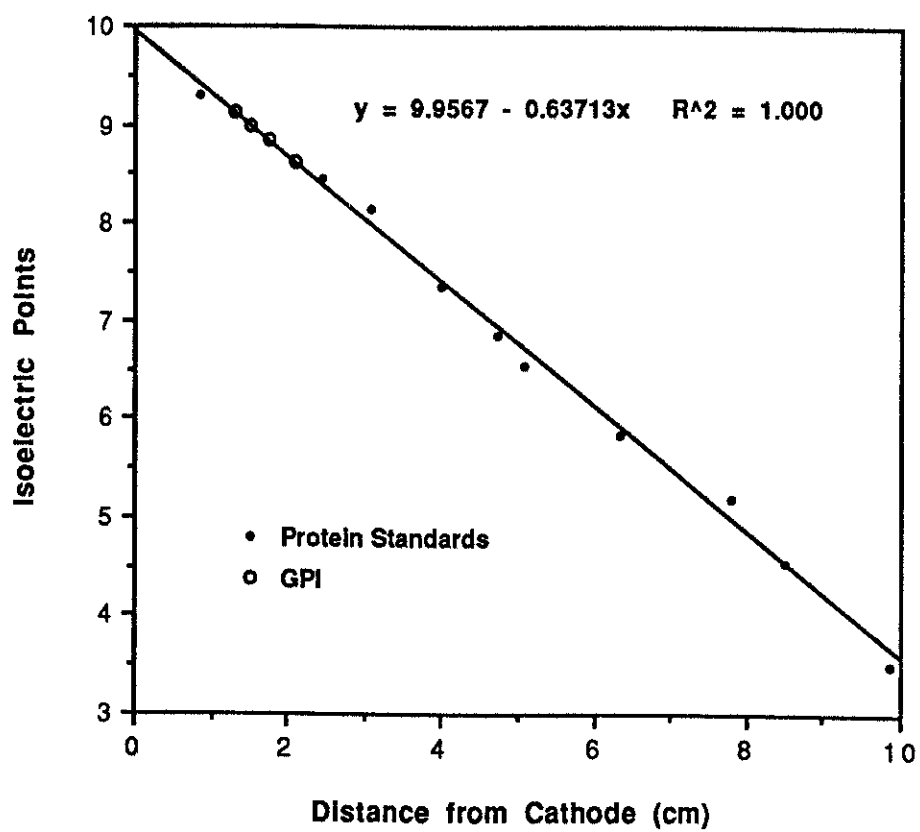


Figure 9. Determination of molecular weights of subunits.

Molecular weights were determined on 10% SDS-PAGE and calculated against standard proteins. The equation and correlation coefficient are for the line fitting through the data points. The standard proteins were from: (a) Bio-Rad; rabbit muscle phosphorylase b (97.4 KDal.), bovine serum albumin (66.2 KDal.), hen egg white ovalbumin (42.7 KDal.), bovine carbonic anhydrase (31.0 KDal), soybean trypsin inhibitor (21.5 KDal), hen egg white lysozyme (14.4 KDal), (b) USBC; cytochrome C hexamer (74.4 KDal.), cytochrome C tetramer (49.6 KDal.), cytochrome C trimer (37.2 KDal.), cytochrome C dimer (24.8 KDal.), cytochrome C monomer (12.4 KDal), (c) Pharmacia; rabbit muscle phosphorylase b (94.0 KDal.), bovine serum albumin (67.0 KDal.), egg white ovalbumin (43.0 KDal.), bovine erythrocyte carbonic anhydrase (30.0 KDal), soybean trypsin inhibitor (20.1 KDal), bovine milk α -lactalbumin (14.4 KDal), (d) Sigma; fructose-6-phosphate kinase (84.0 KDal.), pyruvate kinase (58.0 KDal), fumarase (48.5 KDal.), lactic dehydrogenase (36.6 KDal.).

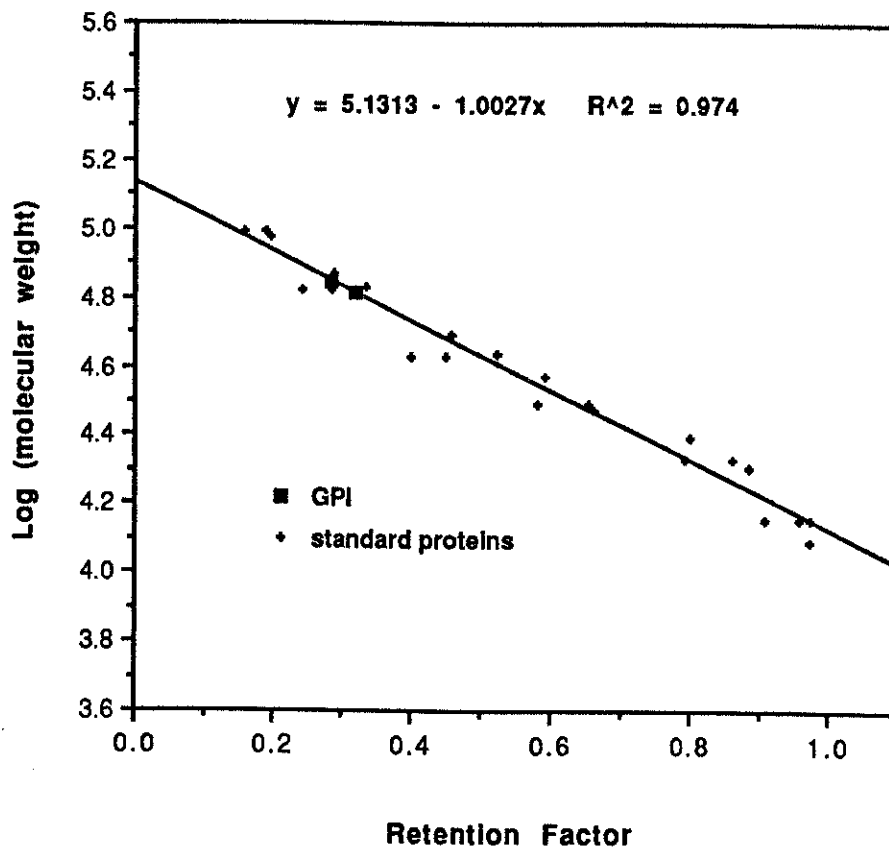


Table III

Properties of GPI Isozymes.

Iso- zyme	pI ^a	Protein recovery ^b	Subunit type ^c	Subunit mwt ^c	Specific activity ^d	N-terminus
1	9.13	44-50	A	63.2 kdal	1099	blocked
2	9.00	35-37	A	63.2	1003	blocked
3	8.84	10-15	A+B	63.2+69.8	549	blocked
4	8.62	1-5	B	69.8	115	blocked

^a The pI was determined by IEF against IEF standards (see Fig. 8)

^b Protein recovery (%) was determined by quantitation of a Coomassie blue stained non-denaturing gradient gel (8-25%) using a laser densitometer.

^c Determined on 10% SDS-PAG. Subunit molecular weights were determined against molecular weight standards (12 to 97 kdal).

^d The isozymes were resolved by preparative IEF and specific activity (units/mg) determined spectrophotometrically.

In contrast, the two minor more acidic isozymes appeared to be composed of subunits of different size. The most acidic isozyme, #4 (pI=8.62) which comprises only 1-5% of the total protein, appeared to be a homodimer of a subunit with a molecular weight of 69.8 KDal. Isozyme #3 with an intermediate pI of 8.84 appeared to be a heterodimer composed of both the A and B subunits (Table III, and Fig. 7).

Immunochemical Properties

The rabbit antiserum against GPI was prepared with human GPI which had been purified using fibrous cellulose phosphate. It specifically reacted with GPI and no cross reaction with other proteins was observed. This antiserum was used as the primary antibody and was followed by second antibody conjugated to the peroxidase ELISA system. As seen in Fig.7, the immunochemical reaction was complete only with the isozymes containing the subunit A and with the subunit A obtained from preparative SDS-PAGE. No immunochemical reaction was observed with subunit B.

Resolution of the subunits

For further characterization of the GPI isozymes, the two types of subunits needed to be separated in large quantities. It was found that the GPI subunits could be

separated by SDS-PAGE and extracted from the gel in two principle ways: 1) simple extraction with an appropriate buffer, and 2) electrophoretic elution (Osterman, 1984, Andrews, 1986). The first method generally required a longer time and resulted in low yields (seldom over 60% and often as little as 15-20 per cent). Moreover, the extract also contained substances leached from the gel matrix. The electrophoretic method normally gave higher recoveries and less contamination by gel-derived material. In this work, the preparative SDS-PAGE was used to separate and elute the GPI subunits. Fig. 10 shows that the two GPI subunits could be isolated successfully by using preparative SDS-PAGE, with at least 90% recovery in only 6 hours. Electrophoretic elution of the protein was found to be much better than methods which extracted protein from gel by diffusion.

Amino Acid Composition

Table IV summarizes the results of amino acid analyses of the two subunits. Amino acid analysis of subunit A gave essentially identical results to the amino acid composition previously reported for human GPI (Lu and Gracy, 1981, Tilley et al., 1974, and Carter and Yoshida, 1969), and showed a high degree of similarity with the enzyme from other species (Pon, 1970, Cini et al., 1988, Chaput et al., 1988). The amino acid analysis of subunit B presented a significant

Figure 10 Separation of GPI subunits by preparative SDS-PAGE.

Samples were electrophoresed in tube gels (1 cm diameter, stacking gel 1 cm, 3% acrylamide; resolving gel 4 cm, 10% acrylamide) at 8 mA and eluted at 10 mL/h flow rate. Fractions (0.75 mL) were collected after tracking dye eluted. The protein concentration was determined by measuring OD 280.

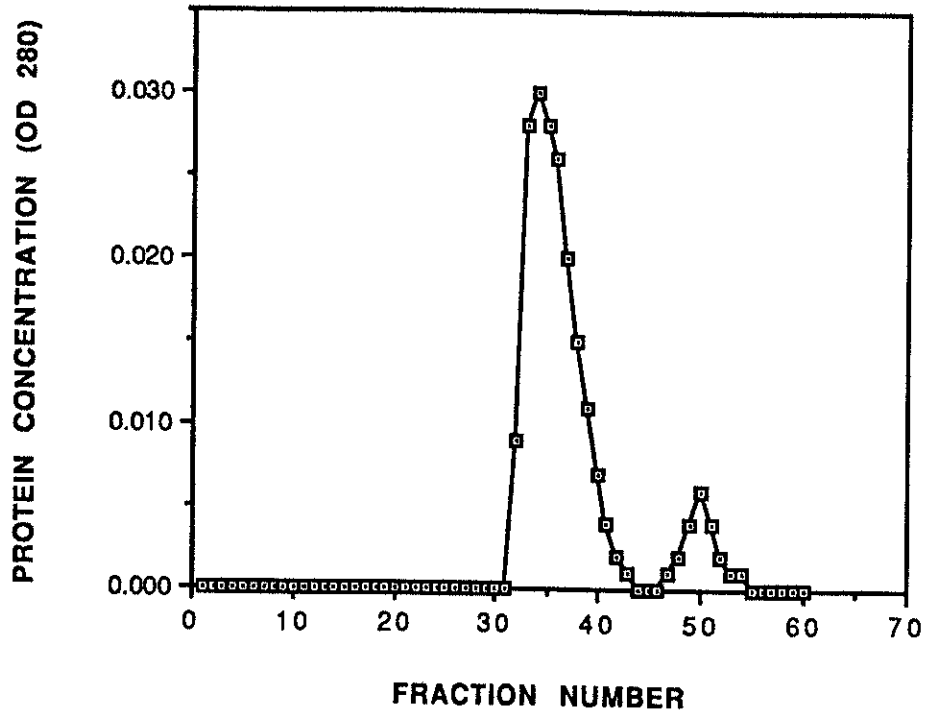


Table IV.
AMINO ACID COMPOSITION OF SUBUNITS^a

Amino Acid	Yeast ^{b,c}	NLK ^{b,d}	Pig ^{b,d}	Rabbit ^e	Bovine ^f	Human ^g	Human ^h	Sub A ⁱ	Sub B ⁱ
ASX	62	55	54	58	58	58	59	62	64
THR	42	35	39	41	34	38	39	33	29
SER	38	35	34	38	34	31	31	27	33
GLX	55	60	61	63	62	65	65	67	69
GLY	41	40	40	44	44	48	43	48	48
ALA	48	42	38	42	41	40	38	43	76
VAL	38	29	30	34	28	32	35	32	36
MET	10	14	14	14	14	12	12	13	12
ILE	30	34	33	35	29	29	33	27	43
LEU	47	55	53	57	54	59	57	55	49
TYR	16	13	12	12	12	14	14	14	17
PHE	34	26	30	30	30	28	28	27	25
LYS	40	38	37	41	32	39	40	31	38
HIS	18	20	20	24	14	24	20	16	14
ARG	10	20	24	21	29	29	30	26	29
PRO	16	23	23	25	ND	ND	24	ND	ND
CYS	0	4	3	6	3	3	6	ND	ND
TRP	9	13	13	12	12	11	12	ND	ND

a). Standard three letter abbreviations are used to designate the amino acids. ND=not determined.

b). The amino acid content was calculated from the protein sequences, c). Green, 1988, d) Chaput, *et al.*, 1988,

e). Pon, 1970, f). Cini *et al.*, 1988, g). Lu & Gracy, 1981, h). Tilley *et al.*, 1974, i). this work

difference from subunit A and other GPI's, especially Ala and Ile.

Cyanogen Bromide Cleavage and Peptide Mapping

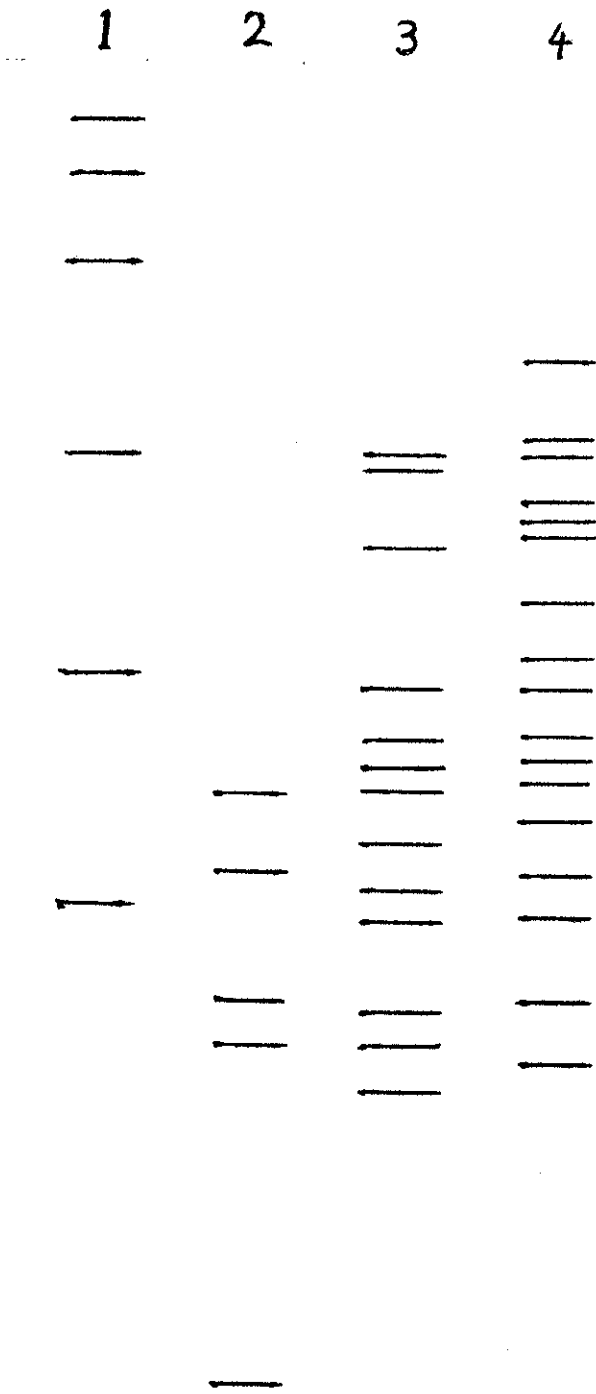
The number of methionine residues in human GPI is approximately 13 for subunit A and 12 for subunit B. Hence, near complete cleavage of the subunits by CNBr were achieved as 15 to 17 peptides for subunit A and B which were observed after 20% SDS-PAGE (Fig.11). These peptides were not identical in number and molecular weight for the two subunits. These data show that the positions of methionine residues in the two subunits were different.

Amino-terminal Analysis

The amino terminal analysis of subunit A and B resulted in no identifiable amino terminal residue. Therefore, it appeared that the amino termini of both subunits were blocked as previously reported for the enzyme from human (Lu and Gracy, 1981), bovine (Cini et al., 1988), and rabbit (Pon, 1970).

Figure 11. Peptide mapping from methionine cleavage of human GPI.

For details of the methionine cleavage by CNBr see Method section. The peptide mapping of human GPI subunits by CNBr cleavage were analyzed on 20% SDS-PAGE. Lane 1 = low molecular weight markers (Pharmacia, 14 to 94 kdal); Lane 2 = SDS molecular weight markers for peptides (Sigma, 16.9 to 2.5 kdal); Lane 3 = human GPI subunit B; Lane 4 = human GPI subunit A.



Changes in Electrophoretic Pattern of Glucose Phosphate
Isomerase as a Function of Storage Time

When the GPI was stored at 4°C in buffer B for periods of up to 3 months, the pI distribution of the isozyme shifted distinctly to the more acidic values, but enzyme catalytic activity was still retained (Fig. 12). The previous studies on GPI have shown that changes in electrophoretic migration can be due to the deamidation of certain asparagine residues (Cini et al., 1988) and/or the oxidation of sulfhydryl residues (Noltmann, 1964, Payne et al., 1972). After storage at 4°C for 6 months and 12 months, the enzyme activities remained approximately 85.8% and 57.4%, respectively. These data agreed with the previous studies on isozymes of GPI in which changes were exhibited in their apparent pI values but catalytic properties were retained (Cini et al., 1988, Tilley et al., 1974). However, *in vivo*, the more acidic isozyme forms of GPI may have been more rapidly degraded since the more acidic forms of proteins generally appeared to be more easily hydrolyzed by proteases (Creighton, 1984, Gracy, 1983).

Figure 12. Electrophoresis pattern of GPI as a function of time of storage.

Incubations were at 4°C for a) 1 month, b) 4 months, c) 18 months in 10 mM TEA, 5 mM BME, and 5 mM EDTA. Samples were analysed by IEF (pH8-11) and visualized by activity staining.

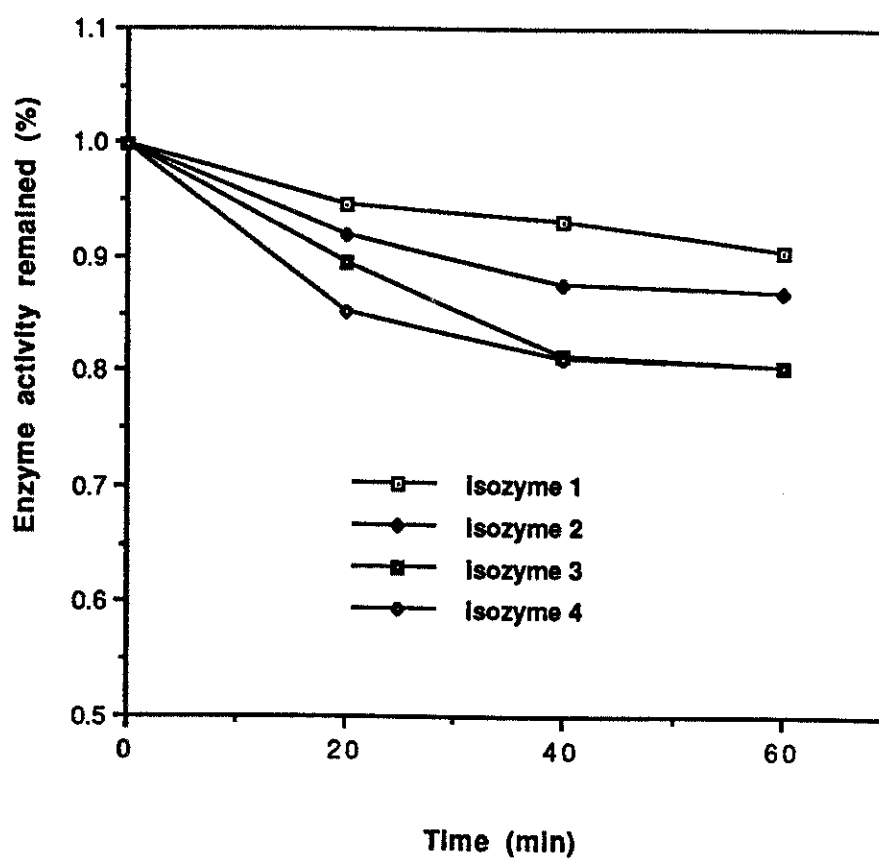


pH Stability of the Isozymes

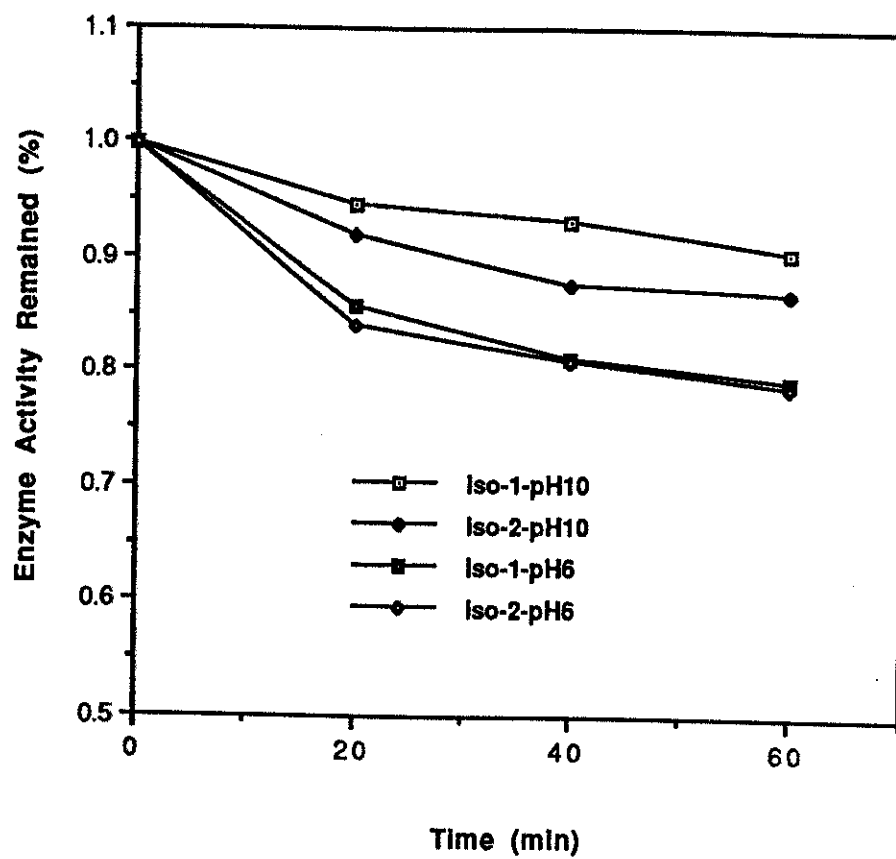
Fig. 13 shows the results of incubation of the isozymes at 37°C at pH 6 and pH 10. At pH 10, the more acidic isozymes were less stable (Fig. 13, A). Comparison of the stability of isozymes #1 and #2 at the two pH values shows that the two most basic forms are less stable at acidic pH than at basic pH (Fig. 13, B). These results agree with the results from bovine GPI isozymes which showed that the more acidic forms had little changes in catalytic properties but exhibit markedly decreased stability (Cini *et al.*, 1988).

Figure 13. The pH stability of GPI isozymes. Isozymes, 0.5-2 $\mu\text{g/ml}$, were incubated in 10 mM TEA, 5 mM BME and 5 mM EDTA at 37°C, (A) at pH 10 in 10 mM CAPS. (B) Comparison of isozyme #1 and #2 at pH 6 and pH 10. Results are from three determinations.

(A)



(B)



CHAPTER IV

DISCUSSION

Purification of Multiple Isoforms of Glucose 6-Phosphate Isomerase

There are numerous reports of the advantages of specific ligand-protein interactions for the purification of proteins. For example, aldolase and fructose 1,6-diphosphatase have been purified by the use of substrate-induced elution of proteins from ion exchange columns (Gracy *et al.*, 1969, Pogell and Sarngadharan, 1971). These methods give rapid and efficient purification of these enzymes with high recovery. Phosphocellulose has traditionally been used for the ion exchange isolation of many proteins. It has also been used to purify GPI by substrate-induced elution (Gracy and Tilley, 1975). During GPI purification, phosphocellulose acts not only as a cation exchanger, but also as a specific affinity column since the properties of phosphocellulose are similar to the substrates of GPI (glucose 6-phosphate and fructose 6-phosphate). Thus, the enzyme specifically binds to the phosphocellulose column and is eluted by its substrates.

There are two kinds of phosphocellulose used to purify

proteins, fibrous and cross-linked spherical particulate phosphocellulose. The fibrous phosphocellulose ion exchangers have poorer flow properties due to their irregular shape. Thus, when this ion exchanger is used, much longer time is needed to wash and remove contaminated proteins and elute the enzyme from the column. This in turn increases the probability of modification by proteolytic digestion and denaturation, and results in lower yields. The cross-linked spherical particulate phosphocellulose has greater uniformity in particulate size, shape and porosity. This leads to improved flow properties and higher capacities for macromolecules, thereby minimizing the time of protein-phosphocellulose and protein-protein interactions during the chromatographic process and consequently enhances the potential for separation and recovery. In the present study we have examined both fibrous and cross-linked spherical particle phosphocellulose. The results are in agreement with the above hypothesis. GPI can be purified more rapidly with higher recovery by the cross-linked spherical particle phosphocellulose, requiring only one-sixth the amount of time and improving recovery by about 35% relative to fibrous phosphocellulose (Table II).

An interesting phenomenon found during the elution was that when GPI was eluted from the phosphocellulose column, the isozymes containing subunit A (higher pI) eluted before the isozymes containing subunit B (lower pI). Based strictly

on principles of ion exchange chromatography one would expect the opposite, i.e. that the protein with lower pI should elute from the cation exchanger prior to the proteins with higher pI. Thus, the isoforms clearly were bound to the phosphocellulose column by means other than ion exchange, probably specific affinity effects. The specific elution with substrate presumably is the result of the formation of a complementary enzyme-substrate complex (Pogell and Sarngadharan, 1971). Thus, these data further support that the phosphocellulose column presents the properties of a specific affinity column, and suggest that subunits A & B have similar but not identical active site structures that result in complementary binding of substrates with different affinities.

Multiple electrophoretic isoforms of GPI have been reported depending upon the species from which the enzyme is isolated (Gracy, 1982, Cini and Gracy, 1986). In the present work, GPI from human placenta was also found to exhibit multiple isozyme patterns upon electrophoresis or IEF. Two different electrophoretic patterns of human placental GPI were obtained depending on the different purification procedures employed (Fig. 7). IEF showed the presence of two isozyme bands when GPI was purified on fibrous phosphocellulose, and four isozyme bands when cross-linked phosphocellulose was used. Thus, the question to be answered

was "are the two additional isoforms artifacts produced by the cross-linked phosphocellulose, or are they lost or inactivated upon chromatography on the conventional fibrous phosphocellulose ion exchanger?". The properties of the isozymes are shown in Table III and Fig.7. The difference in the IEF pattern is not only due to the charge difference, but also the presence of two kinds of subunits with different molecular properties, such as, molecular weight, immunoreactivity, etc. The different properties of the two subunits (see next section) suggest that these isozymes are not artificially produced. Rather, during the more lengthy isolation procedure using fibrous phosphocellulose, the enzyme may be more easily denatured and/or dissociated into subunits (especially the more labile dimers: isozymes #3 and #4), and enzymic activity may not be detected when the enzyme is eluted from the column. This may explain why these isozymes were not previously detected in studies in which the enzyme was purified by chromatography on fibrous phosphocellulose or by even less selective, more lengthy procedures (Lu and Gracy, 1981, Tilley et al., 1974, Carter and Yoshida, 1969, Gibson et al., 1977)

Characterization of the Isoforms and Subunits of Glucose 6-phosphate Isomerase

In this work, four isozyme forms were isolated. The two more basic isozymes (#1 and #2), appeared to be dimers of the same size subunit. Isozyme #1 with pI 9.13 was in agreement with the common major GPI of previous studies (Tilley *et al.*, 1974, Gracy, 1982, Baumann and Brand, 1988). This suggested that isozyme #2 (AA' or A'A', pI 9.0) was formed from isozyme #1 (AA) by posttranslational modification, and the difference was only due to charge. The most acidic isozyme (#4, pI 8.62) was a homodimer with B subunit. Only one isoform (isozyme #3, pI 8.84) appeared to be a heterodimer (AB or A'B). This may have been because the subunit B could only form a heterodimer with the modified (A') or unmodified (A) subunit but not both.

In addition to the differences in physical properties, some differences were found in the catalytic activities and stabilities of the isozymes. Comparing the dimers (BA and BB) that contain the B subunit, with the dimers (AA and AA') containing the A subunit, it was clear that the isoforms containing the A subunits had significantly higher catalytic activity (Table II), and stability (Fig. 13, a) than those containing the B subunit. Both types of dimers containing B subunit (AB and BB) had lower catalytic activity (Table II),

and stability (Fig. 13, a), with the BB homodimer being the lowest. (The possible reasons for these differences are discussed below.)

To further understand the properties of these isoforms, the data from hybridization studies should be considered. In a randomly associating system, two different types of monomers which have the same binding affinity should result in three different dimers at ratio of 1:2:1 (homodimer I:mixed heterodimer:homodimer II) (Widmer et al., 1986, Gilbert and Solter, 1985, Peterson and Wong, 1978, Tilley et al., 1974). However, this pattern was not observed in our experiments. In the case of the four isozymes observed in the present study, the ratio (on a protein basis) was 10:7:3:1 (for isozymes #1,#2,#3,#4, respectively). Hybridization studies of GPIs have shown that active dimers of GPI can be formed from GPI subunits of different species or genetic variants. For example, human and rabbit GPIs can hybridize to form a heterodimer that has enzyme activity (Bruch et al., 1976, Gracy and Schnackerz, 1977). However, the human-rabbit heterodimers dissociate much easier than the rabbit or human homodimers. This suggests that the hybrids do not form all of the intersubunit contacts as do the homodimers. In contrast, human GPI can not hybridize with yeast GPI which is less homologous with mammalian GPI [$< 60\%$ amino acid sequence homology (Chaput et al., 1988, Green,

1988)].

Besides the above hybridization considerations, comparison of the basic properties of the two subunits may also help to further understand these isoforms. The basic physical and chemical properties (e.g. molecular weight, pI, and amino acid composition) of the subunit A (which accounts for approximately 90% of the isolated protein), were found to be in good agreement with previous studies which characterized GPI from human erythrocytes or muscle tissue (Lu and Gracy, 1981, Tilley et al., 1974, Carter and Yoshida, 1969, Tsuboi et al., 1971). In contrast, subunit B (more acidic isozymes), which accounts for less than 10% of the total isolated protein, exhibited a distinctly larger molecular weight (69.8 Kdal), with distinct immunoreactivity and CNBr cleavage peptide mapping, and amino acid composition (Tables III, IV, and Fig.7, 11).

The above data taken together suggest that the two human GPI subunits, A and B, differ in their affinity for each other in forming active dimers. The subunit B may have lower binding affinity to form an active dimer and more easily dissociate to inactive monomer like the labile heterodimer of human and rabbit GPI. The lower specific activity of the isozymes which contain subunit B may also be partly due to the differences in the subunit interface.

Relation of Glucose 6-Phosphate Isomerase and Neuroleukin

A comparison of GPI and NLK is shown in Table V. Since the two proteins have not both been isolated and characterized from the same source, the comparison can only be made between the human GPI and the mouse NLK. From the table we can see that both proteins have blocked N-termini and similar pI values, but they are found in different cellular locations and have very different physiological functions. The other obvious difference is that GPI is a dimer and the dimeric quaternary structure is required for catalytic activity, whereas NLK is a monomer.

It would be interesting to know whether either or both GPI and NLK are glycoproteins. The glycosylation might provide an answer as to how NLK is secreted from the cell, and might also explain the differences between some isoforms of GPI that present variant pI and molecular weight (Baumann and Brand, 1988). Recently the primary structures of GPI from pig muscle and yeasts were determined. For pig GPI, three potential N-linked glycosylation sites (Asn-X-Ser/Thr) were observed, whereas yeast GPI had five (Green, et al., 1988, Chaput et al., 1988). Mouse NLK has 4 potential N-linked glycosylation sites as well (Chaput et al., 1988). Although the potential N-linked glycosylation sites have been recognized in GPI sequences of pig and yeasts, it has not been

Table V
Comparison of Human GPI and Mouse NLK

Property	GPI ^a	NLK ^b
Localization	intracellular	extracellular
Function	enzyme	neurotrophic factor and immunomodulator
Mr (Kdal)	130	56
Quarternary structure	dimer	monomer
pI	9.2	8.5-9.0
N-terminal	blocked	blocked
Nucleotide sequence	(?)	90% homology with pig GPI
Glycosylation	(?)	(?)

a. Gracy, 1982, Lu and Gracy, 1981

b. Chaput, et al., 1988, Gurney et al., 1986, Hallbook,
et al., 1989

determined whether or not GPI is indeed glycosylated.

It has been reported that pig muscle GPI shares 90% nucleotide sequence homology with mouse NLK (Chaput *et al.*, 1988), but the functional and genetic relationship between these proteins is still unknown. One protein-dual function situations are known to exist. For example, it has recently been discovered that several lens structural proteins (crystallins) are active enzymes (Wistow and Piatigorsky, 1987). τ -crystallin is, in fact, an enolase (Wistow and Piatigorsky, 1987, Williams *et al.*, 1985, Giallongo *et al.*, 1981), and ϵ -crystallin is a functional lactate dehydrogenase (LDH), apparently identical to LDH-B4 (Wistow *et al.*, 1987). This raises a question whether GPI and/or NLK represent single gene products with dual functions. Thus, it will be interesting to determine whether: 1) NLK has GPI activity; 2) GPI has neurotrophic and/or immunomodulatory properties ascribed to NLK; and 3) the two proteins are both products of the same gene. The nucleotide sequence homologies suggest that both GPI and NLK could be coded by the same gene. Thus, it has been suggested that NLK (which is a monomer with MW 56 kdal) is derived from GPI subunits (MW 63 kdal) possibly by proteolytic cleavage. Baumann and Brand (1988) have found some cancer-associated variants of GPI with variant molecular weight from gastrointestinal carcinoma. They suggested that the variants were due to a specific intracellular cleavage of

the enzyme in the malignant cells.

That GPI and NLK are coded by two independent but similar structural genes can not be ruled out. Gene duplications may initially release one locus from selection while the other maintains its previous function. The new locus is then free to undergo modifications through chromosomal rearrangements and mutations to assume new functions. Lysozyme and α -lactalbumin are good examples of gene duplication with functional divergence (Creighton, 1983, Campbell and Smith, 1988). Lysozyme functions in the hydrolysis of β -1,4- glycosidic bonds, whereas α -lactalbumin regulates lactose synthesis by binding to galactosyl transferase. Both proteins are derived from a common ancestral gene, and are strikingly similar in protein and gene structures (Campbell and Smith, 1988). During the course of evolution the properties of the two proteins have diverged and α -lactalbumin lost lysozyme activity and became a regulatory protein with respect to lactose synthesis.

Recent studies of GPI and its isoforms emphasize the clinical significance of GPI in diagnosis of cancer. Baumann *et al.* and coworkers (Baumann and Brand, 1988, Baumann *et al.*, 1988) showed that the cancer-associated variants of GPI could be used as tumor markers in monitoring cancer patients, and the assay of serum activity of GPI presented highly diagnostic sensitivity and specificity for screening of gastrointestinal cancer, even for early diagnosis. Since the

cancer-associated variants identified by Baumann and Brand (1988) have the same molecular weight as NLK (56 kdal), the specific cleavage of GPI might be responsible for the conversion of GPI to both trophic factor and cancer-associated variants. These require us to further study the properties of GPI and understand the relationship of these proteins. However, the physiological function of the protein we have designated in the present study as subunit B is unknown. This also raises a question whether NLK could be modified and bound with a subunit of GPI, thus forming a dimer. If this is true, the second question would be what the physiological significance of binding could be, and whether it is involved with cancer-associated variants of GPI. The relation between GPI, subunit B, and NLK is still an open question. Further experiments to test the relation of these proteins to one another as well as other above-mentioned questions are now in progress in this laboratory.

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