THE MEMBRANE ASSOCIATION AND DISSOCIATION OF HUMAN GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE UNDER VARIOUS CONDITIONS OF HEMOLYSIS

Immunochemical evidence for the lack of binding under cellular conditions

D. MARETZKI*, J. GROTH**, A.G. TSAMALOUKAS*, M. GRÜNDEL**, S. KRÜGER* and S. RAPOPORT*

* *Institut für Biologische und Physiologische Chemie der Humboldt-Universität, Hessische Str. 3/4, 104 Berlin, Germany, and*

** *Sektion Biologie der Humboldt-Universität, Invalidenstr. 43, 104 Berlin, Germany*

Received 26 October 1973

1. Introduction

Several authors reported an association of the GAPD to the cell membrane of red cells [1–5]. The varying extent of this association suggested to us a dependence on conditions of hemolysis and recorded the question of its artefactional nature. Therefore we investigated the membrane association and dissociation of the GAPD by means of immunochemical and other techniques under various conditions of hemolysis.

2. Materials and methods

Ghosts from human erythrocytes were prepared by hypotonic hemolysis according to Dodge et al. [6] and from sonicated red cells under isotonic conditions (10 W/cm^2, 1 min in 130 mM KCl, 20 mM NaCl, 2 mM EDTA and 2 mM mercaptoethanol, pH 7.2). In addition sonication was used in combination with hypotonic hemolysis in presence of 2 mM EDTA and 2 mM mercaptoethanol.

Sonicated ghosts were centrifuged in a Spinco model L 2-65 B at 40 000 rpm for 30 min and 4°C. The ghosts were washed five times with hemolysis medium and homogenized repeatedly in a Potter-homogenizer. The ghosts were solubilized by a modified method of Miller [7] (1% Triton X-100, 37°C, 1 hr, 10 mM phosphate buffer, pH 7.4).

2.1. *Preparation of antisera*

Rabbits were injected with a solution of the human GAPD from erythrocytes [8]: 10 mg/ml of the NAD-complex in 5 mM phosphate, pH 7.0; 0.9% NaCl; 0.1 mM dithiothreitol and 0.1 mM EDTA mixed with an equal volume of Freund's complete adjuvant (Difco, Chicago).

Each rabbit was injected witwa total of 6 mg. The first injection of 2 mg was applied in the popliteal lymph nodes. The second injection, 16 days later, subcutaneously with complete adjuvant and a third injection without adjuvant 90 days later.

Antisera to Triton X-100-solubilized ghosts were prepared in a similar manner. Ten mg total membrane protein per rabbit was injected with complete Freund's adjuvant in the popliteal lymph nodes. A second 10 mg portion without adjuvant was given intramuscularly 14 days later. The antisera were collected 10 days after the second or third injection respectively.

Immunodiffusion was carried out in 1% agarose according to Ouchterlony [9]. Immunoelectrophoresis was performed according to Scheidegger [10]. Hemoglobin was estimated by its absorbance at 415 nm. LDH activity was measured according to Phillips [11].

The standard assay for GAPD-activity measurements was as follows: 50 mM imidazole; 150 mM KCl; 1.3 mM NAD; 25 mM phosphate; 2 mM mercaptoethanol; 2 mM EDTA and 20 µl hemolysate at pH 7.2. The reaction

was started by addition of glyceraldehyde-3-phosphate in the range of 0.1–2 mM. The increase in absorbance at 334 nm was recorded with an Eppendorf-photometer equipped with a Phillips-recording apparatus. The initial velocities of NAD reduction were extrapolated.

3. Results

3.1. *Reaction of antiserum to human erythrocyte GAPD*

In the Ouchterlony-test (fig. 1) the antiserum precipitated the purified GAPD and formed well visible lines with various hemolysates. The complete fusion of the precipitates indicates the monospecificity of the antiserum. The precipitates of isotonic hemolysates were always distinctly visible.

As shown in table 1 the GAPD activity in the supernatant of isotonic hemolysates was significantly higher than under hypotonic conditions. These results demonstrated the well-known effect of ionic strength on the GAPD-retention by human ghosts [5].

Table 1
Total activity of the GAPD in various hemolysates at pH 7.2 in presence of 2 mM EDTA and 2 mM mercaptoethanol.

With stroma		Stroma free		
Osmotic	Isotonic (freezing–thawing)	Osmotic	Isotonic (freezing–thawing)	% GAPD-retention
3110	3060	1720 1760	3030 2880	45 38

Isotonic conditions: 130 mM KCl; 20 mM NaCl, pH 7.2; V_{max} was calculated from double reciprocal plots by glyceraldehyde-3-phosphate variation (see method).

Fig. 2 shows a comparison of the double immonodiffusion from ghosts of the hypotonic hemolysates with those obtained under approximately intracellular conditions (ionic strength and pH). The solubilized ghosts were tested against monospecific antiserum to human erythrocyte GAPD. The ghosts obtained by hypotonicity combined with sonication and solubilized, were precipitated in the same way as the purified erythrocyte GAPD. In contrast the solubilized ghosts obtained by isotonic sonication were not precipitated.

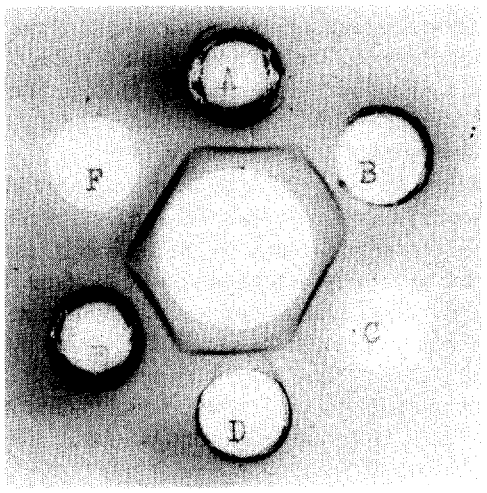

Fig. 1. Double immonodiffusion in 1% agarose: 50 mM glycine–NaOH, pH 9.1; 2 mM EDTA; 2 mM mercaptoethanol. The central trough is filled with antiserum to human erythrocyte GAPD: A, C and E – purified human erythrocyte GAPD; B – hypotonic hemolysate; D – hypotonic sonicated hemolysate; F – isotonic sonicated hemolysate.

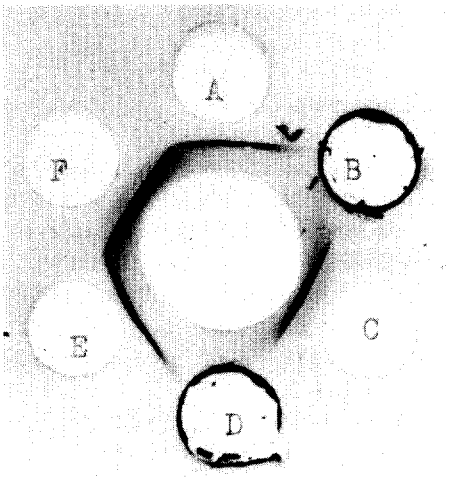

Fig. 2. Double immunodiffisuion, conditions as fig. 1: A and E – hypotonic sonicated ghosts (pH 7.2), Triton X-100 solubilized; B and D – isotonic sonicated ghosts, Triton X-100 solubilized; C and F – purified human erythrocyte GAPD.

3.2. *Fractionation of ghosts*

Ghosts obtained by hypotonic hemolysis and prepared under hypotonic conditions were solubilized with Triton X-100 [7]. They were eluted either with hypotonic or isotonic buffer on a Sephadex G-200 column (fig. 3). The eluate was divided into six fractions. Under hypotonic conditions of elution the GAPD was demonstrated to be associated with molecular fractions higher than 250 000 daltons by the antiserum to erythrocyte GAPD (fig. 4A fractions 1 and 2).

Under isotonic conditions the GAPD was found in fractions 4 and 5 (fig. 4B). This was to be expected according to nearly identical molecular weights of 145 000 daltons for both enzymes.

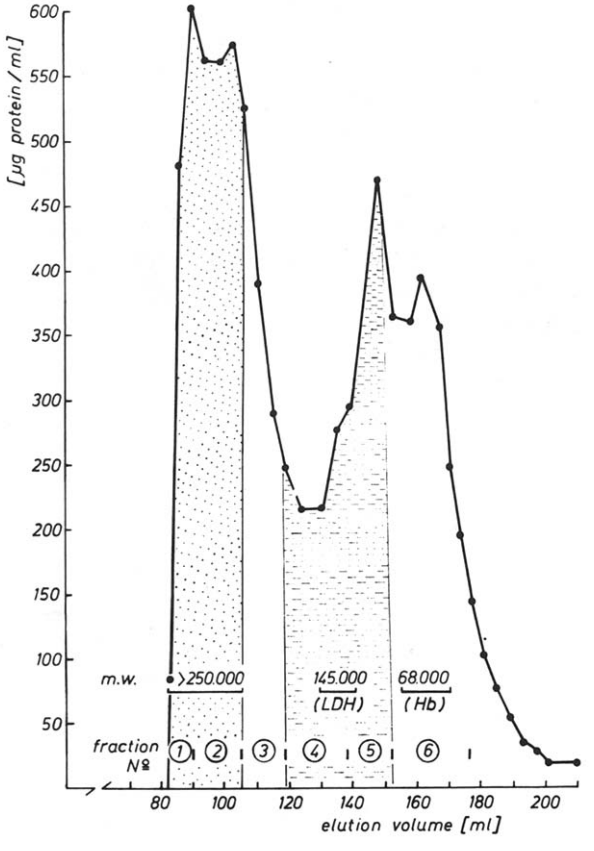

Fig. 3. Gel-filtration on Sephadex G-200 (2.5 × 90 cm column) of Triton X-100 solubilized ghosts. Hemolysis and preparation under hypotonic conditions. Elution into 6 fractions either under hypotonic or isotonic conditions in each case 50–60 mg ghost protein in 5 ml 10 mM phosphate, pH 7.4 and 1% Triton X-100 were put on the column. Hypotonic elution buffer: 2.5 mM phosphate, pH 7.4; 0.2% Triton X-100. Isotonic elution buffer: 125 mM phosphate, pH 7.4; 0.2% Triton X-100.

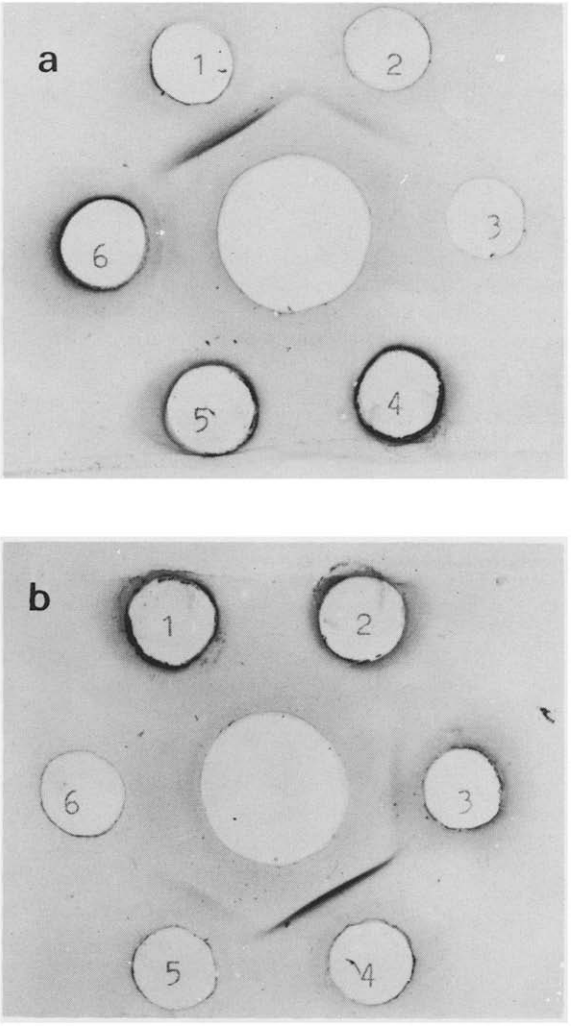

Fig. 4. Demonstration of the GAPD in the 6 fractions of Sephadex G-200 gel filtration (fig. 3) by double immonodiffusion against antiserum to human erythrocyte GAPD: A – precipitates in fractions 1 and 2 under hypotonic conditions of elution; B – precipitates in fractions 4 and 5 under isotonic conditions of elution.

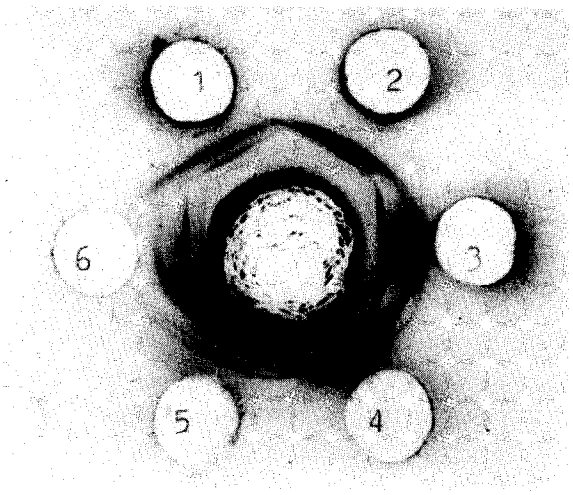

Fig. 5. Heterogeneity of 6 fractions of Sephadex G-200 gel-filtered solubilized ghosts under hypotonic and isotonic conditions (fig. 3). Double immunodiffusion of the fractions against antiserum to whole ghosts (central well).

3.3. *Reaction of antiserum to ghosts*

The fractions of the Sephadex G-200 gel-filtration were tested for heterogeneity by means of antiserum to solubilized human erythrocyte ghosts.

From the Ouchterlony-test (fig. 5) it would appear that fractions 1 and 2 are identical. The purified erythrocyte GAPD was not precipitated by antiserum to solubilized ghosts while the admixture of antiserum to erythrocyte GAPD produced an arc specific for the enzyme (fig. 6).

4. Discussion

Our results demonstrate that under approximate intracellular conditions (ionic strength, pH) the association of GAPD with erythrocyte membrane was not detectable. The large amount of GAPD associated with the membrane under hypotonic conditions can be dissociated by isotonic salt concentrations at pH 7.2. It appears to be an artefact caused by the osmotic hemolysis. This was shown by immunochemical testing of solubilized ghosts and ghost fractions with monospecific antiserum to GAPD.

The nonionic detergent Triton X-100 does not influence the weak interactions between protein and the membrane [12]. The advantage of the immunochemical methods for detection of GAPD lies in the apparent lack of interference by the detergent which is reported to affect the enzyme activity [3, 4]. The lack of GAPD association with the membrane under

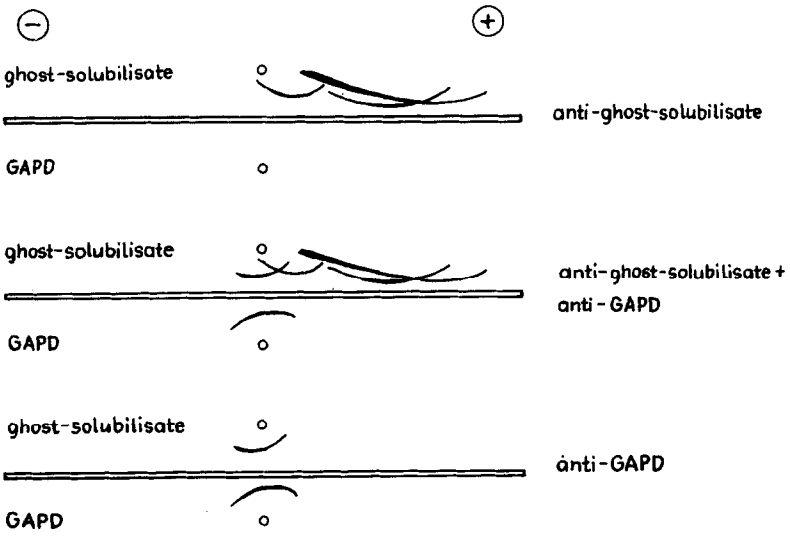

Fig. 6. Immunoelectrophoresis of Triton X-100-solubilized whole ghosts and purified human erythrocyte GAPD. Conditions: 1% agarose; 10–125 mM phosphate, pH 7.4.

intracellular conditions is supported by further observations that whole isotonic hemolysates exhibited a high GAPD-activity in the supernatant (table 1) and a stronger precipitation line than observed under hypotonic conditions (fig. 1).

Shin and Carraway [3] reported 60–80% membrane retention of the GAPD under hypotonic hemolysis conditions whereas we found 38–45%. The differences are due to the addition of 2 mM EDTA to our hemolysis medium. Reynolds and Trayer [15] observed 70–90% solubilization of membrane proteins by 5 mM EDTA.

The possibility that sonication rather than isotonicity was responsible for the lack of membrane association of the GAPD under isotonic conditions is contradicted by the results of the hemolysis by sonication under hypotonic conditions. A large amount of GAPD was found to be associated with hypotonic sonicated ghost preparation.

The ghost fractionation on Sephadex G-200 shows that under isotonic conditions which seems to represent spectrin mainly [3, 14] to which it is associated under hypotonic conditions (fig. 4A).

It was also possible to demonstrate the release of the enzyme from hypotonically obtained ghosts in the immunoelectrophoresis (fig. 6). The liberated GAPD was found as a single line separated from the precipitated ghost-proteins. From these data we conclude that the membrane binding of the GAPD is due to the weak ionic interactions under hypotonicity. GAPD-binding is based on the cationic charge of the erythrocyte enzyme at pH 7.2 (isoelectric point of the human erythrocyte GAPD is 8.2 detected by isoelectric focusing) [13] and the anionic charge of membrane proteins indicated by immunoelectrophoresis (fig. 6).

The amino acid composition of spectrin shows as reported by Marchesi et al. [14] 31.4% glutamate and aspartate and 12.5% arginine and lysine of total amino acids.

A dependence of protein binding to strome on ionic strength (0–75 mosm) and pH (5.4–8.2) was reported by Mitchell et al. [5]. These authors concluded that the enzymes are not structurally important members of the membrane.

Shin and Carraway [3] showed that ATP and 2,3-DPG concentrations promote the release of GAPD from the hypotonic isolated membranes.

The lack of association of GAPD with the membrane under cellular conditions indicates that the kinetic behaviour of the GAPD in the cell corresponds to that in vitro. Furthermore any substrate compartmentation of GAP, NAD, NADH and 1,3-DPG can be excluded.

For investigation of protein binding with erythrocyte membranes it is necessary to use approximated intracellular conditions and therefore sonication under these conditions is recommended for hemolysis and ghost preparation.

Under hypotonic conditions of hemolysis and ghost preparation the enzyme binding is an artefact.

References

[1] Schrier, S.L. (1963) J. Clin. Invest. 42, 756–766.
[2] Tanner, M.J.A. and Gray, W.R. (1971) Biochem. J. 125, 1109–1117.
[3] Shin, B.C. and Carraway, K.L. (1973) J. Biol. Chem. 248, 1436–1444.
[4] Duchon, G. and Collier, H.B. (1971) J. Membrane Biol. 6, 138–157.
[5] Mitchell, C.D., Mitchell, W.B. and Hanahan, D.J. (1965) Biochim. Biophys. Aca 104, 348–358.
[6] Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[7] Miller, D.M. (1970) Biochem. Biophys. Res. Commun. 40, 716–722.
[8] Maretzki, D. and Rapoport, S. (1972) Acta Biol. Med. Germ. 29, 207–221.
[9] Ouchterlony, Ö. (1967) in: Handbook of Experimental Immunology (Weir, D.M., ed.), pp. 655–705, Blackwell, Oxford and Edinburgh.
[10] Scheidegger, J.J. (1955) Intern. Arch. Allerge 7, 103–110.
[11] Phillips, R.E. (1971) in: Manual of Fluorometric Clinical Methods, G.K. Turner Associates.
[12] Mazia, D. and Ruby, A. (1968) Proc. Natl. Acad. Sci. U.S. 61, 1005–1012.
[13] Maretzki, D., Tsamaloukas, A.G., Küttner, G., Krüger, S., Groth, J. and Rapoport, S. (1973) VII[th] International Berlin Symposium on Structure and Function of Erythrocytes, Akademie-Verlag, Berlin.
[14] Marchesi, S.L., Steers, E., Marchesi, V.T. and Tillack, T.W. (1970) Biochemistry 9, 50–57.
[15] Reynolds, J.A. and Trayer, J. (1971) J. Biol. Chem. 246, 7337–7342.