2,3-DIPHOSPHOGLYCERATE SYNTHESIS BY HUMAN WHITE GHOSTS

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1. Introduction

Indirect evidence shows that the human erythrocyte membrane is involved in the fast regulation of DPG-levels* [1,2]. This paper gives direct proof that haemoglobin-free ghosts are capable of active DPG- and ATP-synthesis from FDP. Evidence that this activity is not artifactual is also presented.

2. Materials and methods

White ghosts were prepared from freshly drawn heparinized human blood by hypotonic haemolysis with 0.1 mM EDTA, pH 7.5 followed by 5 washings with 17 mM Tris plus 0.1 mM EDTA, pH 7.5 [3]. Two low-speed centrifugations (3000 rev/min for 10 min in the Sorvall SS-34 rotor) were intercalated in order to eliminate debris and non-haemolized cells. The white ghosts were used intact or after sonication (Branson Sonifier, 20 sec at maximum output). In one experiment, ghosts were prepared from gently sonicated red cells under isotonic conditions according to Maretzki et al. [4]. The composition of the incubation fluids is specified in the legend to table 2. At selected intervals, perchloric acid extracts were made by mixing 2 ml ghost solution with 2 ml 6% (w/v) perchloric acid and neutralized with 10 n KOH. DPG was assayed by the method of Rose and Liebowitz [5], ATP and the glycolytic intermediates were measured according to standard enzymatic techniques [6]. Glycolytic enzyme activities were assayed in sonicated ghosts according to Pescarmona et al. [7]. Total ATPase activity was checked by the assay of inorganic P released and DPG-phosphatase activity was followed by measuring the ^{32}P release from radioactive $D^{32}PG$. Details on both assay methods will be presented elsewhere. Stripped Hb was prepared according to Benesch et al. [8]. Ghosts were counted with a model DN 3 Coulter counter fitted with 50 μ dia aperture tube.

The materials were obtained from the following sources: NAD^+, $NADP^+$, NADH, ATP, ADP, AMP, Tris: Sigma Chem. Co. (St. Louis, Mo., USA); all other biochemicals and crystalline enzymes: C. F. Boehringer (Mannheim, West Germany); all other chemicals were Merck (Darmstadt, West Germany) reagent grade products.

3. Results and discussion

3.1. *DPG formation by white ghosts*

As shown in table 1 aldolase, triosephosphate iso-

* *Abbreviations:* DPG=2,3-diphosphoglycerate; FDP=fructose-1,6-bisphosphate; PFK=6-phosphofructokinase (EC 2.7.1.11); ALD=fructosebisphosphate aldolase (EC 4.1.2.13); TIM=triosephosphate isomerase (EC 5.3.1.1); GAPDH=glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12); PGK=phosphoglycerate kinase (EC 2.7.2.3); PK=pyruvate kinase (EC 2.7.1.40); LDH=lactate dehydrogenase (EC 1.1.1.27); 3-PG=3-phosphoglycerate; GSH=glutathione, reduced form.

Table 1
Enzyme activities in white ghosts in erythrocytes

	Ghosts		Erythrocytes
	μmoles/g dry weight/hr	μmoles/10^{10} ghosts/hr*	μmoles/10^{10} cells/hr
PFK	n.d.	n.d.	230 ± 40 n=6
ALD	3434 ± 240 n=5	44	117 ± 11 n=6
TIM	2105 ± 422 n=6	27	57500 ± 6420 n=6
GAPDH	150450 ± 18380 n=7	1936	3023 ± 648 n=6
PGK	1373 ± 202 n=7	18	4171 ± 678 n=6
PK	n.d.	n.d.	334 ± 29 n=6
LDH	n.d.	n.d.	2261 ± 434 n=6

* Conversion factor: see table 2.
n: number of experiments.
n.d.: not detectable.

Table 2
Production of DPG,ATP and 3-PG by sonicated white ghosts under different incubation conditions

Incubation* medium	pH		DPG	ATP	3-PG
I	7.5	A**	128 ± 11	40 ± 7	198 ± 33
		B	1.6	0.5	2.5
II	7.5	A	173 ± 7	106 ± 17	220 ± 21
		B	2.2	1.4	2.8
III	7.5	A	272 ± 105	520 ± 162	503 ± 98
		B	3.5	6.7	6.5
III	8.5	A	372 ± 60	568 ± 85	635 ± 122
		B	4.8	7.3	8.2

* Composition of the media (mmol/l); I: Na^+ 25, K^+ 142, Mg^{++} 2.5, inorg.phosphate 0.8, HCO_3^- 93, Cl^- 84, NAD^+ 0.5, ADP 0.25, AMP 0.25, FDP 2,3-PG 0.1, GSH 3, TRA–HCl 50, pH 7.5. II: Na^+ 100, K^+ 20, Mg^{++} 2.5, inorg.phosphate 10, SO_4^{2-} 10, Cl^- 90, NAD^+ 0.5, ADP 0.25, AMP 0.25, FDP 2, 3-PG 0.1, GSH 3, TRA–HCl 50, pH 7.5. III: Na^+ 100, K^+ 20, Mg^{++} 2.5, inorg.phosphate 10, SO_4^{2-} 10, Cl^- 90, NAD^+ 2, ADP 1, FDP 2, TRA–HCl 50, pH 7.5 or 8.5.

** A: mean production in μmoles/g dry weight/hr at 37°C (mean of 5 experiments ± SD). B: mean production in μmoles/10^{10} ghosts/hr at 37°C calculated from A (mean conversion factor: $77.7 \cdot 10^{10}$ ghosts/g dry weight).

merase and glyceraldehyde-phosphate dehydrogenase are very active in our ghost preparation: FDP was therefore used as the substrate. Intact white ghosts transformed FDP to DGP,ATP and 3-PG: however, since intact ghosts are only incompletely permeable to substrates, much lower rates than with sonicated ghosts were observed. For this reason our study was performed with sonicated ghosts. As shown in table 2, sonicated ghosts very actively from DPG,ATP and 3-PG from FDP. The amounts formed are dependent on the composition and pH of the incubation fluid: highest rates were obtained with 1 mM ADP and 10 mM inorganic phosphate at pH 8.5. Incubation at nearly intracellular conditions (incubation medium I) reduced DPG production to about $^1/_3$ of maximal rates. Addition of 10 mM inorganic phosphate to medium I almost doubled DPG-buildup (not shown). The rate of DPG-formation was linear up to 60 min in all conditions.

Besides inorganic phosphate, which activates GAPDH by a mass-action effect, ADP and 3-PG play a regulatory role (fig.1); in the absence of ADP, no ATP is formed, due to the block of the PGK-reaction and the strongly reduced formation of DPG is probably due to the limited availability of 3-PG, which is co-substrate for the DPG-mutase reaction. While ADP primarily influences the ATP-buildup, DPG-synthesis is particularly sensitive to very small changes in 3-PG; for example, a drop in 3-PG of about 20 μmol/l is followed by a 50% diminution in DPG-synthesis.

3.2. *ATPase and DPG-phosphatase activity in white ghosts*

Both ATPases and DPG-phosphatase activities were very low in our ghost preparations: maximally 23 μmol/g dry weight/hr and 19 μmoles/g dry weight/hr were split respectively.

3.3. *Possibility of artifacts*

The DPG-synthesis clearly depends on the presence of sufficient GAPDH-activity on the membrane. GAPDH belongs to the tightly bound enzymes [9] and sticks in a specific manner on the inner face of the membrane [10]; however, conditions such as high ionic strength [4], ATP [11] or NAD^+ [12] in millimolar concentrations have been shown to dissociate GAPDH from the membrane. To exclude the possibility that DPG-formation was due to the artifactual presence of GAPDH on the membrane, 1.5 mM ATP was added to ghosts

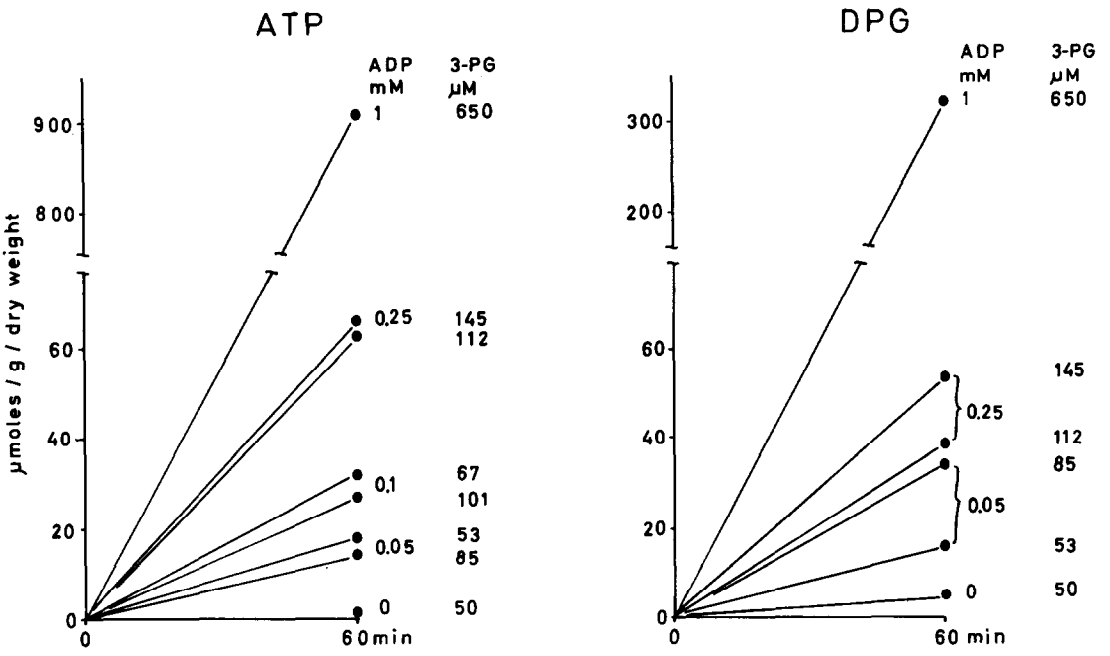

Fig.1. Effect of ADP and 3-PG on the net synthesis of DPG and ATP by white ghosts. The ghosts were incubated 60 min with medium III (see table 2) at pH 7.5 and 37°C.

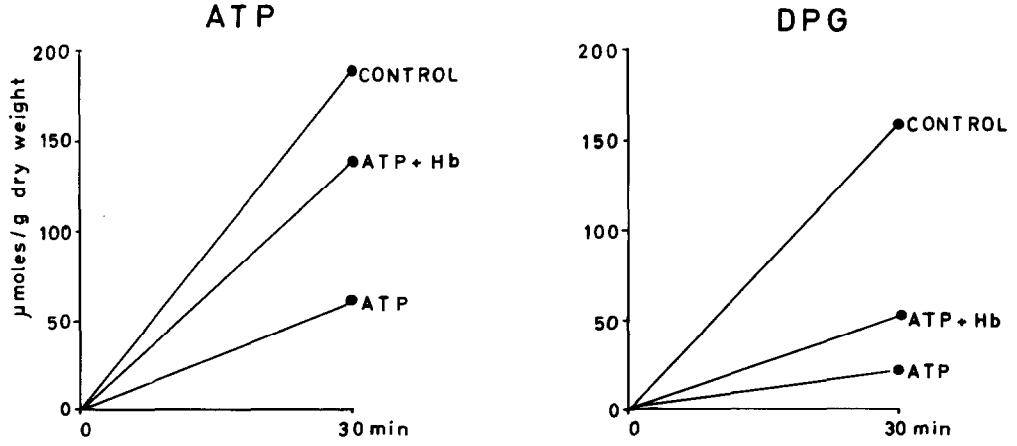

Fig.2. Effect of the pre-incubation with ATP or ATP plus stripped Hb on the net synthesis of DPG and ATP by white ghosts. The ghosts were pre-incubated 13 hr at +4°C and pH 7.5 with 0.1 mM EDTA (controls), 0.1 mM EDTA plus 1.5 mM ATP (ATP), and 0.1 mM EDTA plus 1.5 mM ATP and stripped Hb (ATP + Hb). After two washings with 17 mM Tris plus 0.1 mM EDTA, pH 7.5, the ghosts were incubated 30 min with medium III (see table 2) at pH 7.5 and 37°C.

suspended in 0.1 mM EDTA, pH 7.5 ('leaky ghosts') and washed out after 13 hr at +4°C. As shown in fig.2, the pre-treatment with ATP reduced ATP-formation by about 70% and DPG-synthesis by about 80%. However, if equimolar amounts of ATP and stripped haemoglobin were used, the effects were much less evident: this shows that addition of ATP without haemoglobin is in a sense artifactual as well.

Ghosts prepared by sonication of erythrocytes in isosmotic medium were also used: this treatment ensures complete detaching of GAPDH. Even in these conditions, however, a DPG-synthesis of 34 μmoles/g dry weight/hr was measured.

3.4. *Relevance of red cell membrane in DPG metabolism*

Our data indicate that a substantial portion of the total DPG-production may be synthesized by the membrane system. On a ghost number basis, about 1.6 μmoles DPG/10^{10} red cells/hr (at pH 7.5 and under physiological ion and substrate concentrations) is formed. This amount equals or even exceeds the total DPG-building capacity of the intact human red cell incubated under comparable conditions, assuming a lactate production of 3.3 μmoles/10^{10} red cells/hr and 35–50% of the glycolytic flux going through the DPG-cycle [13]. The DPG-building role of ghosts gives a metabolic sense to the very high activity of the corollary enzymes on the membrane; the separation between building-site (membrane) and storage-site (cytoplasm) explains why the red cell can still synthesize DPG even in the presence of DPG levels that completely inhibit DPG mutase [14].

The ghost preparation described here would seem to be a suitable system for studying the mechanism of action of hormones [15,16] and drugs [17,18] known to influence DPG-levels in vivo.

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