# GLUCOSE-6-PHOSPHATE ISOMERASE FROM EHRLICH ASCITES TUMOR CELLS: PURIFICATION AND SOME PROPERTIES

Klaus-Heinrich RÖHM and Friedhelm SCHNEIDER
*Physiologisch-chemisches Institut der Universität Marburg, Marburg/Lahn, Germany*

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## 1. Introduction

Whereas extensive information is available on glucose-6-phosphate isomerases (EC 5.3.1.9) purified from mammalian tissues such as skeletal muscle of rabbit [1], beef [2] and pig [2] as well as from baker's [3] and brewer's yeast [4] no attempt has been made so far to purify the enzyme from ascites tumor cells which are characterized by high glycolytic rates. Continuing previous experiments on the *in vitro* kinetics of glucosephosphate isomerase and application of the results to the calculation of glycolytic flux e.g. of the anaerobic yeast cells [5] we have attempted to isolate the enzyme from Ehrlich ascites tumor cells. The present paper describes these experiments; some properties of the purified preparation and kinetic parameters of the reaction catalyzed by the ascites tumor enzyme are also given.

## 2. Materials and methods

Glucose-6-phosphate dehydrogenase from yeast, phosphofructokinase from rabbit muscle, glucose-6-phosphate (disodium salt), fructose-6-phosphate (disodium salt), NADP and ATP were purchased from Boehringer, Mannheim. The reference proteins for the molecular weight determination were obtained from the same company except L-asparaginase from *E. coli* which was a gift of Bayer, Wuppertal-Elberfeld.

Buffer substances and most of the other chemicals were of analytical reagent grade from Merck, Darmstadt. The sodium salt of cacodylic acid was purchased from Roth, Karlsruhe, the free acid was prepared by treatment with Lewatit S 100 cation exchanger and recrystallized from ethanol.

*2.1. Protein concentrations* were determined by the method of Lowry [6] or by the Biuret reaction [7].

*2.2. Activity* of glucosephosphate isomerase in the reverse reaction (fructose-6-phosphate → glucose-6-phosphate) was usually determined by coupled enzymatic assay with glucose-6-phosphate dehydrogenase [8] in 50 mM Tris-HCl buffer, pH 8.5, (brought to J = 0.1 by addition of KCl) at 30°. The formation of NADPH was followed at 340 nm in a Zeiss PMQ II spectrophotometer equipped with a recorder. In the forward direction (glucose-6-phosphate → fructose-6-phosphate) the pH-stat assay of Dyson and Noltmann [9] was used. In a total volume of 2 ml the reaction mixture contained KCl (85 mM), $MgSO_4$ (5 mM), ATP (5 mM), phosphofructokinase (1–2 units), glucose-6-phosphate in variable amounts and glucosephosphate isomerase (0.2 units or less). The titrant, usually 5 mM NaOH, and all solutions were flushed with argon to avoid any interference from atmospheric $CO_2$. A standard pH-stat combination (Radiometer, Copenhagen) was applied.

The actual concentrations of all substrate stock solutions were measured by enzymatic assay in each case.

*2.3. Equilibrium constant* of the reaction was obtained by following the approach to equilibrium from both sides. The reaction was started with isomerase, aliquots were removed from the reaction mixture and mixed with perchloric acid. The supernatants obtained by neutralization with KOH and centrifugation were analyzed for glucose-6-phosphate and fructose-6-phosphate.

The kinetic constants and their standard errors were evaluated with the aid of a Telefunken TR 4

digital computer (Zentrale Rechenanlage, University of Marburg) using Cleland's program HYPER [10] in a slightly modified form.

*2.4. Disc electrophoresis* was carried out at pH 5.2 using a system recently described by Pearce et al. [11] with cacodylic acid and 6-amino hexanoic acid as buffer substances. The direction of migration was toward the cathode. With the usual alkaline buffer systems glucosephosphate isomerase shows insufficient mobility because of its high isoelectric point.

*2.5. Isoelectric focussing* was performed in a LKB 8101 column (LKB, Bromma) following the standard procedure recommended by the manufacturer. The carrier ampholyte used covered the pH range from 7 to 9 and was applied in a concentration of 1%.

## 3. Results and discussion

### *3.1. Purification*

All steps were performed at about 4°. The results of a typical purification procedure are summarized in table 1. Cells from 25 mice were combined in this experiment.

Step 1, *Extraction*: Ehrlich ascites tumor cells (the strain was obtained from Karzel [12]) were collected from albino mice 9 days after inoculation. The cells were isolated by centrifugation, washed twice with 0.9% NaCl and stored frozen.

The cells were ground with 100 g of sea sand and 30 ml of extraction buffer (0.15 M sodium acetate, pH 5.0, containing 2% (v/v) glycerol and 1 mM mercaptoethanol) in a mortar for 5 min. Further 170 ml of the same buffer were added, after stirring for 10 min the mixture was centrifuged at 4500 *g* (30 min). The sediment was re-extracted twice with 100 ml buffer each. The combined supernatants were allowed to stand overnight whereby some precipitate was formed.

Step 2, *CM-Sephadex chromatography*: The crude extract was centrifuged at 10 000 *g* for 30 min and the precipitate discarded. The extraction buffer was exchanged for 5 mM sodium phosphate buffer, pH 6.9, (adsorption buffer) by 'diafiltration' (ultrafiltration with simultaneous replacement of solvent by the desired buffer). A hollow fiber device (Amicon, Oosterhout) was used. The resulting solution was stirred with 50 g (wet weight) of CM-Sephadex (Pharmacia, Uppsala) pretreated with adsorption buffer for some days. The gel was collected by filtration and the filtrate treated with further 30 g of CM-Sephadex. The combined gel batches were suspended in 100 ml of adsorption buffer and the mixture poured into a 3 × 15 cm column. After draining off the excess buffer the enzymatic activity was eluted from the column within 400 ml of a linear NaCl gradient (0–100 mM) in adsorption buffer. The active fractions were pooled.

At this step the maintenance of pH and ionic strength is crucial. Even small deviations from the given values will cause failure of binding and/or desorption of the enzyme.

Step 3, *Isoelectric focussing*: The enzyme solution from step 2 (about 100 ml) was reduced in volume by ultrafiltration to 10 ml diluted with water to the initial volume and again reduced to 10 ml. The resulting sample was used to make up the 'light solution' of the sucrose density gradient in isoelectric focussing. The columns were run four days at 500–800 V, fractions of approx. 1 ml were collected and analyzed for protein and enzymatic activity.

The result of a run is shown in fig. 1. Glucosephosphate isomerase activity is resolved into three peaks with isoelectric points of 8.6, 8.8 and 9.0, respectively. The enzyme is essentially pure after step 3, as is demonstrated by disc electrophoresis (see fig. 2). Again three peaks with slightly different mobilities are separated.

Table 1

| Step | Protein (mg) | Activity * (IU) | Specific activity (IU/mg) | Purification | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 1130 | 3150 | 2.4 | 1 | 100 |
| CM-Sephadex | 11 | 1750 | 160 | 67 | 55 |
| Isoel. focussing | 2 | 750 | 380 | 160 | 24 |

* Reverse reaction, pH 8.5, 30°.

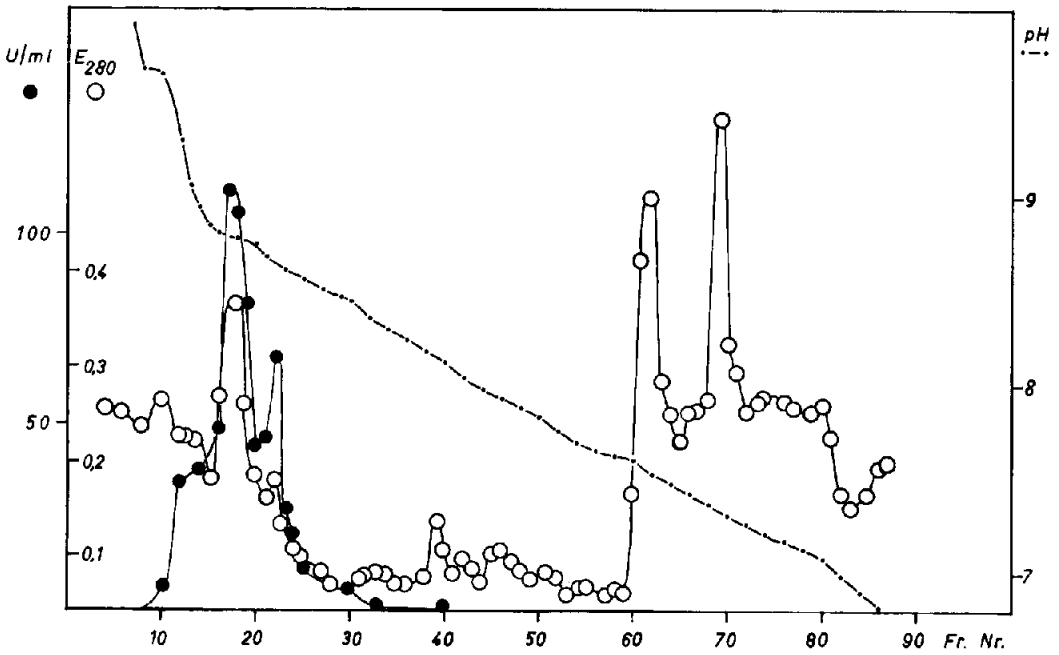


Fig. 1. Elution profile after isoelectric focussing of partially purified glucosephosphate isomerase. pH (.–.–.), the $A_{280}$ (o—o—o) and enzymatic activity (•—•—•) are plotted vs. the fraction number. For details see text.

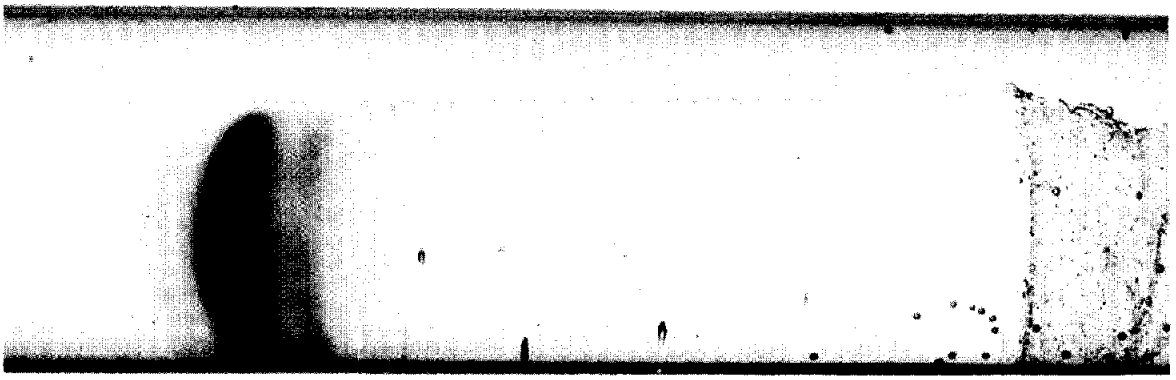

Fig. 2. Disc electrophoresis of glucosephosphate isomerase after isoelectric focussing. Separation at pH 5.2 with the buffer system of Pearce et al. [11]. The sample was an aliquot of the pooled fractions 14–23 (see fig. 1). Stain: Amido black.

Their electrophoretic mobility related to cytochrome *c*, used as marker, is about 0.4 in a 7.5% polyacrylamide gel at pH 5.2.

The most active fractions obtained after step 3 had specific activities of up to 520 U/mg.

### *3.2. Molecular weight*

The enzyme was eluted from a 1 × 42 cm Sephadex G-200 column (equilibrated with 0.1 M sodium phosphate buffer, pH 7.0) immediately subsequent to lactate

Table 2
Kinetic parameters of glucose-6-phosphate isomerase from Ehrlich ascites tumor cells at 30° and pH 8.5.

| | $K_m$* (M) | $V_{max}$** (U/mg) |
|---|---|---|
| Forward reaction (G-6-P → F-6-P) | $2.5 \pm 0.5 \times 10^{-4}$ | 92 ± 4 |
| Reverse reaction (F-6-P → G-6-P) | $1.16 \pm 0.1 \times 10^{-4}$ | 131 ± 3 |

* Mean of 4 independent determinations for each direction.
** Determined with a partially purified enzyme.

dehydrogenase (MG 125 000) with a $K_{av}$ value of approx. 0.35. From this a molecular weight of 110 000 ± 10 000 daltons can be estimated [13]. Further reference proteins used were catalase, aldolase, asparaginase from *E. coli*, bovine serum albumin and ovalbumin. The void volume was determined with dextran blue.

### *3.3. Kinetics*

The Michaelis constants $K_m$ and the maximal velocities $V_{max}$ of the forward and the reverse reaction are summarized in table 2.

The equilibrium constant $K_{eq}$ = [F-6-P]/[G-6-P] was found to be $K_{eq}$ = 0.30 at pH 8.5 and 30°. From the constants given in table 2 a Haldane constant $K_H = V_f K_r / V_r K_f = 0.33$ can be calculated. Therefore the Haldane relationship $K_{eq} = K_H$ does hold with glucosephosphate isomerase from ascites tumor cells as in the case with isomerase from other sources [2, 5].

The enzyme described here is obviously nearly related to glucosephosphate isomerase from rabbit muscle investigated in great detail by Noltman and coworkers [2]. The kinetic constants as well as the chromatographic behaviour are very similar. With both enzymes multiple forms are observed which seem to be however 'pseudo isoenzymes' as was shown for the rabbit enzyme by Blackburn et al. [14]. Finally, the molecular weights determined by gel permeation-chromatography are identical [15].

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