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PURIFICATION OF GLYCOGEN SYNTHETASE-D FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

L. PLESNER, E. SALSAS LEROY*, P. WANG, M. ROSELL PEREZ AND V. ESMANN

The Department of Medicine, Marselisborg Hospital, 8000 Århus C (Denmark) and Catedra de Bioquimica de la Facultad de Farmacia, Universidad de Barcelona, Barcelona (Spain)

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SUMMARY

Suspensions of pure polymorphonuclear leukocytes were prepared from normal human blood. The cells were found to be intact and suited for metabolic studies. Glycogen synthetase-D (UDPG:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) was purified from large batches of cells by a procedure consisting of a differential centrifugation, resulting in a 50 000 × *g* glycogen synthetase pellet, and subsequent ion-exchange chromatography on DEAE-Sephadex. The purification obtained was 2000-fold in protein and 20-fold in glycogen.

It was observed that Tris and EDTA interfere with protein determination and that EDTA is chromatographed on the anion-exchange column, yielding high concentrations of EDTA in the eluate.

The purification in glycogen allowed a study of the influence of both UDPG and glycogen on the enzymatic reaction. K_m for UDPG was 0.042 mM ± 0.003 in six experiments from three preparations of enzyme. Preliminary results have indicated that the influence of glycogen on the reaction rate is apparent only at concentrations of glycogen less than 5 μg/ml.

INTRODUCTION

The enzyme glycogen synthetase (UDPG:α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) has previously been found present in freshly prepared homogenates of human polymorphonuclear leukocytes only in the glucose 6-phosphate-dependent, or D form[1], whereas homogenates of polymorphonuclear leukocytes from insulin-treated human diabetics[2], normal and alloxan diabetic rats[3] and human lymphocytes[4] possessed synthetase-D phosphatase activity, leading to a D to I conversion during incubation of the homogenate. Recently, however, what appears to be a D to I conversion has also been demonstrated in the intact normal human polymorphonuclear leukocyte[5].

Early experiments by ESMANN[6] showed that intact polymorphonuclear leuko-

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cytes incubated with glucose synthesize glycogen for at least 2 h, however, with diminishing rate. The possibility that both the I and D forms of synthetase are active in glycogen synthesis cannot be definitely settled with the present knowledge of the kinetics of the polymorphonuclear leukocyte enzyme. Glycogen synthesis by the D-enzyme has been found in yeast[7].

The present communication describes the purification of synthetase-D from human polymorphonuclear leukocytes, and preliminary kinetic data on the bisubstrate reaction with UDPG and glycogen.

MATERIALS AND METHODS

Preparation of polymorphonuclear leukocytes

For the preparation of one batch of crude enzyme, 1000–2000 ml of freshly drawn blood of compatible serotypes was obtained from the blood bank and processed by a slight modification of the procedure of Rabinowitz[8]. In principle, the erythrocytes of heparinized blood are sedimented at 37 °C over a period of 45 min by the agglomerulating action of dextrane in a final concentration of 1%. The supernatant plasma with the white cells, platelets and remaining red cells was applied to a column of siliconized glass beads and incubated at 37 °C for 30 min. Elution of the column with fresh plasma followed by a Krebs–Ringer bicarbonate buffer with 16.7 mM glucose and 20% plasma removed the lymphocytes and the platelets and remaining red cells from the polymorphonuclear leukocytes and monocytes, which stuck to the glass beads. Subsequent elution of the column with a Tris–phosphate buffer[10] without Ca^{2+} and Mg^{2+}, but with 0.54 mM EDTA and 16.7 mM glucose released the polymorphonuclear leukocytes and in turn also the monocytes. The preparation time was 5 h. To increase the yield of polymorphonuclear leukocytes, we found it profitable to modify the original method by adjusting the flow rate to a maximum of 4 ml/min during the entire elution prior to the collection of polymorphonuclear leukocytes, the latter, however, taking place at a flow rate of 40 ml/min. With this modification, 10 consecutive experiments gave an average recovery of 46.8% (range 36–57) of the polymorphonuclear leukocytes present in the donor blood with an average contamination of 3% mononuclear cells. The preparation contained no platelets and no red cells.

Addition of glucose to the buffers used during the elution procedures ensured that the cells retained their viability and a sufficiently high glycogen content for the purification of synthetase-D. The practically pure suspensions of polymorphonuclear leukocytes prepared in this way are useful not only for enzyme preparations but also for incubation experiments with intact cells. The cells are, however, more prone to clump than in previous preparations of 85% pure polymorphonuclear leukocytes prepared within 45 min[9]. Leukocytes were incubated in a Tris–phosphate buffer with Ca^{2+} and Mg^{2+} and the respiration, determined by direct Warburg manometry, was linear over a period of 3–4 h in 16 consecutive experiments, except in one experiment where heavy clumping occurred. The cells exhibited the usual crowding effect[9], and the oxygen uptake at 10^7 cells/ml and at 37 °C was 160 μmoles/10^{10} polymorphonuclear leukocytes per h, which is similar to previous results on mixed populations prepared within 45 min[9]. Also, in 6 experiments control of leakage of enzymes to the extracellular medium demonstrated that 0–4% of the total intracellular activity of

the enzymes hexokinase and lactate dehydrogenase, determined by conventional enzymatic methods[10], appeared extracellularly after 2–4 h of incubation of the cells in buffer unless clumping occurred, in which case leakage was 10%. Finally, phase-contrast microscopy showed that the cells had retained their morphological integrity and ameboid movements.

The polymorphonuclear leukocytes eluted from the column and used for enzyme preparation were washed once with 50 mM Tris–HCl + 5 mM EDTA (pH 7.8) and centrifuged. The resulting cell button was stored at −19 °C and no loss of synthetase activity was observed after storage for several weeks.

Enzyme purification

All operations were carried out at 0–5 °C. Several batches of cells, obtained as described above, were resuspended in 50 mM Tris–5 mM EDTA (pH 7.8), pooled and ultrasonically disintegrated[1]. The first step in the purification procedure consisted of three subsequent centrifugations at 2000 × *g* for 15 min, 12 000 × *g* for 15 min, and 50 000 × *g* for 60 min, respectively. The last centrifugation sediments glycogen as a pellet and it is important to control the speed of this centrifugation carefully or the main part of the synthetase activity will remain in the supernatant. The 50 000 × *g* pellet can be stored for months at −20 °C without loss of activity.

The second step in the purification procedure was an ion-exchange chromatography. The 50 000 × *g* pellet was resuspended in 1–2 ml of Tris–EDTA buffer with 0.075 M NaCl, mechanically homogenized and applied to a DEAE-Sephadex A-25 anion-exchange column, which previously had been equilibrated by continuous elution with the 50 mM Tris–5 mM EDTA–75 mM NaCl buffer supplied from a beaker (Beaker I) connected to the column by plastic tubing. After application of the sample and passage of the void volume, the NaCl concentration in the eluting liquid was gradually increased by connecting a second beaker, (Beaker II) containing 1 M NaCl in buffer, to Beaker I in such a way that the surfaces of the liquid in the beakers were at the same level. Complete mixing was secured by magnetic stirring in Beaker I. The flow rate from Beaker I through the column was 10 ml/h, which was attained by a Perpex pump (LKB 10200) connected to the column outlet. The steepness of the gradient was determined by the starting volumes in the beakers, smaller volumes yielding steeper gradients. The NaCl concentration must increase by at least 25 mM/h in order to obtain a reasonably narrow peak of enzyme activity. A "broken" gradient (see Fig. 2) was obtained by interrupting the flow from Beaker II to Beaker I for the required period.

Analytical procedures

Glycogen synthetase was determined by the filter paper method of Thomas *et al.*[11] and phosphorylase was assayed with a procedure described elsewhere[12]. The reaction mixture in the latter procedure contained 6 μmoles [U-^{14}C]glucose 1-phosphate (specific activity 10^5 cpm/μmole), 0.9 mg glycogen, 0.09 μmole AMP, 9 μmoles NaF, 6 μmoles piperazine-*N*,*N*′-bis-(2-ethanesulphonic acid) buffer (pH 6.4), and 30 μl enzyme solution in a total volume of 90 μl. After 10-min incubation at 30 °C, 75 μl was withdrawn from the reaction mixture and spotted on a 2 cm × 2 cm of Whatman No. 31 ET filter paper. The paper was then immediately immersed in ice-cold 66% ethanol and washed till the glucose 1-phosphate has been removed from

the papers. After drying, the radioactive glycogen precipitated on the papers was counted in glass scintillation vials containing 10 ml of 0.5% PPO in toluene. The method is analogous to the method described by Thomas *et al.*[11] for the assay of glycogen synthetase. The addition of 1 mM AMP activated the leukocyte phosphorylase approximately 40%.

Glycogen was hydrolyzed by boiling the samples 135 min with HCl in a final concentration of 0.6 M under a nitrogen atmosphere in sealed glass vials to avoid evaporation. The resulting glucose was determined enzymatically with hexokinase and glucose 6-phosphate dehydrogenase. The method allows determination of 15 μg glycogen per ml.

Protein was determined by the method of Lowry *et al.*[13] with human serum albumin as standard. However, determination of protein in the eluate from the DEAE anion-exchange column presented a special problem. In principle, the Lowry method is capable of measuring protein in solution with a sensitivity of 2 μg/ml, but the presence of EDTA and Tris severely interferes with protein determinations, in particular the Lowry method, as has been detailed elsewhere[14]. We have solved the problem by first lyophilizing the eluates and redissolving in a smaller volume in order to facilitate protein precipitation, which was carried out with 5 vol. of 10% trichloroacetic acid followed by centrifugation at 200 000 $\times$ *g* for 2 h. The protein was redissolved in 500 μl 0.2 M NaOH and the extinction measured with double strength Lowry reagents. For the preparation of standard curves, it was found necessary to add glycogen to the standards in the same concentration as in the sample to be measured.

EDTA was determined as follows. To a sample volume of 0.4 ml was added 1 ml of NH_4^+/NH_3 buffer (pH 10). This solution was titrated with a $MgCl_2$ solution after the addition of a few drops of a 0.1% aqueous solution of the indicator Eriochrome. The colour change was from blue to red at the end point of the titration. 1 mmole of EDTA was titrated by 0.93 mmole of $MgCl_2$.

Materials

The columns used for cell separation measured 40 cm with a diameter of 2.5 cm. UDP[^{14}C]glucose and [U-^{14}C]glucose 1-phosphate were purchased from The Radiochemical Centre, Amersham, England, and rabbit liver glycogen, UDPG and AMP from Sigma, St. Louis, Mo., U.S.A. Glucose 1-phosphate, glucose 6-phosphate and enzymes were from Boehringer, Mannheim, D.B.R. DEAE-Sephadex A-25, Sephadex G-200, G-25 and Sepharose 2B, as well as the columns used for ion-exchange chromatography and desalting ($K^{15}/_{30}$, diameter 15 mm, length 30 cm) were products of Pharmacia, Uppsala, Sweden. Piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid) monosodium monohydrate buffer was supplied by Calbiochem, and Eriochrome Black T, C. 1. 203 by Merck, Darmstadt, D.B.R.

RESULTS

Table I and Fig. 1 show the details of a representative purification of synthetase-D in which $5 \cdot 10^9$ of polymorphonuclear leukocytes were processed. The 50 000 $\times$ *g* pellet contains 75% of the glycogen and synthetase activity present in the crude homogenate and represents a 14-fold purification based on protein. The amount of

TABLE I

PURIFICATION TABLE

Fraction	*Vol. (ml)*	*Synthetase-D*		*Protein*			*Glycogen*			*Synthetase-D*		
		Concn (nmoles/ml per min)	*Total (nmoles/min)*	*Concn (mg/ml)*	*Total (mg)*	*Spec. act. (nmoles/mg per min)*	*Concn (mg/ml)*	*Total (mg)*	*Spec. act. (nmoles/mg per min)*	*Recovery (%)*	*Purification*	
											In protein	*In glycogen*
Crude homogenate	24	249	5976	27	648	9.2	2	48	125	100		
2000 × *g* pellet	20	31	620	9	180	3.4	0.38	7.6	82	10		
2000 × *g* supernatant	20	286	5720	22	440	13	2	40	143	96	1.4	1.1
12 000 × *g* pellet	18	42	756	9.5	171	4.4	0.36	6.5	116	13		
12 000 × *g* supernatant	18	263	4734	14	252	19	1.9	34.2	138	79	2	1.1
50 000 × *g* pellet	2	2240	4480	18	36	124	16.8	33.6	133	75	14	1.1
50 000 × *g* supernatant	18	2	36	13	234	0.15	0.05	0.9	40	1		
DEAE fraction	6.7	210	1407	0.0097	0.065	21 600	0.08	0.54	2606	24	2354	21

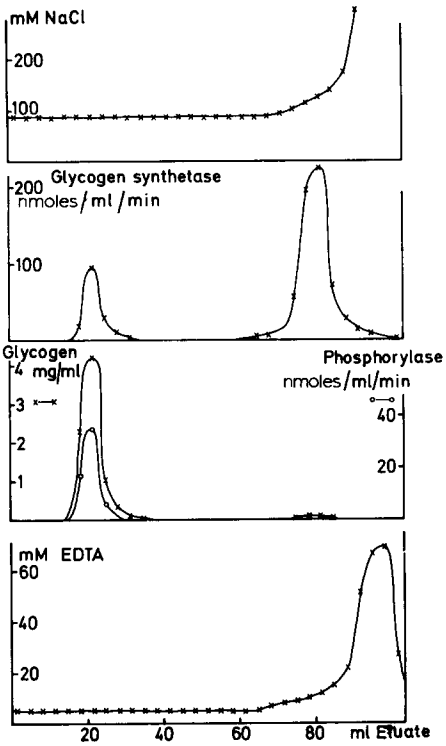

Fig. 1. Elution diagram of a DEAE-Sephadex A-25 chromatography of the 50 000 × *g* pellet from a differential centrifugation of a homogenate of polymorphonuclear leukocytes.

enzyme activity lost in the discarded 2000 × *g* and 12 000 × *g* pellets may be reduced by using more dilute cell suspensions. No I activity, nor any D to I transformation of the enzyme can be demonstrated in the 50 000 × *g* pellet.

The result of the final purification on the DEAE column (Table I) is based upon a protein determination, which was carried out on 3.6 ml of a pool of 6.7 ml eluate, representing almost all of the eluted enzyme activity (Fig. 1). Practically all the glycogen leaves the column in the void volume. The concentration of glycogen in the enzyme fraction is occasionally so low that the accurate determination required for kinetic experiments necessitated lyophilization and redissolving in a smaller volume.

In the experiment shown in Fig. 1 there is no phosphorylase activity present in the synthetase fraction. Already the crude homogenate contains only approx. 10% of the phosphorylase activity normally present in the cells[12] and during the centrifugations and ion-exchange chromatography the recovery of phosphorylase activity steadily declines, so that the amount of phosphorylase found in the anion-exchange eluate is only 2% of the activity present in the crude homogenate. It is not known if inactivated phosphorylase is eluted in the anion-exchange chromatography. However, in some cases, phosphorylase activity is found present also in the synthetase fraction, suggesting that separation of the two enzymes requires special precautions (*cf.* below).

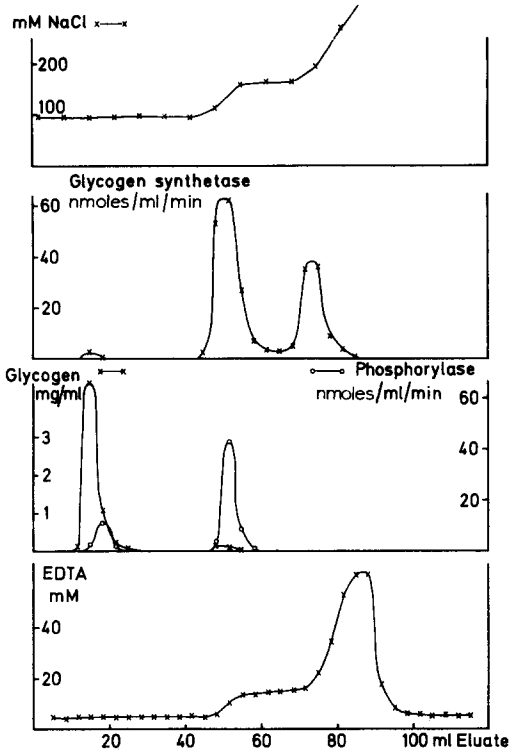

Fig. 2. Elution diagram as in Fig. 1, but with the application of a broken NaCl gradient.

Fig. 2 illustrates the elution pattern from another batch of cells. In this case, a "broken" NaCl gradient was employed for elution of the enzyme, resulting in the recovery of synthetase-D in two peaks, representing 13% and 8% of the activity of the starting material, respectively. Phosphorylase activity was in this case found present in the void volume as well as in the first synthetase peak. This pattern has repeatedly been observed by the application of a broken gradient. The second peak of synthetase-D activity is presumably free of phosphorylase and glycogen. It did not contain enough protein or glycogen in a volume of 2.4 ml for reliable determination.

Both Figs 1 and 2 demonstrate that EDTA is retained on the DEAE column and is eluted by the NaCl gradient along with proteins. The NaCl concentration displacing the enzyme from the columns depends upon the degree of equilibration of the anion exchanger before use.

For kinetic experiments, the eluates with synthetase-D activity are pooled, lyophilized and the excess EDTA and Tris buffer removed by chromatography on a Sephadex G-25 Fine column, which is equilibrated and eluted with 50 mM Tris–HCl–5 mM EDTA (pH 7.8).

Attempts have been made to purify the enzyme further. Using a Sephadex G-200 Fine column the enzyme activity, however, appeared in the middle of the protein peak, and inserting a Sepharose 2B column before the DEAE column gave

some purification with respect to protein, but the procedure led to a considerable loss of activity.

Kinetic studies

The separation of the enzyme from glycogen in the described purification procedure permits the investigation of the enzymatic reaction as a bisubstrate reaction, which will be detailed in a later publication.

However, the influence of the concentration of substrate UDPG on the reaction rate in 6 consecutive experiments is shown in Fig. 3. Initial velocities were measured in the presence of 10 mM glucose 6-phosphate and 10 mM Mg^{2+}. The average K_m for UDPG was 0.042 mM $\pm$ 0.003 (*cf.* legend to Fig. 3 for calculation). The kinetic experiments were carried out at glycogen concentrations between 0.025 and 10 mg/ml. Within this concentration range K_m' of UDPG was not affected.

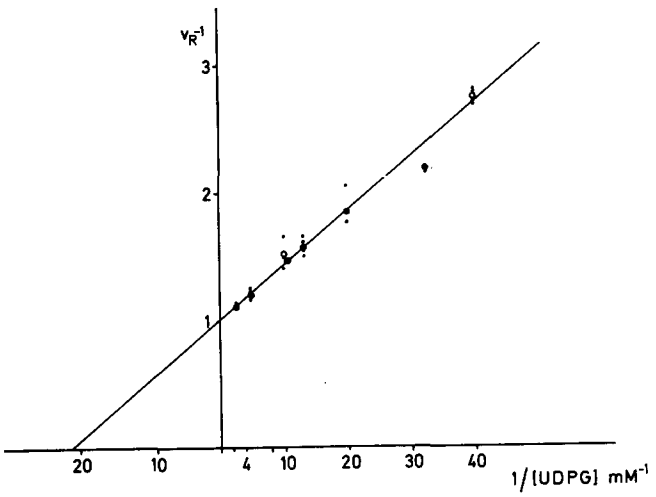

Fig. 3. "Reduced" Lineweaver–Burk plot. v_R is a "reduced" velocity, *i.e.* the measured initial rate divided by the V corresponding to the particular kinetic run. This plot permits experiments with enzymes from different batches and with different specific activities of substrate UDPG to be presented in the same diagram. The line is calculated by fitting a hyperbola according to Wilkinson[15] to the average "reduced" velocities, the reciprocals of which are indicated by circles. These average velocities, in turn, are obtained as (unweighted) means of 1–5 kinetic runs. The dots in the diagram are the reciprocals of these individual (reduced) initial rates. The substrate concentration is in mM.

Working with concentrations of UDPG close to the K_m, the necessary dilution of the enzyme source decreases its glycogen content to nanograms. Preliminary data indicate that a glycogen concentration of less than 5 μg/ml is necessary before significant effect on the reaction rate can be detected.

DISCUSSION

Since, in 1962, the anion-exchange technique was adapted by Rosell Perez[16] for purification of glycogen synthetase, this procedure has been the one most frequently employed. In the present work, two technical problems have been brought

to light. It is detailed in another publication[14] that Tris and EDTA, which are commonly used in enzyme purification procedures, heavily interfere with methods for protein determination and especially make the Lowry method unreliable. This problem has in our experiments necessitated the use of a comparatively large fraction of the purified enzyme for protein determination, which was carried out by precipitating the protein and removing the supernatant containing Tris and EDTA. The precipitation had necessarily to be preceeded by lyophilization and dissolving in a smaller volume in order to obtain complete protein precipitation. The enzyme is stable during the latter procedure.

The other point of technical interest is that EDTA is chromatographed on the ion-exchange column and eluted together with protein. Unpredictably large concentrations of EDTA are present in the eluate, compromizing, among other things, studies on divalent cation activators.

The anion-exchange chromatography is able to separate the bulk of glycogen from synthetase-D, but the purified enzyme is contaminated with a small amount of glycogen and possibly also by the inactive dephospho form of leukocyte phosphorylase, both of which appear removable by the application of a "broken" salt gradient, but only at the cost of more than half of the enzyme activity. The scarcity of material has prevented us from attempting further purification by other methods. The purification obtained, more than 2000-fold in protein and 20-fold in glycogen is, however, sufficient for kinetic analysis.

K_m for UDPG was 0.042 mM $\pm$ 0.003 in the presence of 10 mM glucose 6-phosphate and 10 mM Mg^{2+}, which is more than ten times lower than the previously reported value of 0.6 mM obtained in this laboratory on crude homogenates, and also lower than values reported for other tissues[4,17–20]. The new K_m of 0.042 mM is lower than the concentration (0.1 mM) of UDPG in the cells[21] and supports the possibility that also the D form of synthetase is active in glycogen synthesis in leukocytes. However, information concerning the kinetic properties and actual cell concentrations of activators and inhibitors of the D-enzyme must be at hand before definite conclusions as to the *iv vivo* activity of synthetase-D can be drawn.

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