Biochimica et Biophysica Acta, 1119 (1992) 74–80

BBAPRO 34098

Purification and properties of human glucose-6-phosphate dehydrogenase made in *E. coli*

José M. Bautista *, Philip J. Mason and Lucio Luzzatto

Department of Haematology, Royal Postgraduate Medical School, London (U.K.)

(Received 27 June 1991)

Key words: Glucose-6-phosphate dehydrogenase; Expression; Purification; (*E. coli*)

The cDNA for the X-chromosome encoded human glucose-6-phosphate dehydrogenase (G6PD) has been expressed in *E. coli* and the enzyme purified to homogeneity, using a simple one-step fractionation on 2′5′-ADP-Sepharose. By selecting one of several different expression vectors and by optimizing culture conditions a yield of more than 10 mg of pure enzyme per liter of culture is obtained reproducibly. When the recombinant enzyme and authentic G6PD purified from normal human red cells were compared, they proved to be indistinguishable by the following criteria: electrophoretic mobility in both native and denaturing conditions, the K_m values for glucose 6-phosphate and NADP and the K_i value for NADPH. The recombinant enzyme, unlike the red cell enzyme, retained 100% activity when stored at 4 °C for over 1 year.

Introduction

The human enzyme glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) has been of great interest to haematologists since it was discovered [1] that genetically determined G6PD deficiency in red cells underlies a variety of haemolytic anaemias (reviewed by Beutler, Ref. 2). Subsequently, G6PD emerged as a unique system in human genetics because up to 300 genetic variants were reported, many of them having polymorphic frequencies in various human populations. This high degree of polymorphism is probably a result of malaria selection (reviewed by Luzzatto and Battistuzzi, Ref. 3). The primary structure of human G6PD has been deduced from the nucleotide sequence of cDNA [4], and the structure of the X-linked gene encoding the enzyme has been worked out in detail [5]. Insertion of the G6PD cDNA in eukaryotic [6] and prokaryotic [7] expression vectors leads to the production of functional and immunologically reactive human G6PD. The mutations responsible for a number of polymorphic and deficient G6PD variants have been determined by DNA sequencing [8,9]. Correlation between the amino acid sequence and the properties of the variant enzymes has enabled the identification of some important amino acids [6,10]. However a full understanding of the relationship of structure to function awaits the determination of the tertiary structure. Attempts to crystallize the enzyme have been hampered by the limited amounts of pure protein that can be obtained from human blood.

In the present paper we present a very efficient system for the production of human G6PD in *E. coli* and a simple and rapid method for purifying the enzyme to homogeneity. The biochemical properties of this recombinant G6PD are indistinguishable from those of purified human red cell G6PD and the recombinant G6PD is much more stable than that purified from red cells.

Experimental procedures

Plasmid constructions

All DNA manipulations were carried out by standard procedures [11].

* Present address: Departamento de Bioquímica y Biología Molecular IV, Universidad Complutense de Madrid, Facultad de Veterinaria, Madrid, Spain.

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; rhG6PD, recombinant human glucose-6-phosphate dehydrogenase; RBC-G6PD, human erythrocyte glucose-6-phosphate dehydrogenase; G6P, glucose-6-phosphate. ϵ-ACA, ϵ-amino caproic acid.

Correspondence: P.J. Mason, Department of Haematology, Royal Postgraduate Medical School, Duncane Road, London W12 ONN, U.K.

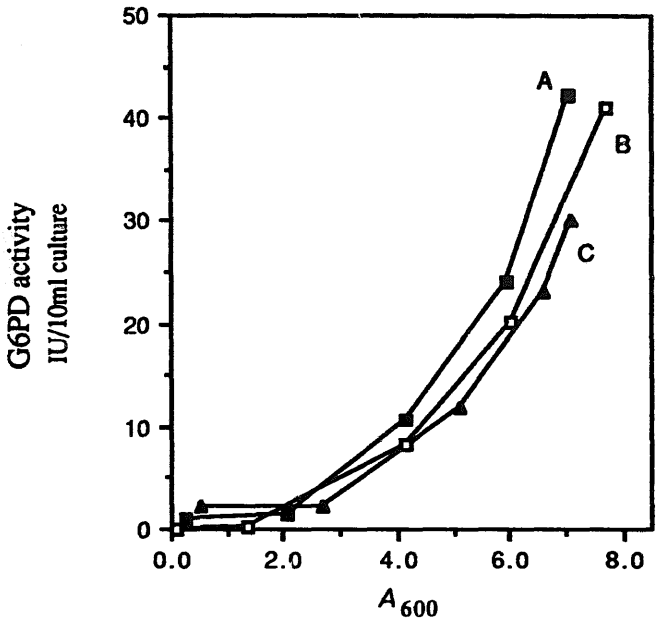


Fig. 1. HB351 pP_LG6PD pcl857 induction at 42 °C.Cells were grown from a single colony overnight at 28°C in 2 ml Super Broth medium plus ampicillin (50 μg/ml) and kanamycin (25 μg/ml) until A_{600} = 10.2. G6PD activity at this point was 0.12 IU/ml culture. Three aliquots of 0.1 ml were inoculated into 10 ml of the same medium and induced as follow: (A) by incubation at 28°C until A_{600} = 0.25 and then raising the temperature to 42°C; (B) by raising the temperature directly to 42°C; (C) by incubation at 28°C until A_{600} = 0.5 and then raising temperature to 42°C.

pP_LG6PD. An M13 subclone containing full length G6PD cDNA [6] was first used as the template for in vitro mutagenesis [12] to convert the sequence around the initiation codon TCATGG to an *Nco*I site CCATGG using oligonucleotide 1,5'AGCACAGCGC-CATGGCAG3'. The G6PD coding sequence was then obtained from the double-stranded form of the resulting clone as an *Xho*I-*Nco*I(partial)fragment, the *Xho*I site having been filled in using the Klenow enzyme. This fragment was ligated into the plasmid pP_Lcmu299 [13] which had been cleaved with *Bam*HI (filled in) and *Nco*I.

pKK233-2 G6PD. Full length G6PD cDNA was also inserted into the inducible expression vector pKK233-2 [14] which contains the inducible tac promotor. Since this plasmid also contains a *Nco*I site immediately downstream of the promotor we inserted the same *Xho*I-*Nco*I(partial) fragment described above into pKK233-2 DNA that had been cleaved with *Hin*d III (filled in) and *Nco*I.

Bacterial cultures and enzyme purification

E. coli strain HB351 (Δ (lac), Δ (zwf-Edd) -Zeb :: Tn 10) [15] was transformed with two plasmids, the expression plasmid pP_LG6PD (selectable with ampicillin) and pc1857 which encodes the mutant cl repressor and kanamycin resistance. Fig. 1 shows the induction of G6PD activity at 42 °C under three different conditions.

1 l of Super Broth medium [16] containing ampicillin (50 μg/ml) and kanamycin (25 μg/ml) was inoculated with 10 ml of an overnight culture of bacteria and incubated at 28 °C with shaking. When the A_{600nm} reached 0.25 the temperature was raised to 42 °C and incubation continued for 14 h. Cells were then collected by centrifugation (5000 × *g* at 4° for 12 min) and resuspended in 300 ml of extraction buffer (Tris-HCl 0.02 M (pH 7.5) containing $MgCl_2$ 3 mM, EDTA 1 mM, β-mercaptoethanol 0.02% and ε-ACA 1 μM), and immediately chilled in ice. Cells were disrupted by sonication at 22 microns in an ice bath by means of 10 bursts of 30 s (interburst 1 min), and the lysate was clarified by centrifugation (60 000 × *g* for 45 min). For rhG6PD purification the supernatant was made 20 μM in glucose 6-phosphate, incubated in ice for 30 min, and then applied to a 2.5 × 15 cm 2',5'-ADP Sepharose 4B column (Pharmacia LKB Biotechnology) equilibrated with either 0.1 M Tris-HCl (pH 7.3) or 0.1 M Hepes (pH 7.3) (containing EDTA 5 mM, β-mercaptoethanol 0.02% and ε-ACA 1 μM) with a flow rate of 50 ml/h. After washing with 1.5 l of equilibration buffer the flow was reduced to 7–10 ml/h and the enzyme was eluted with 40 μM NADP in the same buffer.

G6PD purification from red blood cell lysates was achieved by the same method described above for the purification of the recombinant enzyme.

Protein analysis

SDS-PAGE was performed as previously described [17], and 5–15% gradient gels carried out according to published procedures [18], but replacing sucrose by glycerol at the same concentration in the 15% gel mixture. Cellogel electrophoresis was run in Tris/Borate/EDTA buffer (pH 8.9) and the gel stained for G6PD activity [19].

Enzymatic assays to obtain the K_m value of G6PD for NADP were conducted in duplicate or triplicate using a Perkin-Elmer LS-3B spectrofluorimeter. The precautions to be taken when enzyme kinetic studies are carried out by fluorimetry reported by Dalziel [20] and by Engel and Dalziel [21] were taken into account. The other enzymatic assays were performed measuring the increase in absorbance at 340 nm according to previously published procedures [22]. The buffer used in the kinetic studies was always Tris-Borate 0.25 M (pH 8.0) [23].

Results

Production and purification of recombinant G6PD

Enzymatically active human G6PD coded by the X-linked cDNA has been produced both in cos cells [6]

and in *E. coli* [7]. In both cases the recombinant enzyme had the same electrophoretic mobility as the red cell enzyme. In order to produce large amounts of enzyme for purification three different expression vectors were tested for production of active G6PD (Table I). The plasmid that gives highest expression of human G6PD in *E. coli* was constructed by ligating full length X-linked cDNA [4] into the expression vector pP_Lcmu299 [13], which contains the strong P_L promotor of phage lambda. The initiation codon in this vector is the ATG triplet within a unique *Nco*I CCATGG site. The sequence containing the initiation codon of G6PD was converted from TCATGG to CCATGG by in vitro mutagenesis and the G6PD cDNA was ligated into the expression site as described in Experimental procedures to give plasmid pP_LG6PD (Fig. 2). This plasmid and plasmid pcl857, which encodes the temperature sensitive repressor protein, were co-transfected into *E. coli* strain HB351, which is totally defi-

TABLE I

Production of rhG6PD in E. coli HB351 by using three differents expression vectors

The vectors used for expression of human G6PD were pAC1 (Persico et al., 1989), pKK233-2 and pP_L (see under Experimental procedures), all of which were transfected into the *E. coli* G6PD deficient strain HB351. The production values given are those obtained under the optimal conditions of growth and induction achieved for each construct as follows: pAC1, induction with 2 mM IPTG when A_{600} is 0.6–0.7; pKK233-2 induction with 4 mM IPTG when A_{600} is 0.5–0.65; pP_L induction by raising culture temperature from 28°C to 42°C when A_{600} is 0.2–0.3. The A_{600} of all the cultures at the time of collection of the cells was always between 6.2 and 7.4 for all three constructs. The data presented in this table was obtained from 100 ml cultures.

Expression vector	pAC1	pKK233-2	pP_L
G6PD production *	7.5 ± 1.8 ($n = 2$)	15.4 ± 2.4 ($n = 3$)	70.2 ± 4.9 ($n = 5$)

* Expressed as IU in 100 ml of culture/A_{600}.

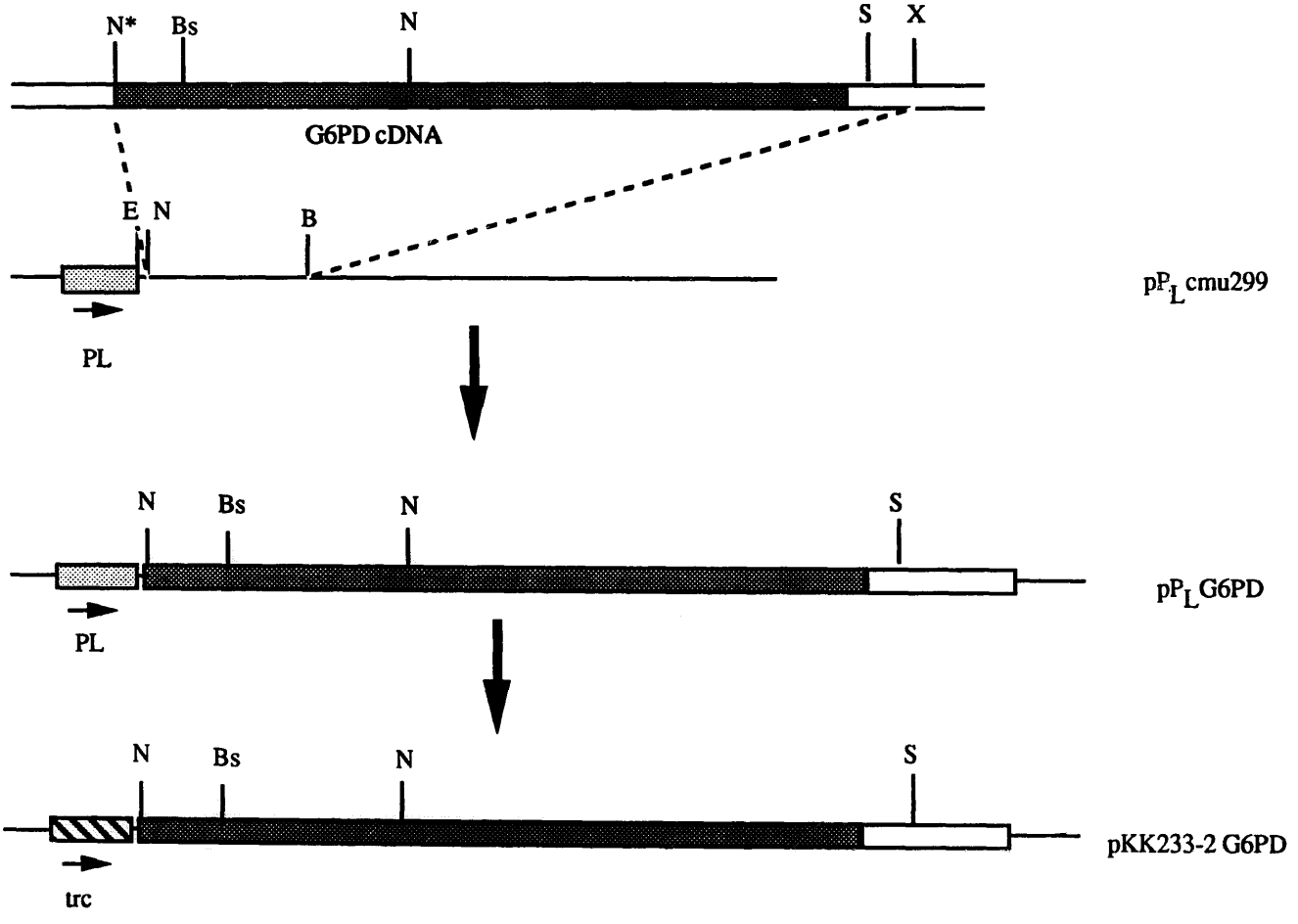
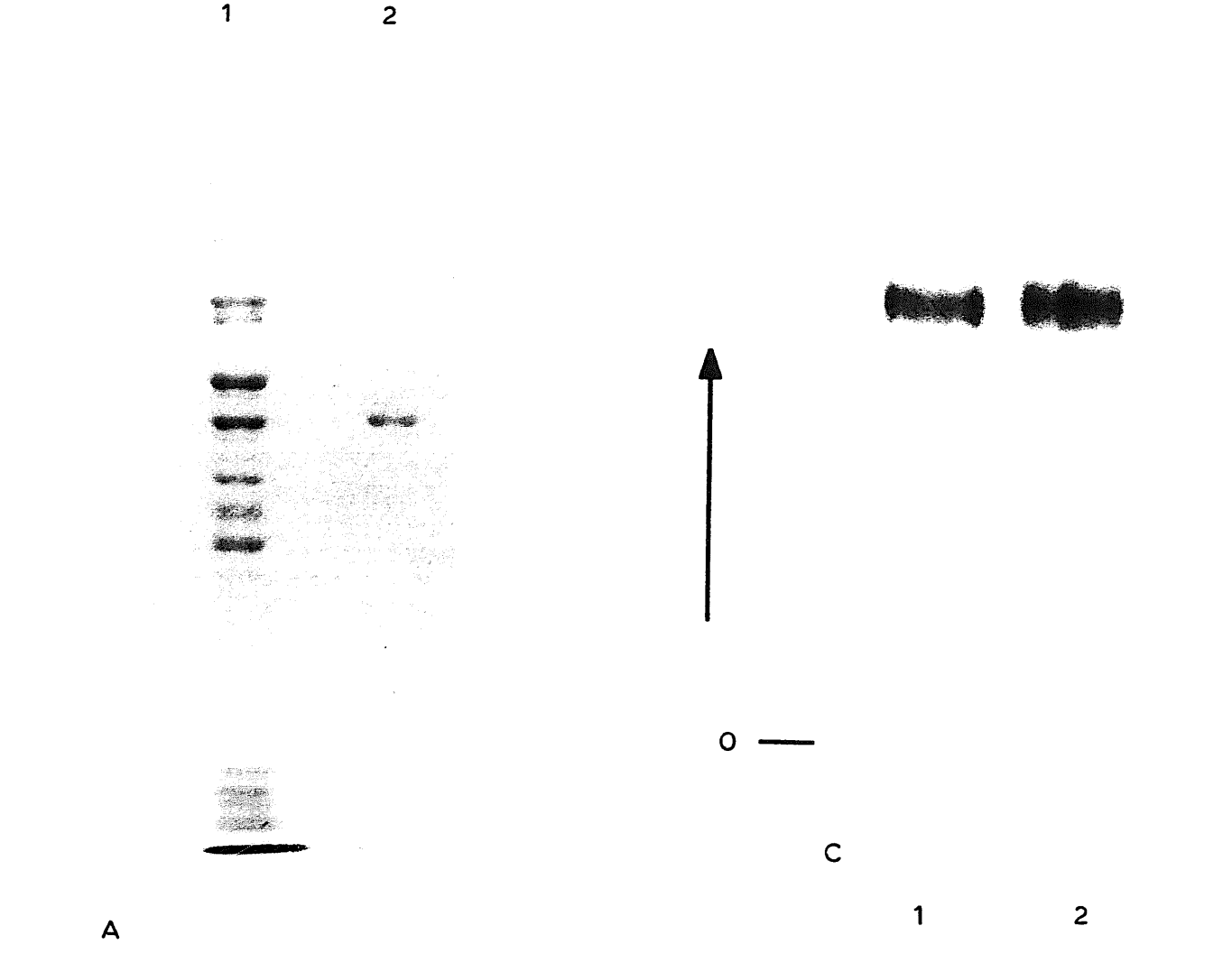


Fig. 2. Plasmid constructs. Plasmids pP_LG6PD and pKK233-2G6PD, used for high level expression of human G6PD in *E. coli* were made by ligating full-length G6PD cDNA into the expression vectors pP_Lcmu299 [13], and pKK233-2 [14], as described in Experimental procedures. Restriction enzyme sites indicated are N, *Nco*I; B, *Bam*HI; Bs, *Bst*EII; X, *Xho*I; E, *Eco*RI; S, *Sma*I; N*, the *Nco*I site containing the G6PD initiation codon that was created by in vitro mutagenesis. PL indicates the position of the leftward phage λ promotor, and trc the trp/lac promoter.

TABLE II

Summary of purification of rhG6PD

Purification step	Activity			Protein		Spec. Act.	
	(vol. (ml))	(I.U./ml)	(total I.U.)	(mg/ml)	(total mg)	(I.U./mg)	(yield %)
Crude extract	300	10.85	3255	1.50	450.0	7.23	100
2′,5′-ADP-Sepharose	60	37.90	2274	0.17	10.2	222.80	82



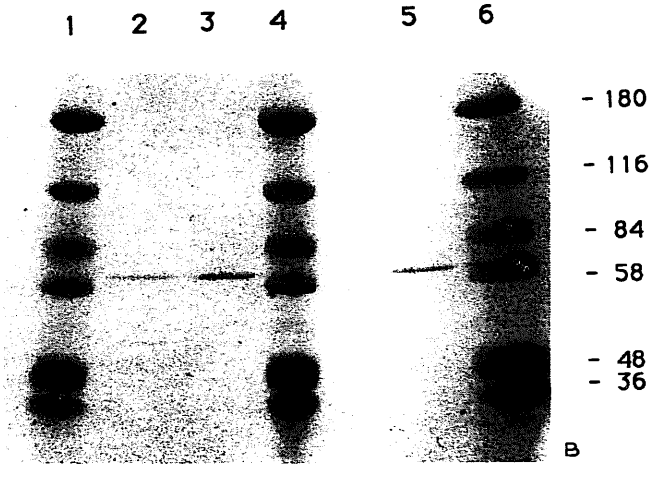


Fig. 3. Purification and properties of recombinant human G6PD. (A) One-step purification of rhG6PD. SDS-PAGE (7.5%) analysis of HB351/pP_LG6PD crude extract (lane 1) and pooled fractions of the eluate from the 2′,5′-ADP-Sepharose column (lane 2). The gel was stained with Coomassie blue. (B) Comparison of rhG6PD and RBC-G6PD by SDS-PAGE. The purified rhG6PD from A. was analysed by 5–15% gradient SDS-PAGE. Lane 1 and 4, molecular weight markers; lane 2, 1 μg RBC-G6PD; lane 3, 1 μg rhG6PD; lane 5, a mixture of 1 μg each of rhG6PD and RBC-G6PD; lane 6, a mixture of 1 μg each of rhG6PD and RBC-G6PD and molecular weight markers. The gel was stained with Coomasie blue. (C) Cellogel analysis of rhG6PD and RBC-G6PD in their native state. After purification fractions were analysed by non-denaturing electrophoresis on cellogel and stained for G6PD activity. Lane 1, 6 ng rhG6PD; lane 2, 6 ng RBC-G6PD. O, origin.

cient in G6PD because of a phage μ insertion [16]. These cells were grown at 28°C to an A_{600nm} of 0.25, and were induced by shifting the temperature to 42°C, and then grown for a further 14 h. Under these conditions 1 l of culture produces about 14 mg of active recombinant G6PD (rhG6PD), equivalent to 3.3% of the soluble protein. The soluble fraction of an extract of the cell pellet was made 20 μM in glucose 6-phosphate and applied to an affinity column of 2',5'-ADP-Sepharose, and eluted with 40 μM NADP. This single step chromatographic fractionation is very efficient (Table II) as it produces a 31-fold purification and 82% of the G6PD activity was recovered from it as pure enzyme (10.2 mg). The purified fraction gives a single band on SDS-PAGE (Fig. 3A and E) and after more than 6 months of storage at 4°C it retains 100% of its activity (Fig. 4).

Recombinant X-linked G6PD and red cell G6PD are indistinguishable

The physical properties of the recombinant enzyme were the same as those of authentic red cell G6PD. By comparison with the appropriate molecular weight markers, the estimated subunit molecular weight of rhG6PD is approx. 59 kDa (Fig. 3B), in good agreement with that of 59.2 predicted from the cDNA sequence [4]. The recombinant protein cannot be resolved from authentic red cell G6PD by SDS-PAGE (Fig. 3B) and the electrophoretic mobility of the two native enzyme species on cellogel, as revealed by staining for enzyme activity, is also identical (Fig. 3C).

TABLE III

Biochemical properties of human G6PD made in E. coli and human red cell G6PD

The number in parentheses represents the number of duplicated experiments used in calculating the given mean.

Properties	Red cell G6PD	Recombinant G6PD
Electrophoretic mobility (%)	100	100
M.W. from SDS-PAGE (kDa)	≈ 59	≈ 59
Specific activity (IU/mg)	180 [a]	220 ± 21 (8)
K_m^{G6P} (μM)	72 [b]	69 ± 3 (5)
K_m^{NADP} (μM)	13 [b]	12 ± 2 (7)
K_i^{NADPH} (μM)	15 ± 2 (3)	14 ± 3 (3)

[a] Cohen and Rosemeyer [24].
[b] Babalola et al. [23].

Enzymatic properties of rhG6PD and red cell G6PD were also compared (Table III). The specific activity of the recombinant protein (220 IU/mg) is a little higher than that of the red cell enzyme, presumably indicating a higher purity. Since the active enzyme is a dimer or a tetramer [24] the fact that the specific activity is maximal must indicate that assembly of the subunits produced in *E. coli* is complete. K_m values for the substrates glucose 6-phosphate and NADP, measured under identical conditions, are very similar for the recombinant and natural enzymes. The same is true for the

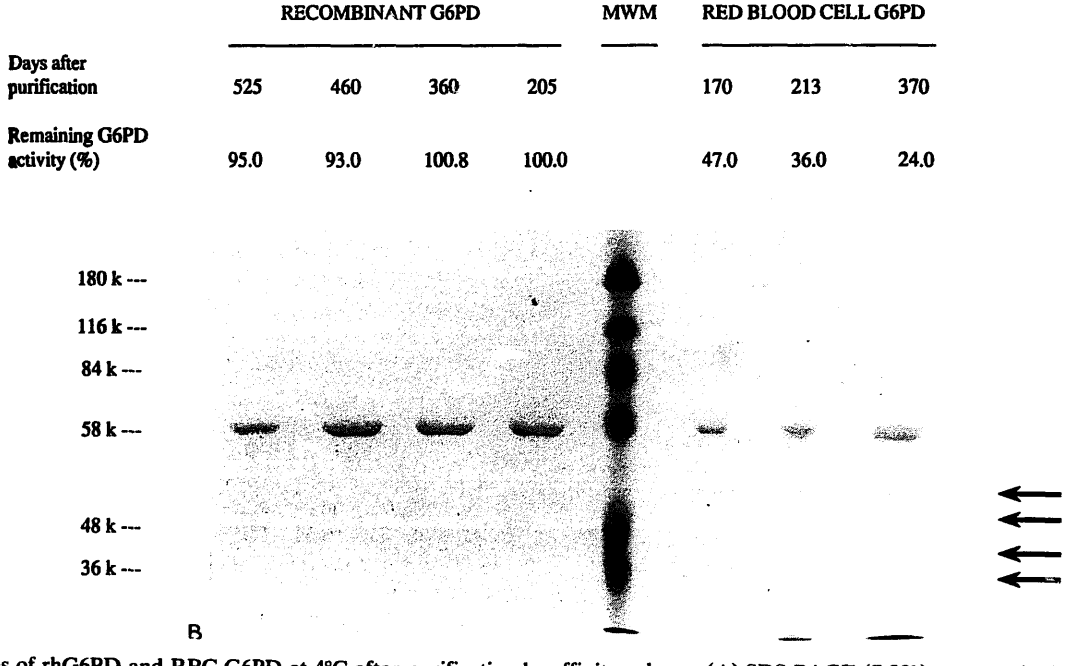


Fig. 4. Stabilities of rhG6PD and RBC-G6PD at 4°C after purification by affinity column. (A) SDS-PAGE (7.5%) pattern obtained immediately after purification of RBC-G6PD (1) and rhG6PD (2). The upper band is G6PD and the lower band in track 1 is an extra band appearing in the affinity column elution of RBC-G6PD. (B) SDS-PAGE (7.5%) pattern of RBC-G6PD and rhG6PD after storage at 4°C for the times indicated at the top of each lane. The percentage remaining G6PD activity of each preparation is shown under the time it was stored. Arrows indicate extra bands appearing in the RBC-G6PD preparations during storage.

NADPH K_i values. These results and those reported elsewhere [25] suggest strongly that rhG6PD made in *E. coli* from the X-linked G6PD cDNA is structurally and functionally identical or very similar to the red cell enzyme.

Discussion

The aim of this work was to establish a reliable system to produce milligram amounts of pure and homogeneous human G6PD for the detailed analysis of its structural and biological properties. Expression of high levels of foreign proteins in *E. coli* often results in the formation of large amounts of insoluble product relative to the soluble protein [26]. Perhaps our most surprising finding is that this relatively large oligomeric protein is largely soluble.

The availability of a G6PD deficient host bacterium [15] was ideal for the production of rhG6PD uncontaminated by *E. coli* G6PD. The same strain was previously used for the same purpose by Persico et al. [7]. The testing of constructs made in different vectors proved useful, because the yield of G6PD obtained with pP_LG6PD was about 10-times higher than that previously reported with pAC1 [7]. The addition of glucose 6-phosphate before loading the crude extract onto the affinity column (S. Pittalis and G. Fiorelli, personal communication) enables the rhG6PD to be eluted with a very low NADP concentration, at which no other proteins are eluted. This modification of the purification procedure originally introduced by De Flora et al [27] has made it much easier to obtain pure G6PD from the bacterial culture. This observation also indicates that binding to the enzyme of the substrate glucose 6-phosphate affects its affinity for the other substrate NADP (in our case for the 2'-5'-ADP moiety of NADP, which is the active group of the resin).

The data reported in this paper indicate that the X-linked cDNA of Persico et al. [7] encodes a protein indistinguishable from red cell G6PD by subunit molecular weight, electrophoretic mobility in the native state, and kinetic properties. The results also show that G6PD in vivo is not subjected to any post-translational modifications that are essential for its activity. Jeffery et al. [28] have shown that in rat liver G6PD the N-terminus is *N*-acetylalanine, which must result from post-translational cleavage of the original N-terminal methionine. We assume the same is true for red cell G6PD. We do not know if the same is true for rhG6PD produced in *E. coli*; but if the N-terminal methionine here is retained, this does not seem to affect either the physical or the enzymic properties of the protein.

The recombinant enzyme is much more stable than that prepared from red cells by the same purification procedure. This could be due to small amounts of contaminating proteinases in the red cell enzyme preparation or to in vivo modifications of the enzyme during red cell ageing.

Since we can now rapidly obtain milligram quantities of pure G6PD, the tertiary structure of this interesting human enzyme should be amenable to analysis. In addition the procedure reported here will lend itself conveniently to the production, by site-directed mutagenesis, of either naturally occurring [8,9] or artificial G6PD variants.

Acknowledgements

We are grateful to E. Remaut and W. Fiers for providing the expression vector pP_Lcmu299. We thank G. Fiorelli for communicating unpublished results. We are grateful to Nick Cross, Nick Foulkes, Margaret Town and Tom Vulliamy for advice, and to Professor K. Dalziel for valuable suggestions on enzyme kinetics. Financial support was received through a programme grant from the Medical Research Council and in part by an EEC project grant. J.M.B was supported by a fellowship from the Ministerio de Educación y Ciencia of Spain and by The British Council.

References

1 Carson, P.E., Flanagan, C.L., Ickes, C.E. and Alving, A.S. (1956) Science 124, 484.
2 Beutler, E. (1978) Topics in Haematology-Hamolytic anaemia in Disorders of Red Cell Metabolism, Plenum, New York.
3 Luzzatto, L. and Battistuzzi, G. (1985) Adv. Hum. Genet. 29, 31–36.
4 Persico, M.G., Viglietto, G., Martini, G., Toniolo, D., Paonessa, G., Moscatelli, C., Dono, R., Vulliamy, T., Luzzatto, L. and D'Urso, M. (1986) Nucleic Acids Res. 14, 2511–2521 and 7822.
5 Martini, G., Toniolo, D., Vulliamy, T.J., Luzzatto, L., Dono, R., Viglietto, G., Paonessa, G., D'Urso, M. and Persico, M.G. (1986) EMBO J. 5, 1849–1855.
6 Mason, P.J., Vulliamy, T.J., Foulkes, N.S., Town, M., Haidar, B. and Luzzatto, L. (1988) Eur. J. Biochem. 178, 109–113.
7 Persico, M.G., Ciccodicola, A., Martini, G. and Rosner, J.L. (1989) Gene. 78, 365–370.
8 Vulliamy, T.J., D'Urso, M., Battistuzzi, G., Estrada, M., Foulkes, N.S., Martini, G., Calabro, V., Poggi, V., Giordano, R., Town, M., Luzzatto, L. and Persico, M.G. (1988) Proc. Natl. Acad. Sci. USA 85, 5171–5175.
9 Beutler, E. (1991) N. Eng. J. Med. 324, 169–174.
10 Hirono, A., Kuhl, W. Gelbart, T., Forman, L., Fairbanks, V.F. and Beutler, E. (1989) Proc. Natl. Acad. Sci. USA 86, 10015–10017.
11 Sambrook, J. Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning, A laboratory manual, Cold Spring Harbor, New York.
12 Zoller, M.J. and Smith, M. (1983) Methods Enzymol. 100, 468–500.
13 Remaut, E., Marmenout, A., Simons, G. and Fiers, W. (1987) Methods Enzymol. 153, 416–431.
14 Amann, E. and Brosius, J. (1985) Gene 40, 183.
15 Fraenkel, D. (1968) J. Bacteriol. 95, 1267–1271.
16 Davis, R.W., Botstein, D. and Roth, J.R. (1980) Advanced Bacterial Genetics, Cold Spring Harbor, New York.
17 Laemmli, U.K. (1970) Nature 227, 681–685.

18 See, Y.P. and Jackowski, G. (1989) in Protein structure: a practical approach (Creighton, T.E. ed.), pp. 1–21 IRL Press, Oxford.
19 Betke, K., Brewer, G.J., Kirkman, H.N., Luzzatto, L., Motulsky, A.G., Ramot, B. and Siniscalco, M. (1967) WHO Tech. Rep. Ser. 366.
20 Dalziel, K. (1962) Biochem. J. 84, 244–254.
21 Engel, P.C. and Dalziel, K. (1969) Biochem. J. 115, 621–631.
22 Battistuzzi, G., Esan, G.J.F., Fasuan, F.A., Modiano, G. and Luzzatto, L. (1977) Am. J. Hum. Genet. 29, 31–36.
23 Babalola, A.O.G., Beetlestone, J.G. and Luzzatto, L. (1976) J. Biol. Chem. 251, 2993–3002.
24 Cohen, P. and Rosemeyer, M.A. (1969) Eur. J. Biochem. 8, 1–7.
25 Mason, P.J., Bautista, J.M., Vulliamy, T.J., Turner, N. and Luzzatto, L. (1990) Cell. 62, 9–10.
26 Marston, F.A.O. (1986) Biochem. J. 240, 1–12.
27 De Flora, A., Morelli, A., Benatti, U. and Giuliano, F. (1975) Arch. Biochem. Biophys. 169, 362–363.
28 Jeffery, J., Soderling-Barros, J., Murray, L.A., Hansen, R.J., Szepesi, B. and Jornvall, H. (1988) Proc. Natl. Acad. Sci. USA 85, 7840–7843.