FEBS Letters 412 (1997) 126–130 FEBS 18910

Expression, purification and characterization of GDP-D-mannose 4,6-dehydratase from *Escherichia coli*

Laura Sturla, Angela Bisso, Davide Zanardi, Umberto Benatti, Antonio De Flora, Michela Tonetti*

Institute of Biochemistry, University of Genova, Viale Benedetto XV, 1, 16132 Genova, Italy

Received 4 June 1997

Abstract GDP-D-mannose dehydratase (GMD) catalyzes the first step of the pathway that converts GDP-D-mannose to GDP-L-fucose in bacteria, plants and mammals. Recently, the gene coding for GMD has been identified and sequenced in *E. coli*. Based on this sequence, we have expressed and purified GMD in *E. coli* as a glutathione transferase (GST) fusion protein. The fused GST-GMD protein and the thrombin-cleaved GMD were then characterized. The catalytically active form of both enzyme species seems to be a hexamer of 410 and 250 kDa, respectively. The GST-GMD fusion protein has a K_m of 0.22 ± 0.04 mM and a specific activity of 2.3 ± 0.2 μmol/h/mg. Ca^{2+} and Mg^{2+} activate GMD, while GDP-L-β-fucose, the end-product of the pathway, inhibits it specifically. The GST-GMD fusion protein contains one mole of tightly bound $NADP^+$ per mole of hexamer. Apparently, this $NADP^+$ is involved in the catalytic mechanism of GMD.

Key words: GDP-D-mannose dehydratase; GDP-L-fucose biosynthesis; L-Fucose metabolism; $NADP^+$; (*Escherichia coli*)

1. Introduction

L-Fucose is a 6-deoxy-monosaccharide widely occurring in glycoconjugates from many different sources, from microorganisms to plants and animals. L-Fucose is formed from GDP-D-mannose via a metabolic pathway identified by Ginsburg both in bacteria and animal tissues [1–3], which leads to the formation of GDP-L-β-fucose. This latter compound is the substrate for many different fucosyltransferases, that are responsible for the incorporation of L-fucose in glycoproteins and glycolipids [4,5].

The first step of this metabolic pathway is believed to be the dehydration of GDP-D-mannose, with the formation of the quite unstable intermediate GDP-4-keto-6-deoxy-D-mannose [1–3,6–8]. The second part of the pathway consists of an epimerization at C-3 and C-5 followed by an NADPH-dependent reduction of the 4-keto group, leading to GDP-L-fucose formation [9]. We have recently isolated and sequenced the enzyme, formerly named FX [10,11], which is responsible for the second part of this pathway in human cells and which displays both the epimerase and the reductase activities [12].

The enzyme catalyzing the first reaction, i.e. GDP-D-mannose 4,6-dehydratase (EC 4.2.1.47) (GMD), has been obtained to different degrees of purity from both bacteria and mammals [6,13]. The mechanism of the GMD-catalyzed reaction has been proposed to involve a protein-bound pyridine dinucleotide as responsible for the transfer of C-4 hydrogen to the C-6 position of the deoxy-monosaccharide [1,6], similarly to what has been demonstrated for the TDP-D-glucose dehydratase involved in TDP-L-rhamnose synthesis in bacteria [14]. NAD^+ was postulated to play this role with the GMD purified from porcine thyroid [6], while $NADP^+$ has been more recently implicated with the *Klebsiella pneumoniae* enzyme [13]. Another apparent difference concerning GMD purified from the two above sources is its oligomeric structure, reported to be hexameric for the thyroid enzyme [6] and dimeric for the *K. pneumoniae* protein [13].

The nucleotide sequence of the gene coding for GMD and the amino acid sequence of GMD protein have not yet been identified. A putative sequence of GDP-D-mannose 4,6-dehydratase in *E. coli*, named *orf0.0*, was proposed on the basis of its close relationship to sequences found in gene clusters responsible for other metabolic pathways (i.e. colitose and perosamine synthesis), in which GMD activity is involved [15]. Recently, Stevenson et al. [16] demonstrated that the *gmd* gene, corresponding to *orf0.0*, which is contained in the *E. coli* K12 *wca* gene cluster responsible for colanic acid production, once cloned in a *wca*-deleted strain, is able to restore GMD activity in the crude lysate from the deficient cells. This finding strongly supported the identification of the previously proposed sequence. However, the molecular and enzymatic properties of the *E. coli* GMD are so far completely unknown.

In this paper we report the expression of GMD in *E. coli* as GST-fusion protein and its structural and enzymatic characterization. Our results, which confirmed the enzymatic activity of the *gmd* gene product and demonstrated the presence of tightly protein-bound $NADP^+$, should allow the complete in vitro reconstruction of the pathway from GDP-D-mannose to GDP-L-fucose and accordingly the elucidation of its kinetic and regulatory properties.

*Corresponding author. Fax: (39) (10) 354415.
E-mail: toninodf@unige.it

Abbreviations: GDP, guanosine diphosphate; GST, glutathione-*S*-transferase; GMD, GDP-D-mannose 4,6-dehydratase; TDP, thymidine diphosphate; PCR, polymerase chain reaction; LB broth, Luria-Bertani broth; IPTG, isopropyl-β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid

2. Materials and methods

2.1. Expression and purification of recombinant GDP-D-mannose 4,6-dehydratase (GMD)

Chromosomal DNA was prepared from *E. coli* (strain INVF1, Invitrogen) as described [17]. The following primers, used to amplify the *gmd* gene, were designed on the basis of the sequence reported by

PII S0014-5793(97)00762-X

Stevenson et al. [16] (GenBank accession no. U38473) and were obtained from TibMolBiol, Genova, Italy:

5′-AATTAGATCTATGTCAAAAGTCGCTCTCATCACC (forward)
5′-AATTCTCGAGTTATGACTCCAGCGCGATCGCC (reverse).

Three μg of chromosomal DNA were amplified with the following PCR conditions: 94°C for 30 s, 64°C for 30 s, 72°C for 75 s for 15 cycles, 1.5 mM $MgCl_2$. For protein expression the Glutathione-*S*-Transferase (GST) Gene Fusion System (Pharmacia, Milan, Italy) was used. The 1134 base-pair PCR product was digested with *Bgl*II and *Xho*I restriction enzymes (Amersham, Milan, Italy) and ligated into *Bam*HI and *Xho*I sites of pGEX-4T-2 plasmid (Pharmacia), which contains a region coding for the thrombin protease recognition site, using standard protocols [17]. This plasmid was chosen because no thrombin cleavage sites were present on the GMD sequence. After ligation, the plasmid was transfected into *E. coli* for protein expression. The plasmids obtained from single colonies were then sequenced using Sequenase 2.0 (Amersham) to verify the presence of the correct insert. *E. coli* cells were then grown overnight in LB broth [17] at 37°C. The overnight culture was diluted 1 : 20 in LB and grown at 25°C to reach an optical density of 0.6 at 660 nm. The cells were then induced for 18 h with 0.1 mM IPTG at 25°C.

Cells were collected by centrifugation, washed with ice-cold H_2O and then lysed by sonication in 100 mM Tris-HCl, pH 8.3, containing 150 mM NaCl, 1% Triton X-100, 2 mg/ml lysozyme, 3 mM PMSF, 5 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 10 mM EDTA (all from Sigma, Milan, Italy). The crude extract was clarified by centrifugation at $10\,000\times g$ for 10 min.

The GST-GMD fused protein was purified from the supernatant by affinity chromatography, using GST-Sepharose-4B (Pharmacia) and following the manufacturer's instructions. GST-GMD fused protein was eluted directly from half of the GST-Sepharose using 10 mM GSH at 25°C. The remaining matrix with the bound fusion protein was treated with 50 U/ml thrombin (Pharmacia), which recognizes a cleavage sequence inserted between GST and GMD, for 16 h at 25°C. This treatment released the native GMD, while GST remained bound to the matrix. Thrombin was then removed from the eluate by treatment with benzamidine-Sepharose-6B (Pharmacia).

2.2. Characterization of recombinant GMD

The recombinant proteins were analyzed by discontinuous SDS-PAGE [18] in order to check purity and molecular mass. Size exclusion chromatography was performed on an HP1090 HPLC system (Hewlett Packard, Walbronn, Germany) with a TSK-gel $G3000SW_{XL}$ column (5 μm particle size, 7.8×300 mm, TosoHaas, Montogomeryville, PA, USA), mobile phase 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1 M Na_2SO_4, at a flow rate of 0.7 ml/min. Molecular mass markers for denaturing gel electrophoresis and for size exclusion chromatography were obtained form Sigma.

2.3. Enzyme assays

Both the GST-GMD fusion protein and the thrombin-cleaved GMD were analyzed for the ability to convert GDP-D-mannose (Sigma) to GDP-4-keto-6-deoxy-D-mannose. The standard assay was performed at 37°C in 50 mM Tris-HCl buffer, pH 8.0, with 1 mM $MgCl_2$ (unless otherwise specified), using different concentrations of GDP-D-mannose containing GDP-D-[U-^{14}C]mannose (Amersham) with a final specific activity of 6.2 mCi/mmol. The reaction mixture was deproteinized and the identity of the GMD product, i.e. GDP-4-keto-6-deoxy-D-mannose, was evaluated both by HPLC analysis, using a Packard Flow-One detector for radioactivity, and by TLC analysis, after $NaBH_4$ reduction and acid hydrolysis, as previously described[12]. Protein concentration was determined according to Bradford [19].

The amount of NAD^+ or $NADP^+$ bound to the GST-GMD fused protein was determined by a sensitive enzymatic cycling assay procedure [20], using ethanol and alcohol dehydrogenase for NAD^+ analysis and glucose 6-P and glucose 6-P dehydrogenase for $NADP^+$, respectively. All the reagents used for these assays were obtained from Sigma. Before performing the analyses, the protein was extensively dialyzed to remove any trace of residual GSH, which proved to interfere with the colorimetric assay.

3. Results

Approximately as much as 15 mg of the GST-GMD fusion protein could be obtained from 1 liter of *E. coli* culture. The induction for longer periods of time at lower temperature and IPTG concentration (see Section 2.1), compared to standards protocols [17], was essential to avoid precipitation of the fused protein inside the cells and to obtain a functional enzyme. After affinity purification using GST-Sepharose, the fusion protein migrated as a single band with an apparent molecular mass of 68 kDa (Fig. 1, lane 2), consistent with the sum of the molecular masses of GST and GMD, as predicted by translation of the corresponding nucleotide sequences. Thrombin cleavage of the matrix-bound GST-fusion protein and subsequent removal of thrombin by treatment with benzamidine-Sepharose produced a single band of approximately 42 kDa (Fig. 1, lane 1), which is in agreement with the expected M_r for the native GMD and compares well with the M_r of the porcine thyroid enzyme [6].

Gel permeation analysis was used to determine the native molecular mass of both the GST-fused and the cleaved GMD. As shown in Fig. 2A, HPLC size exclusion chromatography of the GST-fused GMD yielded three distinct peaks. Comparison of the elution volumes of these peaks with those of known molecular weight standards suggested the presence of a hexameric structure (expected M_r, 410 kDa), of a trimer (205 kDa) and of the single monomer corresponding to the GST-GMD fusion protein (68.4 kDa), respectively. Out of the three peaks, only peak 1 (see Fig. 2A) exhibited enzymatic activity, while the other two were catalytically inactive, nor did they regain activity upon supplying $NADP^+$ or NAD^+ to the corresponding fractions. After thrombin cleavage, size exclusion HPLC chromatography revealed the presence of two peaks, with apparent molecular weights of 250 and 130 kDa, respectively, again suggesting the presence of a hexameric and of a trimeric structure deriving from the 42 kDa monomer (Fig. 2B). Similarly to the findings obtained with the GMD-fusion protein, enzymatic activity was observed in peak 1 only.

Both the GST-fusion protein and the thrombin-cleaved GMD exhibited enzymatic activity, converting GDP-D-man-

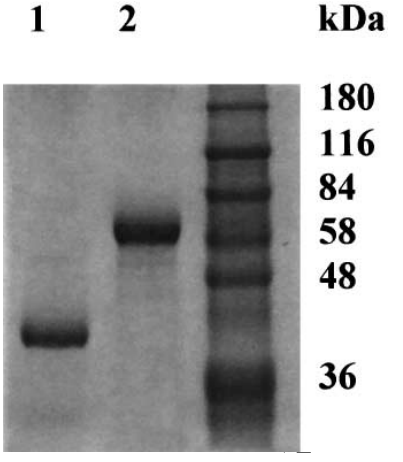

Fig. 1. SDS-PAGE analysis [18] of the thrombin-cleaved GMD (lane 1) and of GST-GMD fusion protein after purification on GST-Sepharose (lane 2). Five μg of protein were loaded and the gel was stained with Coomassie Brilliant Blue R-250.

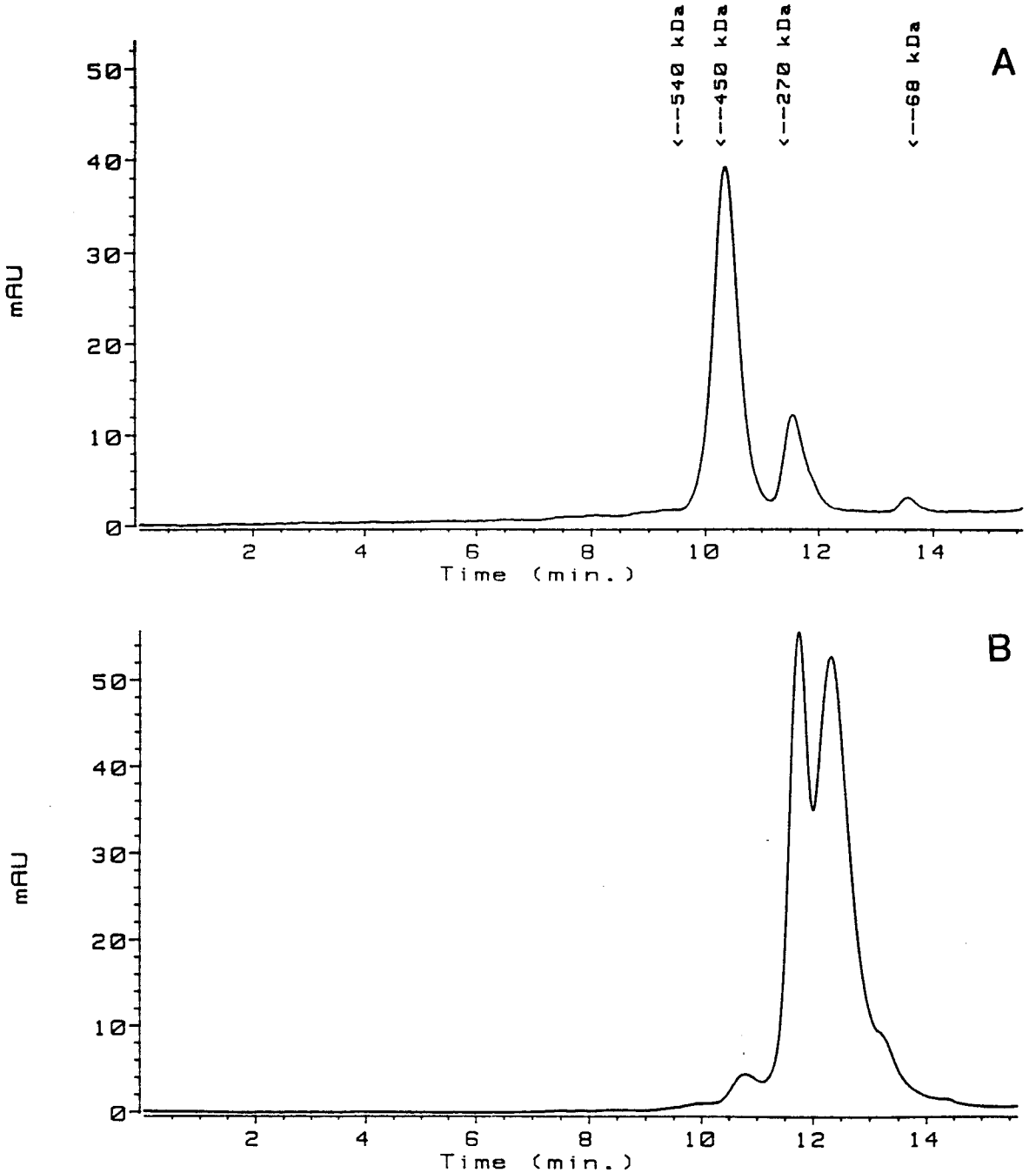

Fig. 2. Size exclusion chromatography, performed against known molecular weight standards, of purified GST-GMD fused protein (A) and of the thrombin-cleaved GMD (B). Molecular mass standards: urease (hexameric form), 540 kDa; ferritin, 450 kDa; urease (trimeric form), 270 kDa; bovine serum albumin, 68 kDa.

nose to GDP-4-keto-6-deoxy-D-mannose, as determined by HPLC and TLC analyses [12]. The instability of the thrombin-cleaved GMD, which was not prevented by addition of thiol compounds, antioxidants or chelating agents (see Section 4), prompted us to study the functional activity of the GST-fusion protein, which could be assayed immediately after purification from GST-Sepharose and which proved to be mainly in the hexameric form by size exclusion HPLC analysis (Fig. 2A). The K_m, determined in 50 mM Tris-HCl, pH 8.0, in the presence of 1 mM $MgCl_2$, was 222 ± 44 μM and the specific activity was 2.3 ± 0.2 μmol/h/mg protein. The GST-fused GMD exhibited a pH optimum at 8.0 (Fig. 3A) and was remarkably stimulated by both $MgCl_2$ and $CaCl_2$, as shown in Fig. 3B.

GMD has been previously reported to require NAD^+ as cofactor for an internal oxido-reduction reaction between C-4 and C-6 of the GDP-bound sugar [6]. Conversely, GMD purified from *K. pneumoniae* has been shown to have a strict requirement of exogenous $NADP^+$ for activity [13]. In our system, the activity of the recombinant *E. coli* protein, both as GST-fused and cleaved GMD, was unaffected by the addition of exogenous NAD^+ or $NADP^+$. However, the cycling

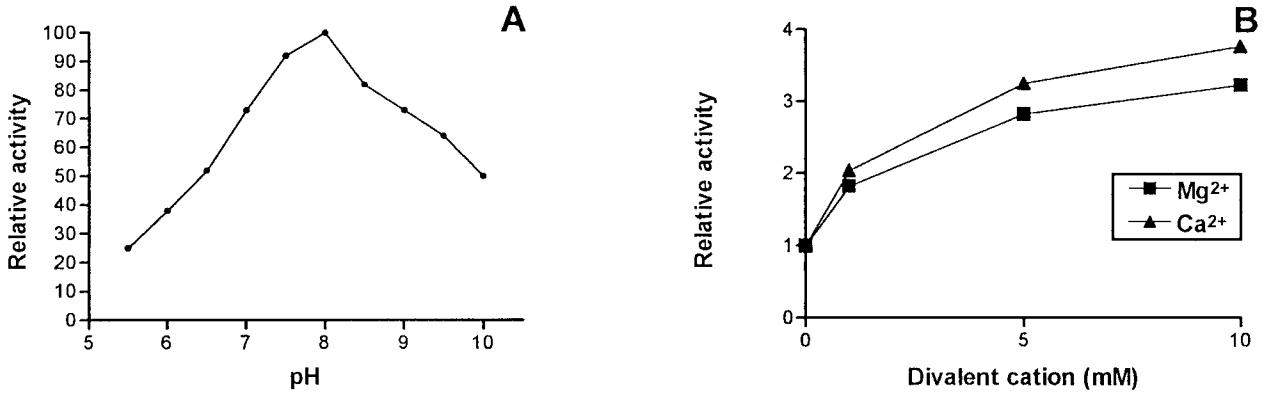

Fig. 3. (A) Effect of pH on the activity of GST-GMD fused protein. Data are expressed as relative activity, assigning a value of 100 to the maximal activity observed (see Section 2.3). (B) Activity of GST-GMD fused protein in the presence of different concentrations of divalent cations. A value of 1 was assigned to the activity observed in the absence of both Ca^{2+} and Mg^{2+}.

assays indicated that $NADP^+$, and not NAD^+, is firmly bound to the protein. The molar ratio of $NADP^+$/GST-GMD fusion protein obtained with this sensitive assay procedure was 1.18 ± 0.24, suggesting that one molecule of the dinucleotide is bound to one molecule of native hexamer. No $NADP^+$ was found when the catalytically inactive protein (obtained after prolonged storage at 25°C), was analyzed, supporting the view of a strict requirement of tightly bound $NADP^+$ for the mechanism of the enzymatic reaction.

GMD purified from porcine thyroid was reported to be susceptible to inhibition by GDP-L-β-fucose [6]. Table 1 reports the effects of this and other GDP-monosaccharides on GST-GMD fused protein activity. GDP-L-β-fucose, which is the terminal product of this metabolic pathway, showed a significant inhibitory activity, while GDP-L-α-fucose had no effect, thus suggesting a specific mechanism of feedback inhibition. A low extent of inhibition was also observed with GDP-D-glucose, while GDP alone was ineffective.

4. Discussion

The metabolic pathway from GDP-D-mannose to GDP-L-fucose, initially identified and partially characterized by Ginsburg in the sixties [1–3], is remarkably conserved in the evolution. This fact emphasizes its importance for the biosynthesis of GDP-L-fucose which in mammalian cells can be formed, although to a comparably much lower extent, also by a 'salvage' pathway from GTP and L-fucose 1-P [21,22].

Table 1
Effects of GDP-monosaccharides on GST-fused GMD activity

	Concentration (μM)	% Inhibition
Control (no inhibitor)		0
GDP-L-β-fucose	10	0
	100	54 ± 15
	250	73 ± 4
GDP-L-α-fucose	10	0
	100	0
	250	0
GDP-D-glucose	100	0
	250	11 ± 4
	500	34 ± 12
GDP	100	0
	250	0
	500	0

GST-GMD fusion protein was incubated for 15 min with 10 μM GDP-D-mannose in 50 mM Tris-HCl, pH 8.0 and 1 mM $MgCl_2$ in the presence of different concentrations of guanine nucleotides. Results are expressed as percent of inhibition compared to the control incubated in the absence of inhibitors.

Some uncertainties still remain on the properties of the GDP-D-mannose to GDP-L-fucose pathway. Although GMD has been purified from both bacterial and animal sources, the nucleotide or amino acid sequences have never been determined. A putative nucleotide sequence has been postulated for the *E. coli* GMD, but only recently did it receive a biochemical validation [16]. Our data provide final evidence that the product of the *gmd* gene is indeed GDP-D-mannose 4,6-dehydratase and supply further insight on the molecular and functional properties of this enzyme. Furthermore, the present results allow large amounts of enzyme to be obtained, compared to the quite lower quantities available upon purification from porcine thyroid [6], from *K. pneumoniae* [13] and from bovine and human tissues (Tonetti, M. and Sturla, L., unpublished data). Thus, it will be possible to scale up the preparation of its enzymatic product, the GDP-4-keto-6-deoxy-D-mannose, a very labile intermediate, which is the substrate of the second enzyme (FX) involved in the pathway form GDP-D-mannose to GDP-L-fucose. This should lead to a more detailed characterization of the epimerase-reductase activity displayed by FX protein. In particular, the availability of both enzyme proteins involved in the pathway, i.e. the expressed GMD and the purified FX protein [12], makes it possible for the first time to reconstruct this pathway in vitro and to identify discrete mechanisms of reaction and specific sites of regulation (see below). For instance, the established presence of tightly GMD-bound $NADP^+$, coupled with the known NADPH requirement of the epimerase/reductase that catalyzes the formation of the end-product (and feedback inhibitor) GDP-L-fucose, seems to suggest a key role of the cellular $NADP^+$/NADPH redox system in the regulation of the complete pathway. In any case, the occurrence of tightly apoenzyme-bound $NADP^+$ playing both a structural and a catalytic role is strongly reminiscent of the long known property previously established for human erythrocyte glucose 6-P dehydrogenase [23,24].

The finding that $NADP^+$ is the cofactor essential for GMD activity is in contrast with the generally accepted notion which considers NAD^+ to be involved in the reaction, by analogy with TDP-D-glucose 4,6-dehydratase and CDP-D-glucose 4,6-

dehydratase [14,25]. Our results are partially in agreement with the data obtained for GMD purified from *K. pneumoniae*, which requires the exogenous addition of $NADP^+$ [13]. This enzyme, however, seems to be structurally different from the *E. coli* GMD, since it was reported to be a dimer composed by two identical subunits of 55 kDa, while the recombinant protein and also GMD purified from porcine thyroid were found to be hexamers [6].

Attention is being paid to elucidate the structural basis of the instability of enzymatic activity of the thrombin-cleaved GMD. This rapid inactivation is in agreement with the results reported by Broschat et al. [6] on the lability of GMD during purification from porcine thyroid and with our data on the poor stability of both human erythrocyte and bovine pancreatic GMD at different degrees of purification (Tonetti, M. and Sturla, L., unpublished data). Notwithstanding this problem, which might underlie specific regulatory mechanisms of GMD activity, the present characterization of the GST-GMD fusion protein seems to provide useful information on the native enzyme, similarly to other fusion proteins generated and used to investigate the catalytic properties [26] and the subcellular compartmentalization [27] of specific enzymes. Complete reconstruction of a species-homologous pathway from GDP-D-mannose to GDP-L-fucose would be more informative than studies on heterologous enzyme proteins. To this purpose and to complete the present data, expression in *E. coli* of the previously sequenced *wcaG* gene [15], which shows a remarkably high sequence homology with FX protein, i.e. the epimerase/reductase activity that catalyzes GDP-L-fucose synthesis, is currently underway in our laboratory.

Acknowledgements: This work was partially supported by the Associazione Italiana per la Ricerca sul Cancro, by the CNR Target Project on Genetic Engineering and by MURST.

References

[1] Ginsburg, V. (1960) J. Biol. Chem. 235, 2196–2201.
[2] Ginsburg, V. (1961) J. Biol. Chem. 236, 2389–2393.
[3] Foster, D.W. and Ginsburg, V. (1961) Biochim. Biophys. Acta 54, 376–378.
[4] Feizi, T. (1990) Trends Biochem. Sci. 15, 330–331.
[5] Feizi, T. and Childs, R.A. (1987) Biochem. J. 245, 1–11.
[6] Broschat, K.O., Chang, S. and Serif, G.S. (1985) Eur. J. Biochem. 153, 1693–1697.
[7] Bulet, P., Hoflack, B., Porchet, M. and Verbert, A. (1984) Eur. J. Biochem. 144, 255–259.
[8] Ripka, J., Adamany, A. and Stanley, P. (1986) Arch. Biochem. Biophys. 249, 533–545.
[9] Chang, S., Duerr, B. and Serif, G. (1988) J. Biol. Chem. 263, 1693–1697.
[10] Morelli, A. and De Flora, A. (1977) Arch. Biochem. Biophys. 179, 698–705.
[11] Morelli, A., Benatti, U., Radin, L., Wrigley, N.G. and De Flora, A. (1977) FEBS Lett. 80, 1–4.
[12] Tonetti, M., Sturla, L., Bisso, A., Benatti, U. and De Flora, A. (1996) J. Biol. Chem. 271, 27274–27279.
[13] Yamamoto, K., Katayama, I., Onoda, Y., Inami, M., Kumagai, H. and Tochikura, T. (1993) Arch. Biochem. Biophys. 300, 694–698.
[14] Zarkowsky, H., Lipkin, E. and Glaser, L. (1970) J. Biol. Chem. 245, 6599–6606.
[15] Aoyama, K.M., Haase, A.M. and Reeves, P.R. (1994) Mol. Biol. Evol. 11, 829–838.
[16] Stevenson, G., Adrianopoulos, K., Hobbs, M. and Reeves, P.R. (1996) J. Bacteriol. 178, 4885–4893.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Laemmli, U.K. (1970) Nature 227, 6805–6806.
[19] Bradford, M.B. (1976) Anal. Biochem. 72, 248.
[20] Zocchi, E., Polvani, C. and Guida, L. (1990) Biochem. Biophys. Res. Commun. 173, 179–185.
[21] Ishihara, H., Schachter, H. and Heath, E.C. (1972) Methods Enzymol. 28, 399–402.
[22] Ishihara, H. and Heath, E.C. (1972) Methods Enzymol. 28, 403–406.
[23] Wrigley, H.G., Heather, J.V., Bonsignore, A. and De Flora, A. (1972) J. Mol. Biol. 68, 483–499.
[24] De Flora, A., Morelli, A. and Giuliano, F. (1974) Biochem. Biophys. Res. Commun. 59, 406–413.
[25] Matsuhashi, S. and Strominger, J.L. (1966) Methods Enzymol. 8, 310–315.
[26] Inageda, K., Takahashi, K., Tokita, K., Nishima, H., Kanaho, Y., Kukimoto, I., Kontani, K., Hoshino, S. and Katada, T. (1995) J. Biochem. 117, 125–131.
[27] Zhou, J., Bai, Y. and Weiner, H. (1995) J. Biol. Chem. 270, 16689–16693.