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Identification of glucoselysine-6-phosphate deglycase, an enzyme involved in the metabolism of the fructation product glucoselysine

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The metabolism of the glycation product fructose-ε-lysine in *Escherichia coli* involves its ATP-dependent phosphorylation by a specific kinase (FrlD), followed by the conversion of fructoselysine 6-phosphate into glucose 6-phosphate and lysine by fructoselysine-6-phosphate deglycase (FrlB), which is distantly related to the isomerase domain of glucosamine-6-phosphate synthase. As shown in the present work, several bacterial operons comprise: (1) a homologue of fructoselysine-6-phosphate deglycase; (2) a second homologue of the isomerase domain of glucosamine-6-phosphate synthase, more closely related to it; and (3) components of a novel phosphotransferase system, but no FrlD homologue. The FrlB homologue (GfrF) and the closer glucosamine-6-phosphate synthase homologue (GfrE) encoded by an *Enterococcus faecium* operon were expressed in *E. coli* and purified. Similar to FrlB, GfrF catalysed the reversible conversion of fructoselysine 6-phosphate into glucose 6-phosphate and lysine. When incubated with fructose 6-phosphate and elevated concentrations of lysine, GfrE catalysed the formation of a compound identified as 2-ε-lysino-2-deoxy-6-phospho-glucose (glucoselysine 6-phosphate) by NMR. GfrE also catalysed the reciprocal conversion, i.e. the formation of fructose 6-phosphate (but not glucose 6-phosphate) from glucoselysine 6-phosphate. The equilibrium constant of this reaction (0.8 M) suggests that the enzyme serves to degrade glucoselysine 6-phosphate. In conclusion, GfrF and GfrE serve to metabolize glycation products formed from lysine and glucose (fructoselysine) or fructose (glucoselysine), via their 6-phospho derivatives. The latter are presumably formed by the putative phosphotransferase system encoded by *gfrA–gfrD*. The designation *gfr* (glycation and fructation product degradation) is proposed for this operon. This is the first description of an enzyme participating in the metabolism of fructation products.

Key words: Amadori product, fructation, fructoselysine, glucosamine-6-phosphate synthase, glycation, Heyns product.

INTRODUCTION

Aldoses react spontaneously with amino compounds to form Schiff bases, which spontaneously undergo an isomerization reaction (Amadori rearrangement), leading to the formation of 1-amino-2-keto sugar derivatives. These compounds, termed fructosamines when the reacting sugar is glucose or mannose, may form from any kind of primary amine, including the N-terminus and the lysine side-chains of proteins (reviewed in [1–3]). Fructose and other ketoses similarly react with amines, but in their case several rearrangements are possible, leading to the formation of 2-amino-aldose derivatives (either with a glucosamine or a mannosamine configuration) or to 2-amino-3-hexulose derivatives [4,5]. Spontaneous reactions of amines with glucose or fructose are often designated glycation and fructation respectively.

Fructosamines are metabolized by, and support the growth of, some fungi and bacteria (reviewed in [6]). Two different types of pathways have been identified. The first one involves H_2O_2-producing oxidases that (generally) catalyse the cleavage of the bond between the amino group and the first carbon of the sugar portion, releasing glucosone [7–11]. The second type of pathway, described in *Escherichia coli* and *Bacillus subtilis*, is initiated by the phosphorylation of the 6th carbon of the fructose moiety by an ATP-dependent kinase belonging to the PfkB (phosphofructokinase B) family ([12–13a]). The resulting fructosamine 6-phosphate derivative is next cleaved to a free amino acid and glucose 6-phosphate by a 'deglycase' distantly related to the isomerase domain of GLMS (glucosamine-6-phosphate synthase). Until now, no enzyme has been known to metabolize fructation products.

While searching bacterial genomes for putative fructosamine-6-phosphate deglycases, we noted the existence of operons encoding not only a putative fructosamine-6-phosphate deglycase, but also a second homologue of the isomerase domain of GLMS, more closely related to it. The purpose of the present work was to identify the biochemical function of this novel protein.

EXPERIMENTAL

Materials

Auxiliary enzymes were from Roche Applied Science Biochemicals. Radiochemicals, Sephacryl S-200 and DEAE-Sepharose were from Amersham Biosciences. Fructoselysine 6-phosphate and fructoseglycine 6-phosphate were synthesized as described previously [13]. AG 50W-X4 (100–200 mesh) and Biogel P2 fine were purchased from Bio-Rad.

Expression and purification of the proteins

A 5′ primer containing the putative ATG codon (A**CATATG**CTGAAATTCAATGAAGAAGAAC for *gfrF* or G**CATATG**TTTACGATGCAAGATTATATCT for *gfrE*) in an NdeI site (indicated in bold) and a 3′ primer containing the putative stop codon

Abbreviations used: *gfr*, glycation and fructation product degradation; GLMS, glucosamine-6-phosphate synthase; HMBC, heteronuclear multiple bond connectivity; ORF, open reading frame; PfkB, phosphofructokinase B; PTS, phosphotransferase system.

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(G**GGATCC**TAATAATCAAACTGACGATAGTAG for *gfrF* or C**GGATCC**TTATAATTTACTTTTCAAGACTGTATC for *gfrE*) flanked by a BamHI site were used to amplify genomic DNA by PCR from *Enterococcus faecium* with 2.5 units of *Pwo* polymerase. PCR products of 1002 and 1047 bp respectively were obtained, subcloned into pBlueScript and checked by sequencing. NdeI–BamHI fragments containing the whole ORFs (open reading frames) were obtained from partial digestions and inserted in pET-3a. These vectors were used to transform *E. coli* BL21(DE3)pLysS. The resulting bacteria were grown in 250 ml of M9 medium containing 100 mg/l ampicillin and 25 mg/l chloramphenicol. Bacteria were induced to express the genes, and extracts were prepared and centrifuged as described previously [12]. The resulting supernatants were stored at −70 °C in 10 % glycerol. For the purification of the expressed proteins, 12.5 ml of supernatant were diluted 5-fold in buffer A (20 mM Tris, pH 7.8, 1 mM dithiothreitol, 5 μg/ml leupeptin, 5 μg/ml antipain and 1.3 M glycerol) and loaded on to a DEAE-Sepharose column (10 ml) equilibrated with 20 mM Tris, pH 7.8. The column was washed with 75 ml of buffer A and protein was eluted with a linear NaCl gradient (0–0.5 M in 2 × 50 ml of buffer A). Fractions of 2 ml were collected and stored at −70 °C. GfrE was eluted from the column at 50 mM NaCl and the deglycase (GfrF) at 150 mM NaCl. For the latter, an additional purification step was required to separate it from glucose-6-phosphate isomerase. DEAE-Sepharose peak fraction (0.5 ml) was applied on a Superdex 200 column (1 cm × 30 cm) equilibrated with a buffer containing 25 mM Hepes, pH 7.1, 1 mM dithiothreitol and 100 mM NaCl. Fractions of 0.5 ml were collected and stored at −70 °C in 10 % glycerol.

Preparation and NMR analysis of the condensation product of fructose 6-phosphate and lysine

A mixture (25 ml) containing 10 mM fructose 6-phosphate, 250 mM lysine, 10 mM Hepes, pH 7.1, 5 mM $MgCl_2$ and 2.5 mg of GfrE was sterilized by filtration on a 0.22-μM membrane and incubated overnight at 30 °C. The incubation medium was mixed with 2.1 ml of 60 % $HClO_4$ and the supernatant was neutralized with 3 M K_2CO_3. The sample was centrifuged at 10000 ***g*** for 10 min, and the supernatant was diluted 2-fold in 5 mM Hepes, pH 7.1, and loaded on to a 25-ml column of AG 50W-X4 (Na^+ form) equilibrated in the same buffer. The flow-through and one volume of washing were pooled, and the pH was adjusted to 1.5 with concentrated HCl. The sample was applied on to a 5-ml AG 50W-X4 column (H^+ form) equilibrated with water. The column was washed with 40 ml of water and the compound was eluted with 0.25 M NH_4OH. The fractions (6 ml) containing the compound (detected enzymatically with GfrE, see the next paragraph) were pooled and concentrated to a volume of 0.6 ml. The sample was diluted with one volume of water and loaded on to a Biogel P2 fine column (0.9 cm × 55 cm) equilibrated with water. The appropriate fractions (0.6 ml) were pooled and freeze-dried for NMR analysis. Approx. 30 μmol of compound free from fructose 6-phosphate (as determined spectrophotometrically with glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase, see below), lysine and ammonia were obtained in this way. The sample was dissolved in 0.6 ml of ^2H_2O and transferred into a 5-mm NMR tube for spectroscopic analyses. The pH of the final solution was 6.2. All NMR spectra were acquired on a Bruker DRX500 spectrometer at 30 °C. One-dimensional ^{1}H-NMR spectra were acquired with water presaturation, using a 6 μs pulse width, corresponding to a 60° flip angle and a repetition delay of 5 s. Proton chemical shifts were relative to 3-(trimethylsilyl)propanesulphonic acid (sodium salt), designated at 0.015 p.p.m. Two-dimensional spectra were performed using standard Bruker pulse programs.

Glucoselysine-6-phosphate and fructoselysine-6-phosphate deglycases assays

The reactions were assayed either spectrophotometrically (at 340 nm) through the formation of glucose 6-phosphate or fructose 6-phosphate, or with a radiochemical assay measuring the condensation reaction. The mixture (1 ml) for the spectrophotometric assay contained 50 mM Hepes, pH 7.1, 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM NADP, 0.1 mM fructoselysine 6-phosphate or glucoselysine 6-phosphate, 5 μg of glucose-6-phosphate dehydrogenase, 10 μg of glucose-6-phosphate isomerase (if needed) and appropriate amounts of the enzymatic preparation to obtain linear slopes. The same mixture was used for an end-point assay of (purified) glucoselysine 6-phosphate. In this case, 25 μg of purified GfrE was added to initiate the reaction. The condensation reactions were measured as described previously [13], except that, where needed, [^{14}C]fructose 6-phosphate (prepared by phosphorylation of [^{14}C]fructose with hexokinase) was substituted for radiolabelled glucose 6-phosphate.

RESULTS

Identification of operons encoding a new protein related to GLMS

BLAST searches with the sequences of *E. coli* and *B. subtilis* fructosamine-6-phosphate deglycases were carried out to find putative fructosamine operons in other bacterial genomes. This approach allowed us to identify additional operons that contained a putative deglycase and a putative fructosamine 6-kinase of the PfkB family (e.g. in *Clostridium acetobutylicum*, *Salmonella* serotype Typhimurium, *Rhodobacter sphaeroides*, *Ent. faecium*; Table 1 and results not shown). We also found deglycase-containing operons (Figure 1 and Table 1) that lacked a PfkB homologue, but encoded elements of a putative PTS (phosphotransferase system) and a protein (hereafter called GfrE) of ≈350 amino acids, similar to the isomerase domain of GLMS, and being, in fact, more closely related to this domain than fructosamine-6-phosphate deglycases are.

As shown in Figure 2, the number of identities of this protein with *E. coli* GLMS amounted to 73 and 72 for GfrE from *Ent. faecium* and *Salmonella* Typhimurium, as compared with 54 and 56 for the fructoselysine-6-phosphate deglycase of *E. coli* and its *Ent. faecium* homologue (GfrF). However, the GfrE sequences lacked many of the conserved residues of the isomerase domain of GLMS (underlined in the GLMS sequence) and, unlike GLMS, did not comprise a glutaminase domain. The glutaminase domain of GLMS provides the ammonia that reacts with the keto-group of fructose 6-phosphate to form a Schiff base, which is secondarily isomerized to glucosamine 6-phosphate by the isomerase domain (reviewed in [14,15]). These findings suggested that GfrE did not catalyse the synthesis of glucosamine 6-phosphate, but a related reaction involving presumably an amino compound and fructose 6-phosphate or glucose 6-phosphate. To identify this reaction, we overexpressed and studied the properties of the GLMS homologue (GfrE) found in a *Ent. faecium* operon (Figure 1). We also overexpressed the fructosamine-6-phosphate deglycase homologue (GfrF) of the same operon as it was expected to have a related function.

Over-expression and purification of GfrF and GfrE

The two ORFs of interest were amplified by PCR and inserted into a pET-3a plasmid for expression in *E. coli* BL21(DE3)pLysS.

Table 1 Composition of operons containing a fructoselysine-6-phosphate deglycase homologue

Each line corresponds to a putative operon containing a fructoselysine-6-phosphate deglycase homologue. Note that the genomes of *Ent. faecium* and *Salmonella* Typhimurium contain 2 and 5 of such operons respectively. The presence of homologues for PTS IIc and other components is indicated by the presence of a value in the Table. This value corresponds to the percentage identity with *Ent. faecium* GfrB, GfrE or GfrF, or with *E. coli* FrlB or FrlD. The accession numbers are given for the GfrE and/or GfrF homologues. P, phosphate.

Species	Presence of a homologue of . . . / Identity (%) with . . .	PTS IIc GfrB	Glucoselysine-6-P deglycase GfrE	Fructoselysine-6-P deglycase GfrF	Fructoselysine-6-P deglycase FrlB	Fructoselysine 6-kinase FrlD	Accession number (gi) of GfrE and GfrF homologues
Ent. faecium		100	100	100	25	–	68195898 and 68195899
Ent. faecalis		79	51	80	23	–	29343940 and 29343939
Salmonella Typhimurium		69	47	33	26	–	16423106 and 16423107
Salmonella Typhimurium		38	24	25	26	–	16419082 and 16419081
Fusobacterium nucleatum		68	45	33	20	–	19714141 and 19714140
Listeria monocytogenes		50	35	56	23	–	16411452 and 16411451
L. innocua		51	34	56	22	–	16414621 and 16414620
E. coli K12		–	–	25	100	100	14917085
E. coli O157:H7		–	–	24	98	96	37999731
Shigella flexneri		–	–	25	100	99	30043606
Clostridium acetobutylicum		–	–	25	24	31	15026593
B. subtilis		–	–	28	29	32	2635758
Salmonella Typhimurium		–	–	27	28	30	16422163
Ent. faecium		–	–	50	20	41	68195378
Ent. faecium		–	–	24	27	29	68194996
Ent. faecium		36	–	36	22	–	68196659
Ent. faecium		35	–	25	25	–	68196111

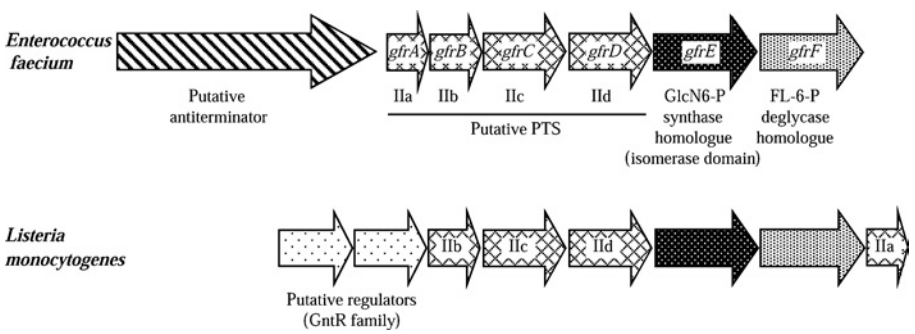

Figure 1 Organization of bacterial operons comprising a homologue of fructoselysine-6-phosphate (FL-6-P) deglycase associated with a closer homologue of the isomerase domain of glucosamine-6-phosphate (GlcN6-P) synthase and a putative PTS

Operons with a structure similar to that found in *Ent. faecium* (GfrE, accession number gi 48825763) are found in *Salmonella* Typhimurium (gi 1676778) and *Ent. faecalis* (gi 29343940). Operons similar to the one found in *Listeria monocytogenes* (gi 16804038) are found in *L. innocua* (gi 16414621) and *Pediococcus pentosaceus* (gi 48870616).

Expression was carried out for 20 h at 18 °C in M9 medium. As shown in Figure 3, the putative deglycase (GfrF) was produced essentially in a soluble form, as indicated by the presence of a $\approx$38 kDa band in lane 4. Part of GfrE was also soluble (visible as a $\approx$39 kDa band in lane 2), the majority being, however, present in the pellet (lane 6) (Figure 3). In both cases, the expression was strongly induced by IPTG (isopropyl β-D-thiogalactoside).

Both proteins were purified by chromatography on DEAE-Sepharose. GfrE was eluted at the beginning of the salt gradient (with $\approx$50 mM NaCl), and was therefore substantially purified after this single step, being free from glucose-6-phosphate isomerase (which could interfere in the assays, see below). GfrF, which was eluted at 150 mM NaCl, was further purified by chromatography on Superdex 200, being then nearly completely separated from glucose-6-phosphate isomerase.

Identification of the reactions catalysed by GfrF and GfrE

We showed previously that the activity of the deglycases of *E. coli* and *B. subtilis* could be conveniently detected by allowing the reaction to proceed in the non-physiological direction [12,13] as follows. Incubation of [^{14}C]glucose 6-phosphate with an elevated concentration ($\geqslant$50 mM) of the appropriate amino acid leads to the formation of a radiolabelled product, which is easily detected, after enzymatic removal of the phosphate group, by chromatography on a cation-exchanger at acidic pH. As we expected that the closer GLMS homologue might physiologically produce fructose 6-phosphate, we tested the formation of radiolabelled cationic derivatives from both [^{14}C]glucose 6-phosphate and [^{14}C]fructose 6-phosphate in the presence of GfrF and GfrE. The two amino acids that were chosen initially were lysine and glycine, substrates (in the reverse reaction) of the fructosamine-6-phosphate deglycases of *E. coli* and *B. subtilis* respectively [13]. As shown in Figure 4, the putative deglycase (GfrF) formed a cationic ^{14}C-labelled compound from lysine and glucose 6-phosphate. The small amount of radioactive product (2%) observed with fructose 6-phosphate is presumably due to the presence of residual glucose-6-phosphate isomerase activity ($<$1% of the deglycase activity). By contrast, GfrE catalysed the formation of a radioactive product from lysine and fructose 6-phosphate, but not from glucose 6-phosphate.

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GfrE-Ef ---------MFTMQDYIYE-EKEVLSTILKKN------DFSTR-----EH  29
GfrE-St --------MSPTMLTYINE-ESDVLANIIRRHRQS-LEEVSRF-----AS  35
Glms-Ec YDAGDKGIYRHYMQKEIYE-QPNAIKNTLTGRISHGQVDLSELGPNADEL 288
GfrF-Ef -----------MLKFNEEE-QIKAIKGALALRPQVEEIIDKLY-------  31
FrlB-Ec ----MLDIDKSTVDFLVTENMVQEVEKVLSHDVPLVHAIVEEM------V  40

GfrE-Ef MKKTVNLLILATGSSYNACLAAKPALESYGD-LTVDIQEPFYFFNYG-KL  77
GfrE-St QKTLRRILILATGSSLNAAFCARYFFERCG--ISIDIKEPYTFSQYE-NS  82
Glms-Ec LSKVEHIQILACGTSYNSGMVSRYWFESLAG-IPCDVEIASEFRYRK-SA 336
GgrF-Ef AEKFDAVYYLGIGGTYASSMQAVTYMNGKSN-LPVFVQHAAEYYTTGNKR  80
FrlB-Ec KRDIDRIYFVACGSPLNAAQTAKHLADRFSD-LQVYAISGWEFCDNTPYR  89

GfrE-Ef SPSIDTVIAVSQSGKSASTVEAVKMIQKQGLP-VVAITNDVQSPLALEAA 126
GfrE-St DPQADMVIAISQSGKSASTLEAMRKVQAQGRP-VFALTADPQSPLGKASD 131
Glms-Ec VRRNSLMITLSQSGETADTLAGLRLSKELGYLGSLAICNVPGSSLVRESD 386
GfrF-Ef LTKDSIVILSSVTGTTQEVVKAVEQIKKVGAT-LIGFIDKANSKLSQLCD 129
FrlB-Ec LDDRCAVIGVSDYGKTEEVIKALELGRACGAL-TAAFTKRADSPITSAAE 138

GfrE-Ef QIIDLGAGVESVGFVTKGYSATVLQLLLLGIGIGISKNKISKKIEQDYMQ 176
GfrE-St YPLDILTGIESVGFVTRGFSATVLNLLLIALLIARQQQRLTESQVEEYVA 181
Glms-Ec LALMTNAGTEIGVASTKAFTTQLTVLLMLVAKLSRLKG-LDASIEHDIVH 435
GfrF-Ef FVVTYPAPGTEQ------------IKFFMAADRLMYLNGEFEAYSEYYEQ 167
FrlB-Ec FSIDYQADCIWEIH----------LLLCYSVVLEMITRLAPNAEIGKIKN 178

GfrE-Ef QLRKIINHLPAIIQKTEEFFDGYQSLFRLAQRFVTIGYGPNWGTAKE-AE 225
GfrE-St QLQRIAATLPLVIVRTEAFIHQHQAVLRNGTRFVATGYGALVGVAKE-LE 230
Glms-Ec GLQALPSRIEQMLSQ-DKRIEALAEDFSDKHHALFLGRGDQYPIALE-GA 483
GfrF-Ef LERYLPTGLVEAEKKADAFGLSFAEKHRRDSMHYFIGAGNQWGAVYSYAM 217
FrlB-Ec DLKQLPNALGHLVRTWEEKGRQLGELASQWPMIYTVAAGPLRPLGYKEGI 228

GfrE-Ef TKLTETIRVPSQGFELEAYMHGPYLEADASHLLFFIEGESVNKERSQKLQ 275
GfrE-St TKFTETVRVPSSGFELEAYMHGPYLEANAEHVMFFFEDR--PDARSRALR 278
Glms-Ec LKLKEISYIHAEAYAAGELKHGPLALIDADMPVIVVAPNNELLEKLKSNI 533
GfrF-Ef CYWEEQSWLPSKSIHAAEFLHGTLEIVEETTPVTLFLGEDEQRVLSERVA 267
FrlB-Ec VTLMEFTWTHGCVIESGEFRHGPLEIVEPGVPFLFLLGNDESRHTTERAI 278

GfrE-Ef RYMSRYVGETLTITTKKAR-NEKTLGLAIEC---DEYLSVLALVVPFQLF 321
GfrE-St EYMTPAVAKTFTLTLAKAAQDDQTLALDVAV---DHHFSPLLLIVPVQLM 325
Glms-Ec EEVRARGGQLYVFADQDAGFVSSDNMHIIEMPHVEEVIAPIFYTVPLQLL 583
GfrF-Ef KLLPKICSNYTLIDTKDYPVEGIS--EKYRG----RVLSFLLMHVVTQRI 311
FrlB-Ec NFVKQRTDNVIVIDYAEISQGLHP-----------WLAPFLMFVPMEWL 316

GfrE-Ef AYKTAVAKGIDLNKKIFEDFDTVLKSKL 349
GfrE-St AFHIASLKGIDLSVRIFDDFDRVLKSKI 353
Glms-Ec AYHVALIKGTDVDQPRNLAKSVTVE--- 608
GfrF-Ef DAHVEQLNCHPLEIRRYYRQ-FDY---- 334
FrlB-Ec CYYLSIYKDHNPDERRYYGGLVEY---- 340
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Figure 2 Alignment of *Ent. faecium* and *Salmonella* Typhimurium GfrE with the isomerase domain *E. coli* GLMS and with fructoselysine-6-phosphate deglycases

The aligned sequences are as follows: GfrE from *Ent. faecium* (GfrE-*Ef*, gi 48825763) and *Salmonella* Typhimurium (GfrE-*St*, gi 16767783); the isomerase domain of *E. coli* GLMS (Glms-*Ec*, gi 3915705); *E. coli* fructoselysine-6-phosphate deglycase (FrlB-*Ec*, gi 14917085) and *Ent. faecium* GfrF (GfrF-*Ef*, gi 48825764), which is shown in the present paper to be also a fructoselysine-6-phosphate deglycase. The residues identical with *E. coli* GLMS are boxed in grey. The conserved residues among GLMS sequences [15] are underlined in the GLMS sequence. Amino acids that are strictly conserved in 12 GfrE homologues (including those listed in Table 1), but not found in GLMS, are underlined in the GfrE sequences.

The action of the putative fructosamine 6-phosphate deglycase (GfrF) as a fructoselysine-6-phosphate deglycase was confirmed by measuring spectrophotometrically the formation of glucose 6-phosphate. The specific activity that was observed at 100 μM fructoselysine 6-phosphate (0.4 μmol/min per mg) is similar to that observed with *E. coli* FrlB.

Identification of the product formed from lysine and fructose 6-phosphate by GfrE

The product of condensation formed from lysine and fructose 6-phosphate by GfrE was produced in sufficient amount (30 μmol) to allow structural characterization. Four possible structures were considered for the new sugar derivative: (i) a glucosyl structure, (ii) a mannosyl structure, and (iii and iv) two optical isomers of a ketonyl derivative (Figure 5). The analysis of the one-dimensional ^{1}H spectrum (Figure 6) revealed the presence of two characteristic signals at 5.5 and 5.0 p.p.m. (a region where the anomeric signals of hexopyranoses typically occur), with intensities of 0.80 and 0.19 respectively (as compared with the signal of the ε-protons of the lysine residue). The presence of these signals is not compatible with hypothetical derivatives (iii) and (iv) (Figure 5), since no

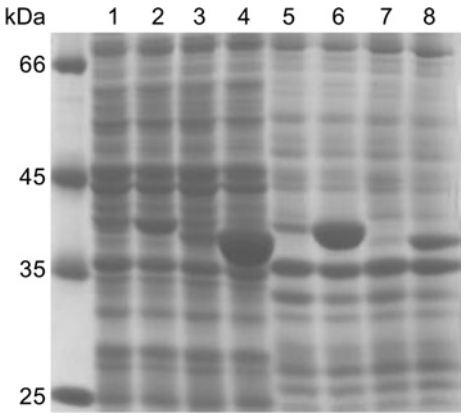

Figure 3 SDS/PAGE analysis of extracts of bacteria expressing GfrE and GfrF

Bacteria containing the GfrE (lanes 1, 2, 5 and 6) or the GfrF (lanes 3, 4, 7 and 8) expression vectors were incubated without (lanes 1, 3, 5 and 7) or with (lanes 2, 4, 6 and 8) IPTG (isopropyl β-D-thiogalactoside) for 20 h at 18°C in M9 medium. Bacterial extracts were prepared as described in the Experimental section and centrifuged. Samples (15 μl) of the supernatants (lanes 1 to 4) and pellets (resuspended in the initial volume of buffer, lanes 5 to 8) were loaded on to the gel.

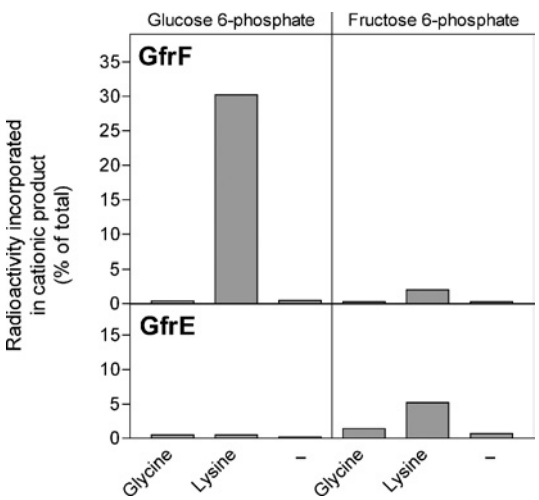

Figure 4 Identification of the reaction catalysed by GfrF and GfrE

The reaction was measured in the non-physiological direction by incubating 5 μg of each enzymatic preparation with [^{14}C]glucose 6-phosphate or [^{14}C]fructose 6-phosphate (0.1 mM) and 50 mM glycine or lysine. Reaction products were analysed by chromatography on AG 50W-X4 (H^+) columns after enzymatic removal of the phosphate group. Controls without enzymes (results not shown) gave conversions of < 0.7 % of total radioactivity. -, without glycine and lysine.

proton in those structures is predicted to display such chemical shifts. Instead, the values of the chemical shifts and their intensities are compatible with the presence of the two anomeric forms of a hexose. The measurement of the coupling constants of these signals (corresponding to the coupling between protons 1 and 2 of the hexose) showed values of 3.4 and 8.3 Hz for the signals at 5.5 and 5.0 p.p.m. respectively. This precludes the possibility of a mannosyl derivative: in a mannosyl-like structure the proton bound to carbon 2 is in an equatorial position and therefore displays small scalar couplings of similar magnitude (1–3 Hz) both in the α and β configurations. By contrast, a glucosyl residue presents its proton 2 in an axial position, which results in coupling

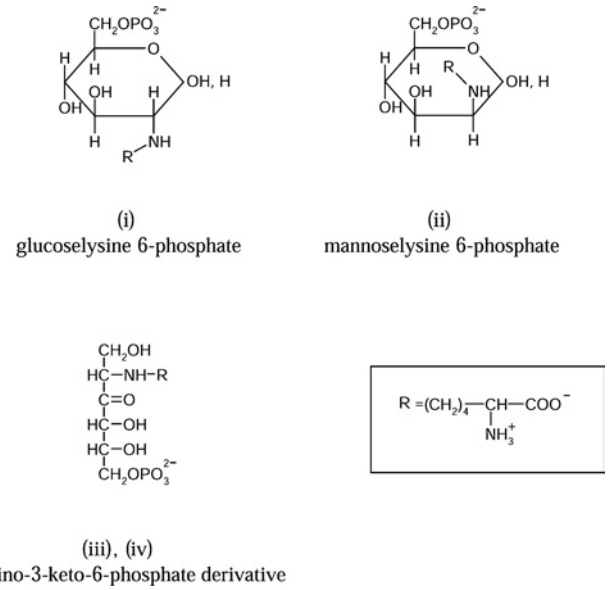

Figure 5 Structures of potential products of the condensation of fructose 6-phosphate and lysine

Only one of the two potential 2-lysino-3-keto-6-phosphate derivatives is shown.

constants of approx. 7–9 Hz in the α configuration and 3–4 Hz in the β configuration. The compound appears therefore to be 2-ε-lysino-2-deoxy-6-phosphoglucose (glucoselysine 6-phosphate).

To confirm this conclusion, we used two-dimensional NMR spectroscopy. The COSY spectrum (proton–proton direct correlation; results not shown) allowed us to assign the signals in the one-dimensional spectrum. The relative intensities, coupling constants and chemical shifts of all assigned signals are in accordance with the values expected from lysine and glucosyl residues. The only exception is position 6 of the hexose, which is shifted towards the lower field, but this is the expected consequence of a substitution with an electrophilic group, such as a phosphate group. In fact, a proton–phosphorus correlation spectrum (results not shown) revealed the presence of a connectivity between a phosphorus signal in the phospho-monoester region and the proton signals at position 6 of the hexose. A carbon–proton HMBC (heteronuclear multiple bond connectivity) spectrum showed correlations between carbon ε of the lysine and the proton at position 2 of the hexose in both α and β configurations, indicating that the lysine residue is bound to the second carbon of the glucose moiety through its ε-amino group (results not shown).

Further characterization of GfrE

We also observed that GfrE did not act on [^{14}C]fructose 6-phosphate when lysine was replaced by alanine, arginine, glutamine, glutamate, isoleucine, serine or valine (all tested at 50 or 100 mM). With 50 mM lysine, the conversion of fructose 6-phosphate amounted to 5 % after 30 min in the presence of 35 μg/ml GfrE, and this was not changed by prolonging the incubation time to 3 h or by increasing the concentration of enzyme by 5-fold (results not shown). This indicated that the conversion of 5 % corresponded to the thermodynamic equilibrium of the reaction, which was calculated to be 0.8 M for the ratio [fructose 6-phosphate] × [lysine]/[glucoselysine 6-phosphate].

The activity of GfrE was also determined spectrophotometrically (in the presence of glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase) through the production of fructose 6-phosphate from glucoselysine 6-phosphate. A K_m value of 0.4 mM and a V_{max} of 3 μmol/min per mg protein were observed. No change in A_{340} was observed if glucose-6-phosphate isomerase was omitted from the assay, indicating that fructose 6-phosphate, not glucose 6-phosphate, was produced by the enzyme. No reaction was observed with GfrE when fructoselysine 6-phosphate was used instead of glucoselysine 6-phosphate, indicating that the enzyme is not able to catalyse the isomerization of fructoselysine 6-phosphate and glucoselysine 6-phosphate. The enzyme was also inactive on glucosamine 6-phosphate (tested at 0.5 mM).

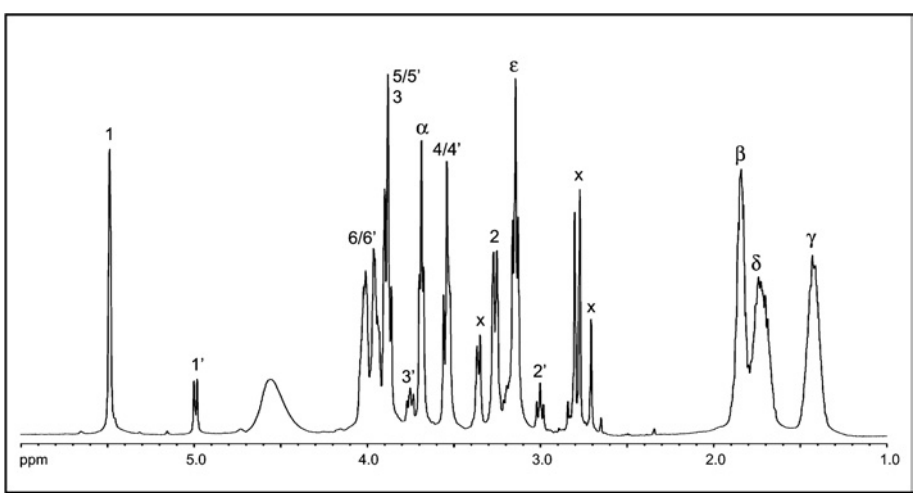

Figure 6 One-dimensional ^{1}H-NMR spectrum obtained with the product of condensation of fructose 6-phosphate and lysine

Signals arising from the lysine moiety are labelled with Greek letters and those arising from the sugar moiety are labelled numerically, with a 'prime symbol' for the β-anomer. This spectrum shows the presence of two characteristic resonances at 5.5 and 5.0 p.p.m., corresponding to both anomeric forms of a hexose, excluding the possibility of a ketonyl structure. The signals labelled 'x', which do not present any COSY connectivities and only correlated among themselves in the HMBC spectrum, derive from a contaminant whose structure was not determined.

DISCUSSION

The enzyme encoded by *gfrE* catalyses the conversion of glucoselysine 6-phosphate into lysine and fructose 6-phosphate. This was demonstrated by taking advantage of the fact that the reaction catalysed by GfrE is easily reversible *in vitro*. The product formed from fructose 6-phosphate and lysine was characterized by NMR and shown to be a glucosamine-6-phosphate-like compound, in which the amine bound to C2 is the ε-amine of lysine. This finding is consistent with GfrE being closely related to the isomerase domain of glucosamine-6-phosphate synthase. The latter catalyses the isomerization of the Schiff base, resulting from the condensation of fructose 6-phosphate and ammonia (derived from glutamine) to glucosamine 6-phosphate. The equilibrium constant of the GfrE reaction is about 0.8 M, which suggests that the physiological role of this enzyme is to cleave glucoselysine 6-phosphate to lysine and fructose 6-phosphate. It can indeed be calculated that, at a lysine concentration of 1 mM, the fructose 6-phosphate to glucoselysine 6-phosphate ratio is 800 at thermodynamic equilibrium.

The proposal that GfrE serves to metabolize glucoselysine 6-phosphate is consistent with the association of its gene with ORFs encoding a fructoselysine 6-phosphate deglycase and a putative PTS. PTSs catalyse the entry and the phosphoenolpyruvate-dependent phosphorylation of carbohydrates in bacteria (reviewed in [16]). The association of the *gfr* PTS with enzymes metabolizing glucoselysine 6-phosphate and fructoselysine 6-phosphate suggests that it serves to phosphorylate both glucoselysine and fructoselysine on their sixth carbon. The 'bifunctionality' of the *gfr* PTS would be consistent with its belonging to the same family as PTS II^{Man}, which is known to phosphorylate both an aldohexopyranose (mannose) and a ketohexofuranose (fructose) on their sixth carbon [16]. All this suggests that the operon described in the present work serves to metabolize glycation products formed from lysine and glucose (fructoselysine) or fructose (glucoselysine), two sugars that are abundant in free form in vegetables and fruits, where their concentration may reach $\approx$7 % of the fresh mass (i.e. $\approx$400 mM) [17]. The designation *gfr* (glycation and fructation product degradation) has been chosen to describe this function.

Operons with a similar gene composition are found in several bacterial genomes. It is likely that some of them encode enzymes that allow the metabolism of glycation and fructation products derived from other amino acids than lysine. This could be an explanation for the existence of two distantly related *gfr* operons in *Salmonella* Typhimurium. The genome of the latter species also comprises an operon with a putative ATP-dependent fructosamine-6-kinase and a fructosamine-6-phosphate deglycase (but no PTS and glucoselysine-6-phosphate deglycase homologue), which suggests that glycation products may be an important energy substrate for this bacterium. This must be also the case for *Ent. faecium*, the genome of which has as many as five deglycase-containing operons (Table 1).

Glucoselysine-6-phosphate deglycase is the closest known homologue of the isomerase domain of glucosamine 6-phosphate synthase, sharing with it several of the residues that bind its hexose-phosphate substrate or participate in catalysis. This is the case for Ser^{303}, Ser^{347}, Ser^{349} and Thr^{352} (the numbering in this paragraph refers to *E. coli* GLMS), the side chains of which interact with the phosphate group of the substrate; for Thr^{302} (replaced by a serine in GfrE), which forms hydrogen bonds with the hydroxy group present on C4 of the substrate; for His^{504}, which is thought to participate in the opening of the fructose 6-phosphate ring by removing a proton from the hydroxy group bound to C2 and returning it to O5; and for Glu^{488}, which appears to play an important role in sugar isomerization by performing a proton transfer between C1 and C2 of the sugar-amine-phosphate [18].

However, many conserved residues in GLMS are not found in glucoselysine-6-phosphate deglycases. This is most importantly the case for the whole glutaminase domain, but also for many scattered residues in the isomerase domain. Of note is the absence of Lys^{603}, which is thought to form a Schiff base intermediate with fructose 6-phosphate in GLMS [18]. A Schiff base is presumably directly formed with the reacting free amino acid in the 'reverse reaction' of glucoselysine-6-phosphate deglycase. Reciprocally, GfrE and other putative glucoselysine-6-phosphate deglycases comprise several strictly conserved residues (e.g. Arg^{233}, Glu^{242}, Glu^{249}, Ser^{347} and Lys^{348} in *Ent. faecium* GfrE) that are not found in GLMSs, and may therefore participate in aspects of substrate binding and catalysis that are specific to the new enzyme.

In conclusion, GfrE catalyses the cleavage of glucoselysine-6-phosphate to lysine and fructose 6-phosphate. It is the first enzyme to be described as participating in the metabolism of fructation products. As a close homologue of glucosamine 6-phosphate synthase, its study may be helpful to understand some of the unresolved aspects of the reaction catalysed by this enzyme.

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