Identification of enzymes acting on α-glycated amino acids in *Bacillus subtilis*

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Abstract **We have characterized the *Bacillus subtilis* homologs of fructoselysine 6-kinase and fructoselysine-6-phosphate deglycase, two enzymes that specifically metabolize the Amadori compound fructose-ε-lysine in *Escherichia coli*. The *B. subtilis* enzymes also catalyzed the phosphorylation of fructosamines to fructosamine 6-phosphates (YurL) and the conversion of the latter to glucose 6-phosphate and a free amino acid (YurP). However, their specificity was totally different from that of the *E. coli* enzymes, since they acted on fructoseglycine, fructosevaline (YurL) or their 6-phosphoderivatives (YurP) with more than 30-fold higher catalytic efficiencies than on fructose-ε-lysine (6-phosphate). These enzymes are therefore involved in the metabolism of α-glycated amino acids.**

Keywords: Fructosamine; Amadori product; Glycolysis; Sugar kinase; Sugar isomerase

1. Introduction

Fructosamines, the Amadori products resulting from the condensation of glucose with amines, are metabolized by microorganisms through two distinct types of enzymes. On the one hand, fructosyl amine oxidases catalyze the oxidative cleavage of fructosamines or other ketoamines either between the nitrogen and the sugar moiety, or between the nitrogen and the aglycone portion [1–6]. The oxidation product then spontaneously hydrolyzes to glucosone and a free amine in the first case, or to a free ketoamine and an aldehyde in the second case. These oxidases generally display a rather broad substrate specificity. A completely different type of metabolism has been demonstrated in the case of fructose-ε-lysine in *Escherichia coli* [7]. This bacterium, which can grow on fructose-ε-lysine as sole energy source, converts this Amadori product to fructose-ε-lysine 6-phosphate by a kinase designated FrlD, and then to free lysine and glucose 6-phosphate by a 'deglycase' (FrlB). Both enzymes appear to be specific for fructose-ε-lysine (FrlD) or its 6-phospho-derivative (FrlB). In the present work, we show that the *Bacillus subtilis* proteins YurL and YurP, which share about 30% sequence identity with FrlD and FrlB [7], catalyze similar reactions but show totally distinct substrate specificities.

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Abbreviations: fructoseglycine, *N*-α-(1-deoxyfructosyl)glycine; Fructosevaline, N-α-(1-deoxyfructosyl)valine; fructoseglycylglycine, *N*-α-(1-deoxyfructosyl)glycylglycine; fructose-ε-lysine, *N*-ε-(1-deoxyfructosyl)lysine; DMF, 1-deoxy-1-morpholinofructose; IPTG, isopropyl-1-thio-β-D-galactopyranoside

2. Materials and methods

2.1. Materials

Alkaline phosphatase (grade II, from calf intestine), glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), hexokinase (from yeast overproducer), lactate dehydrogenase (from rabbit muscle) and pyruvate kinase (from rabbit muscle), from Roche Applied Science Biochemicals, were desalted by centrifugation before use. *E. coli* fructoselysine 6-kinase and fructoselysine-6-phosphate deglycase were prepared as previously described [7]. Fructose-ε-lysine [8,9], fructoseglycine [10], fructosevaline, and fructoseglycylglycine [10,11] were synthesized as indicated. Their purity was checked by paper chromatography and mass spectrometry, and their titer was estimated by measuring the reducing power [12] with DMF as a standard or (in the case of fructose-ε-lysine) enzymatically with fructoselysine 6-kinase [7], with similar results. DMF was from Sigma. Crude preparations of fructosamines were obtained by incubating 1 M glucose in the presence of 0.2 M amino acids (0.15 M in the case of phenylalanine and leucine) for 24 h at pH 7 and 80 °C.

Fructoseglycine 6-phosphate and fructosevaline 6-phosphate were synthesized by phosphorylating 100 μmol of fructoseglycine or fructosevaline with 200 or 500 μg of purified *B. subtilis* fructosamine kinase (YurL), respectively. The phosphorylation was carried out for 2 h at 30 °C in 5 ml of 50 mM HEPES, pH 7.1, containing 40 mM ATP-Mg. The incubation mixture was acidified with 2.5 ml of ice-cold 10% (w/v) $HClO_4$, centrifuged at $10\,000 \times g$ for 10 min and the supernatant was neutralized with 3 M $KHCO_3$. After a 10-min centrifugation, the supernatant was diluted with 5 volumes of water and loaded onto an AG 1-X8 column (Cl^- form, 15 ml) equilibrated with water. The column was washed with 75 ml of water and the phosphorylated compounds were eluted with a linear NaCl gradient (0–0.5 M in 2×75 ml of water). Fractions containing fructoseglycine 6-phosphate (assayed by measuring P_i [13] after hydrolysis with alkaline phosphatase) and fructosevaline 6-phosphate (assayed by measuring inorganic phosphate as above or spectrophotometrically with glucose-6-phosphate dehydrogenase and *B. subtilis* deglycase [7], with similar results) were pooled, concentrated and desalted by gel-filtration on Biogel P2.

Fructoselysine 6-phosphate was prepared with fructoselysine 6-kinase and purified on AG 1-X8 (Cl^- form) buffered with 20 mM sodium acetate, pH 5.0 [7]. [^{14}C]Glucose 6-phosphate was synthesized from [^{14}C]glucose and ATP with hexokinase, and purified by chromatography on AG 1-X8 (Cl^- form).

2.2. Expression and purification of the kinase (YurL)

A 5′ primer containing the putative ATG codon (C**CATATG**AAATTGATTGCGGTTGGAG) in a *Nde*I site (in bold) and a 3′ primer containing the putative stop codon (A**GGATCC**TTATAGTATTCTCGTTTTTTCACT) flanked by a *Bam*HI site were used

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to PCR-amplify genomic DNA from *B. subtilis* with 2.5 U of *Pwo* polymerase. This PCR product was subcloned in pBlueScript, from which a *Nde*I–*Bam*HI fragment was then transferred in pET-3a (Novagen). The resulting vector was used to transform *E. coli* BL21(DE3)pLysS [14]. The bacteria were grown in 0.4 l M9 medium supplemented with 0.5 mg/l biotin, 0.5 mg/l thiamine, 2 g/l glucose, 1 g/l Casamino acids, 0.1 g/l ampicillin and 25 mg/l chloramphenicol. The culture was grown at 37 °C and expression of the protein was induced by the addition of 0.4 mM IPTG when A_{600} reached 0.5–0.6. After 16 h at 37 °C, the cells were collected by centrifugation, resuspended in 20 ml of buffer A (20 mM HEPES, pH 7.1, 5 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 5 μg/ml leupeptin/antipain, and 1 mg/ml lysozyme) and submitted to three cycles of freezing and thawing. The bacterial extract was incubated on ice for 1 h with 2 mg of DNaseI in the presence of 10 mM $MgSO_4$ and centrifuged for 30 min at $10\,000\times g$. The resulting supernatant was diluted 4-fold with buffer B (25 mM HEPES, pH 7.1, 1 mM dithiothreitol, and 5 μg/ml leupeptin/antipain) and loaded onto a DEAE-Sepharose column (15 ml) equilibrated with 20 mM HEPES, pH 7.1. The column was washed with 75 ml of buffer B and the retained proteins were eluted with a linear NaCl gradient (0–0.5 M in 2×75 ml of buffer B). Fractions of 3 ml were collected and stored at −70 °C in 10% glycerol.

2.3. Expression and purification of the deglycase (YurP)

A pET-15b vector (Novagen) comprising the YurP open reading frame was prepared as described above, using the following primers in the PCR: G**CATATG**AGTCAGGCCACAGCAAA and T**GGATCC**-TCAATACTCAACTTTCCACATGTA. The protein was expressed as a His-tag fusion protein in the same conditions as described above. The cells derived from a 0.4-l culture were collected by centrifugation, resuspended in 20 ml of buffer A without EDTA and dithiothreitol, and extracted as described for the kinase. The bacterial extract was centrifuged for 30 min at $10\,000\times g$. After addition of NaCl to a final concentration of 300 mM and adjustment of the pH to 7.0, the supernatant (5 ml) was mixed with 3 ml (wet gel volume) of Co^{2+}-Affinity resin (Talon®; BD Biosciences Clontech) equilibrated with buffer C (25 mM HEPES, pH 7.1, 0.5 mM PMSF, and 300 mM NaCl). The binding was performed at 4 °C for 1 h, under gentle agitation. The gel was washed twice with 30 ml of buffer D (buffer C with 1 μg/ml leupeptin/antipain) and 3 times with 20 ml of buffer D containing 10 mM imidazole. The His-tag fusion protein was eluted with 3×4.5 ml of buffer D containing 150 mM imidazole. These fractions were stored at −70 °C in 10% glycerol.

2.4. Measurement of enzyme activities

All enzyme assays were carried out at 30 °C. The assay mixture for the kinase (1 ml) contained 25 mM HEPES, pH 7.1, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.25 mM phospho(enol)pyruvate, 0.15 mM NADH, 1 mM ATP-Mg, 1 mM fructosamine, 10 μg pyruvate kinase and 5 μg of lactate dehydrogenase. The enzyme (0.8 μg in 5 μl) was added to initiate the reaction, which was followed by measuring A_{340}. For the measurement of the deglycase activity, the assay mixture (1 ml) contained 25 mM HEPES, pH 7.1, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.25 mM NAD and 2.5 U/ml glucose-6-phosphate dehydrogenase. The enzyme (3–65 μg in 5–50 μl) was added to initiate the reaction, which was followed by measuring A_{340}.

2.5. Reversibility of the reaction catalyzed by the deglycases

[U-^{14}C]Glucose 6-phosphate (40 000 cpm) and 0.1 mM unlabeled glucose 6-phosphate were incubated at 30 °C for 60 min in a mixture (0.2 ml) containing 25 mM HEPES, pH 7.1, 1 mM $MgCl_2$, 32 μg of deglycase (from *B. subtilis* or *E. coli*, [7]) and 50 mM of the indicated amino acid. The samples were further incubated with 10 μg of alkaline phosphatase for 30 min to dephosphorylate the fructosamine 6-phosphates that had formed and thereby facilitate their retention on cation-exchanger. Samples were deproteinized by the addition of 0.1 ml of ice-cold 10% (w/v) $HClO_4$ and centrifugation; 0.25-ml portions of the supernatants were diluted with 0.75 ml water and applied to AG 50W-X4 columns (H^+ form, 1 ml). The columns were washed twice with 2 ml of water and 12 ml of 1 M NaCl. The collected fractions were mixed with Optima Gold (Packard) scintillation fluid and counted for radioactivity.

3. Results and discussion

3.1. Properties of the kinase

Extracts of cells expressing YurL, the *B. subtilis* homolog of fructoselysine 6-kinase, contained large amounts of a soluble protein with the expected size of 31 kDa (Fig. 1A, lane 2). This protein was barely detectable in extracts of non-induced cells. It was purified by chromatography on DEAE-Sepharose, representing more than 80% of total protein in the peak fraction of the eluate (Fig. 1A, lane 5). The kinetic properties were studied on this fraction. About 100 mg of purified protein was obtained from an 0.4-l culture.

The enzyme phosphorylated all fructosamines that we tested but with very different affinities (Table 1). The best substrates

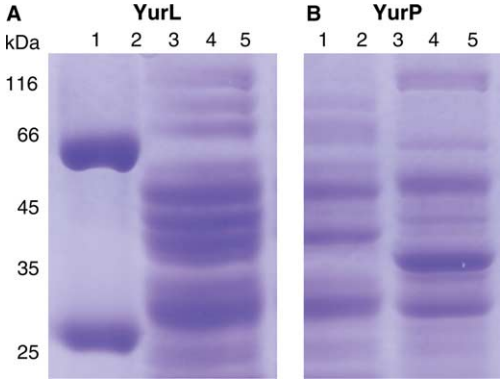

Fig. 1. SDS–PAGE analysis of extracts from bacteria expressing the kinase YurL (A) and the deglycase YurP (B) and of the purified proteins. Bacteria containing the appropriate expression vectors were incubated without (lanes 1 and 3) or with (lanes 2 and 4) IPTG. Bacterial extracts were prepared and centrifuged at $10\,000\times g$ for 30 min. Twenty μl of the supernatants (lanes 1 and 2) and pellets (resuspended in the initial volume of buffer, lanes 3 and 4) was loaded. Lanes 5: enzyme purified by chromatography on DEAE-Sepharose (YurL; 20 μl) or by Co^{2+}-affinity chromatography (YurP; 40 μl).

Table 1
Specificity of the fructosamine kinases from *B. subtilis* and *E. coli*

Substrate	*B. subtilis* (YurL)			*E. coli* (FrlD)		
	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
Fructosevaline	0.1	3	15	>20	0.04[a]	0.001
Fructoseglycine	0.6	10	8.3	80	1	0.006
Fructoseglycylglycine	6	10	0.83	>20	9[a]	0.23
Fructose-ε-lysine	14	7	0.25	0.02	30	750
DMF	47	5	0.05	24	6.5	0.14

The kinetic properties were determined on purified preparations. Values shown are means of three independent determinations. SEM values (not shown) were lower than 10% of the means.
[a] Activity at 20 mM of substrate.

were fructosevaline ($K_M = 100$ μM) and fructoseglycine ($K_M = 600$ μM), two derivatives of α-amino acids in which the α-carboxylic group is free. At least 10-fold lower affinities were found with the dipeptide derivative, fructoseglycylglycine, and with fructose-ε-lysine, indicating the importance of a free carboxylic group close to the glycated amine. An even lower affinity was observed with DMF (1-deoxy-1-morpholinofructose), a tertiary amine that does not contain any carboxylic group. The V_{max} values did not differ by more than 3-fold between the different substrates. Of interest, the catalytic efficiency (k_{cat}/K_M ratio) of the *B. subtilis* enzyme with fructose-ε-lysine as a substrate was at least 30-fold lower than with fructosevaline or fructoseglycine. The specificity of YurL differs therefore markedly from that of its *E. coli* ortholog, which displays a catalytic efficiency about 3000-fold higher for fructose-ε-lysine than for its second best substrate (Table 1).

Although the assays that we describe above do not allow us to conclude that the phosphorylated carbon is the sixth carbon of the deoxyfructose moiety, the fact that the phosphorylation product can be converted to glucose 6-phosphate and an amino acid (see next paragraph) is a strong indication for this. In addition, the kinase is homologous to *E. coli* FrlD, which has been shown to catalyze the phosphorylation of the sixth carbon of fructose-ε-lysine. Furthermore, it belongs to the PfkB/ribokinase protein family [15–17], which comprises enzymes that phosphorylate a hydroxymethyl group bound to a furanose ring.

To investigate further the specificity of the kinase, we measured its ability to phosphorylate crude glycation products obtained after a 24 h incubation at 80 °C of M glucose with each of the standard amino acids (less tryptophan and tyrosine) at a concentration of 150 mM (phenylalanine and leucine) or 200 mM (all others). YurL-dependent ADP formation from ATP was observed in the presence of all incubation products except those corresponding to glutamine and proline. When the incubation products obtained with glycine, alanine, valine, leucine, isoleucine, phenylalanine, methionine, and arginine were used, the reaction catalyzed by YurL rapidly reached a plateau, allowing us to calculate that the concentration of fructosamines that had been formed ranged from 4 to 14 mM, depending on the amino acid. Knowing these concentrations, we tentatively determined K_M and V_{max} of YurL for the corresponding fructosamines. These were (mean of two independent values): 0.5 mM and 12 U/mg for fructoseglycine, 0.09 mM and 4 U/mg for fructosealanine, 0.09 mM and 4 U/mg for fructosevaline, 0.15 mM and 6 U/mg for fructoseleucine, 0.05 mM and 3 U/mg for fructoseisoleucine, 0.05 mM and 4 U/mg for fructosephenylalanine, 0.1 mM and 6 U/mg for fructosemethionine, and 0.3 mM and 11 U/mg for fructosearginine. These values have to be taken with caution, as some other ketoamines, which could potentially interfere in the assay, may have formed during the incubation. However, their validity is suggested by the fact that the K_Ms obtained with the crude preparations of fructoseglycine and fructosevaline agree with the values observed with pure fructoseglycine or fructosevaline. Reaction of YurL with products obtained with other amino acids was slower, suggesting that they were poorer substrates. Taken together, these data indicate that the fructosamine kinase from *B. subtilis* preferentially phosphorylates fructosamines derived from hydrophobic amino acids.

3.2. Properties of the deglycase

Preliminary expression attempts indicated that the His-tagged version of YurP was produced in larger amounts in soluble form (Fig. 1B) than the unmodified protein (not shown). The modified protein was therefore purified to homogeneity by Co^{2+}-affinity chromatography. About 50 mg of purified protein was obtained from a 0.4-l culture.

The products of the phosphorylation of fructoseglycine and fructosevaline by YurL, and fructose-ε-lysine 6-phosphate, prepared with FrlD, were tested as substrates for YurP (Table 2). The fructosevaline derivative was the best substrate both in terms of K_M and V_{max}. The second best substrate was fructoseglycine 6-phosphate for which the enzyme displayed a ~3-fold higher K_M value and a 5-fold lower V_{max}. Fructose-ε-lysine 6-phosphate was the poorest substrate with a 50-fold lower V_{max}, a 24-fold higher K_M, and a more than 1000-fold lower catalytic efficiency than observed with fructosevaline 6-phosphate. By contrast, the catalytic efficiency of the *E. coli* deglycase was at least three orders of magnitude lower on fructosevaline 6-phosphate and fructoseglycine 6-phosphate than on fructose-ε-lysine 6-phosphate (Table 2).

The activity of the fructoselysine-6-phosphate deglycase was previously measured in the non-physiological direction by using [^{14}C]glucose 6-phosphate as a tracer and high concentrations of lysine [7]. We have followed the same strategy to study the specificity of the *B. subtilis* enzyme. As shown in Fig. 2, glycation products containing from 4% to 20% of the initial radioactivity were observed after a 1 h incubation with [^{14}C]glucose 6-phosphate and various amino acids (glycine, valine, lysine, glutamine, methionine, arginine, and isoleucine). Except for lysine, prolonging the incubation did not yield higher conversion extents, indicating that the thermodynamic equilibrium had been reached. By contrast, the *E. coli* enzyme catalyzed a significant conversion of [^{14}C]glucose 6-phosphate only in the presence of lysine. Taking into account the fact that lysine can be glycated on both its α- and ε-amino groups, these results underline the marked difference in specificity between the two enzymes.

Table 2
Specificity of the fructosamine-6-phosphate deglycases from *B. subtilis* and *E. coli*

Substrate	*B. subtilis* (YurP)			*E. coli* (FrlB)		
	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
Fructosevaline-6-P	0.05	0.5	6.5	nd	0.0008[a]	0.0005
Fructoseglycine-6-P	0.16	0.1	0.41	nd	0.002[a]	0.0013
Fructose-ε-lysine-6-P	1.2	0.01	0.005	0.4	1	1.6

The kinetic properties were determined on purified preparations. Values shown are means of two independent determinations, which did not differ by more than 10%.
[a] Activity at 1 mM of substrate; nd: not determined.

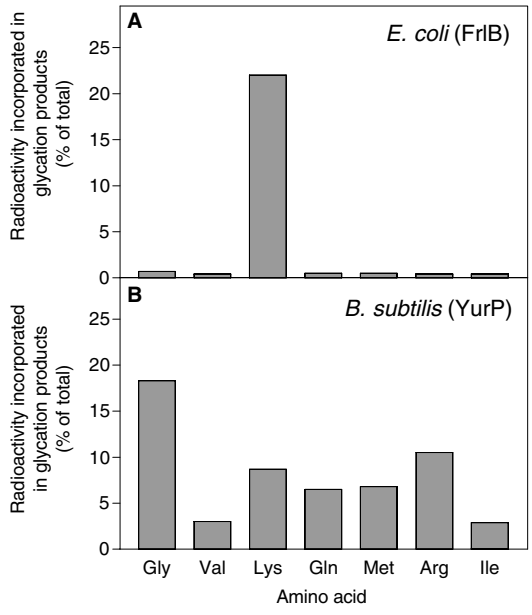

Fig. 2. Reversal of the reaction catalyzed by FrlB and YurP. Radiolabeled glucose 6-phosphate and the indicated amino acids were incubated with the deglycase of *E. coli* (A) or the deglycase of *B. subtilis* (B). Reaction products were analyzed by chromatography on AG 50W-X4 (H^+) columns.

In conclusion, the properties of the two enzymes of *B. subtilis* indicate that they are responsible for the metabolism of fructosamines bound to the α-amino group of amino acids. Both enzymes are relatively non-specific, which indicates that they may metabolize a number of different glycated amino acids.

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