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Purification and characterization of the *Escherichia coli* thermoresistant gluconokinase encoded by the *gntK* gene

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Abstract **A thermoresistant gluconokinase encoded by the *gntK* gene of *Escherichia coli* K-12 was purified and characterized. The K_m values of the purified enzyme for gluconate and ATP are 42 μM and 123 μM, respectively, and the activity was not altered by the presence of pyruvate. The enzyme was shown to function as a dimer with two identical subunits of 18.4 kDa. These characteristics appear to be distinct from those of the gluconokinase reported by E.I. Vivas, A. Liendo, K. Dawidowicz, and T. Istúriz (1994) J. Basic. Microbiol. 16, 117–122.**

Key words: Gluconokinase; *gntK* gene; *gntV* gene

1. Introduction

The initial step of the gluconate metabolism in *Escherichia coli* is its entry into the cells and its subsequent phosphorylation to gluconate-6-phosphate [1], which is followed by catabolism through the Entner-Doudoroff pathway and through the pentose phosphate pathway [2]. The initial step is carried out by two different systems, GntI and GntII, whose genes were reported to be located around 75 min and 96 min on the *E. coli* genome, respectively [3–6]. The GntI system that functions predominantly consists of a high affinity gluconate permease, a low affinity gluconate permease, and a thermoresistant gluconokinase, which are encoded by the *gntT, gntU,* and *gntK* genes, respectively [6–8]. These genes were cloned and characterized ([9], Izu et al., submitted and unpublished). While the GntII system that functions as a subsidiary consists of another gluconate permease and a thermosensitive gluconokinase encoded by the *gntS* and *gntV* genes, respectively [6–8]. The GntII was demonstrated to be repressed by the presence of pyruvate [3,8]. Recently, a thermoresistant gluconokinase was purified from *E. coli* and characterized [10], and another permease encoded by the *gntP* gene with the high affinity for gluconate was uncovered in *E. coli* [11].

To elucidate the physiological role of such a complex gluconate utilization, it may be crucial to characterize the product as well as the expressional regulation of each gene involved in the gluconate utilization. Here, we purified and characterized the *E. coli* thermoresistant gluconokinase (EC 2.7.1.12) encoded by the *gntK* gene, and compared its characteristics with those of the thermoresistant gluconokinase reported by Vivas et al. [10].

2. Materials and methods

2.1. Purification of gluconokinase encoded by gntK

YU120 [Δ*pts gntT*] cells harboring pGNT5, which bears the *E. coli* *gntK* gene (Izu et al., submitted), were grown in LB for 15 h, harvested, washed twice with 0.85% NaCl, and suspended with 20 mM Tris-HCl buffer (pH 7.0). The suspended cells were disrupted by passing thrice through a French pressure cell press (16000 p.s.i.), and after removing cell debris by centrifugation at 8000×*g* for 10 min, crude extracts were obtained. The extracts were then applied onto a DEAE-Toyopearl column equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was washed with 10 bed volumes of the same buffer. The enzyme was eluted by a gradient composed of 4 bed volumes of the same buffer and 4 bed volumes of the same buffer containing 0.3 M NaCl. Active fractions eluted at about 0.15 M NaCl were pooled and dialyzed against 20 mM Na-acetate (pH 6.0). The dialyzed material was applied on a phosphocellulose column equilibrated with 20 mM Na-acetate buffer (pH 6.0). The column was then washed with 10 bed volumes of the same buffer and further with 10 bed volumes of the same buffer containing 0.3 M NaCl. The enzyme was eluted by a gradient composed of 4 bed volumes of the same buffer containing 0.3 M and 0.7 M NaCl. Active fractions eluting at about 0.55 M NaCl were pooled and concentrated by ultrafiltration with a Toyo UK50 membrane filter. The resultant material was found to have a homogeneity of more than 95%, judging from SDS-15% PAGE.

2.2. Enzyme assay

Gluconokinase activity was measured at 25°C by the assay system with pyruvate kinase and lactate dehydrogenase (Boehringer Mannheim, Mannheim, Germany) as described [12] in 50 mM Tris-HCl (pH 7.0) containing 5 mM phosphoenolpyruvate, 65 mM KCl, 20 mM $MgSO_4$, 90 μM NADH, 3.2 mM ATP, and 1 mM sodium gluconate, or by another assay system with 6-phosphogluconate dehydrogenase (Boehringer Mannheim) [13] in 50 mM Tris-HCl (pH 7.0) containing 20 mM $MgSO_4$, 400 μM $NADP^+$, 3.2 mM ATP, and 1 mM sodium gluconate. The former was used for the assay in enzyme purification, whereas the latter was used for determining kinetic parameters or optimum temperature, or testing enzyme thermoresistance or effect of pyruvate on the enzyme activity. K_m values for gluconate and ATP were determined in the presence of 3.2 mM ATP or 1 mM gluconate. The kinetic constants were estimated by a program, EnzymeKinetics (Trinity Software Technical Support, NH, USA). According to Ref. [3], thermoresistance was examined by measuring the remaining activity at 25°C after 3 h incubation at 30°C in 0.2 ml volumes of enzyme solution (250 μg/ml in 20 mM Tris-HCl, pH 7.0), and the activity was compared with that of the same solution without the treatment. Thermoresistance was also examined by measuring activity at 25°C after 20 min or 40 min incubation at 25°C, 30°C, 40°C, or 50°C in the same enzyme solution. For determining optimal temperature, assay solution only containing buffer was preincubated for 20 min at different temperatures and after addition of other components, the reaction was started by the addition of enzyme. The background activity was also obtained by performing the same procedure without gluconate. All these assays were carried out at least three times.

2.3. N-terminal sequencing

N-terminal sequencing was performed on an automatic gas phase sequenator, PSQ2 (Shimadzu, Kyoto, Japan). The purified gluconokinase (100 μg) was subjected to SDS-12% PAGE and transferred to a polyvinylidene difluoride membrane. Using a single band obtained

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from the membrane, the N-terminal amino acid sequence was determined.

2.4. Determination of a native M_r

The purified gluconokinase (244 μg) was applied onto a TSK gel G3000 SW column (7.5×60 cm). HPLC (Shimadzu) was performed with 50 mM potassium phosphate (pH 7.0) containing 100 mM NaCl as a HPLC running buffer under the conditions of pressure 63 kg/cm and flow rate 0.6 ml/min. Standard proteins were also applied onto the same column under the same condition.

2.5. Database searching

Database searching was carried out using GENETYX (Software Development, Tokyo, Japan).

3. Results and discussion

3.1. Purification of thermoresistant gluconokinase encoded by the gntK gene

Purification of the thermoresistant gluconokinase in *E. coli* was performed with crude extracts prepared from YU120 cells harboring an expression plasmid of the *gntK* gene, pGNT5 (Izu et al., submitted). The crude extracts were passed through a DEAE-Toyopearl column followed by a phosphocellulose column (Table 1). The gluconokinase was finally purified 62.5-fold to the crude extracts with a specific activity of 250 U/mg of protein in an overall recovery of 55%. The final material was compared with other fractions by SDS-15% PAGE (Fig. 1). An 18.4-kDa band corresponding to that deduced from the nucleotide sequence of the *gntK* gene was found in more than 95% purity. To estimate the catalytic form of the gluconokinase, the purified protein was subjected to HPLC analysis under the conditions of 50 mM potassium phosphate (pH 7.0) containing 100 mM NaCl. The estimated M_r was 38 kDa in contrast with 18.4 kDa estimated by SDS-PAGE. Therefore, the thermoresistant gluconokinase is assumed to function as a dimer composed of two identical subunits.

The N-terminal 10 amino acid sequence of the purified GntK was determined. The resultant sequence, Ser-Thr-Thr-Asn-His-Asp-His-His-Ile-Tyr, completely agreed with that deduced from the nucleotide sequence of *gntK* (Izu et al., submitted) except for the first Met, which may be posttranslationally removed in cells. The coding sequence of the *gntK* gene is thus initiated with an unusual initiation codon of TTG and consists of 176 codons including a TAA stop codon according to the nucleotide sequence, and the gene product is calculated to have a M_r of 19.5 kDa, which is consistent with that obtained from SDS-PAGE. From database searching was found a hypothetical gene product, designated GntV, with 47% identity to GntK. Its gene is located between the *pepA* and *leuX* genes on the genome sequence. These facts suggest that the *gntV* gene may encode the thermosensitive gluconokinase of the GntII system.

At the N-terminal portion of the enzyme, a sequence from positions 16 to 38 homologous to the ATP-binding consensus sequence, Val-X-Gly-Ser-Gly-X-Ser-X_2-Ala-X_{11}-Leu-Asp [14],

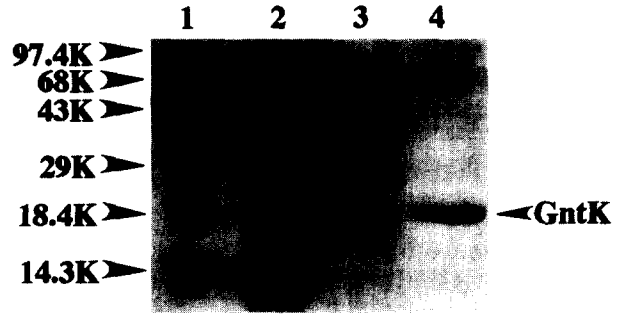

Fig. 1. SDS-PAGE of the thermoresistant gluconokinase encoded by the *gntK* gene. Samples through the purification process of the thermoresistant gluconokinase were analyzed by SDS–15% PAGE. Lanes 2–4 showed crude extracts (60 μg), the active fraction from DEAE-Toyopearl column chromatography (67 μg), and the active fraction from phosphocellulose column chromatography (12 μg). Bands corresponding to the thermoresistant gluconokinase are indicated by an arrowhead. Molecular weights of the prestained standard markers (lane 1) are shown in a kilo scale on the left: phosphorylase *b* (97400), bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (29000), β-lactoglobulin (18400), and lysozyme (14300).

was found, suggesting that ATP in the kination reaction binds to the domain including the sequence. Such a sequence was also found at the N-terminal portion of the hypothetical GntV.

3.2. Characterization of the E. coli thermoresistant gluconokinase

To examine thermoresistance of the purified gluconokinase, we measured the remaining activity after the enzyme was treated as reported by Istúriz et al. [3]. 100% of the gluconokinase activity was retained after 30 min incubation at 30°C, whereas, under the same condition, the thermosensitive enzyme was reported to lose the activity more than 75% [3]. Additionally, we tested its thermoresistance after 20 min or 40 min incubation at 25°C, 30°C, 40°C, or 50°C, and found that only at 50°C, the activity was reduced to be 63% and 50% after 20 min and 40 min incubation, respectively, compared with that at 25°C. Optimum temperature of the purified enzyme was also determined to be 45°C; in comparison with the activity (325 U/mg) at 45°C, the activities at 25°C, 30°C, 40°C, 50°C, 55°C, and 60°C were 75%, 86%, 97%, 85%, 57%, and 6%, respectively. Therefore, these results indicate that the purified enzyme is a thermoresistant gluconokinase from *E. coli*.

The kinetic properties of the purified gluconokinase were examined. The K_m values for gluconate and ATP were estimated to be 42 (4.0, standard deviation) μM and 123 (3.5) μM, respectively, and the V_{max} to be 285 U/mg. It was reported that pyruvate acts as a metabolic repressor for the GntII system [3,8], and that another *E. coli* thermoresistant gluconokinase purified by Vivas et al. showed 3.9- and 4.4-

Table 1
Purification summary of the thermoresistant gluconokinase encoded by the *gntK*

Step	Total protein (mg)	Total activity (U)	Spec. act. (U/mg)	Purification (fold)	Yield (%)
Crude extract	180	720	4.0	1	100
DEAE-Toyopearl	10.6	472	44.5	11.1	65.5
Phosphocellulose	1.57	393	250	62.5	54.6

Table 2
Characteristic comparison of the thermoresistant gluconokinases purified from *E. coli*

	GntK	Reported gluconokinase[a]
Native M_r	38 kDa (homodimer)	100 kDa (homotrimer)
Subunit M_r	18.4 kDa	29.5 kDa
Thermoresistance	stable	stable
Optimum temperature	45°C	55°C
K_m for gluconate	42 (4.0) [b]μM	20 μM
K_m for ATP	123 (3.5) [b]μM	45 μM
Effect of pyruvate	no effect	increased K_m for gluconate and ATP

[a]Reported by Vivas et al. [10].
[b]Experiments were performed four times and the values with standard deviation were shown.

fold increase in the K_m values for gluconate and ATP, respectively, in the presence of 1 mM pyruvate [10]. We thus also examined the effect of pyruvate on the enzyme activity. When pyruvate was added in the assay mixture at the concentrations of 1 mM and 10 mM, the K_m values for gluconate and ATP were not changed compared with that in the absence of pyruvate. Therefore, the results indicate that pyruvate has no effect on the activity of the thermoresistant gluconokinase from *gntK*.

3.3. Comparison of GntK with a thermoresistant gluconokinase reported recently

Vivas et al. [10] also purified and characterized a thermoresistant gluconokinase from an *E. coli* K-12 derivative strain, Ca26 (HfrH, *gntR20, gntV18, thiA, strA*) that constitutively produces the thermoresistant gluconokinase [3]. However, the characteristics of the reported enzyme are different from those of GntK purified in this study (Table 2): first, the specific activity of the reported enzyme was 30 U/mg of protein, whereas that of the purified GntK was 250 U/mg of protein. Second, the native M_r of the reported enzyme was 100 kDa with three identical subunits of 29.5 kDa, which completely differs from that deduced from the *gntK* nucleotide sequence, but the native M_r of GntK was 38 kDa with two identical subunits of 18.4 kDa. Third, the K_m values of the reported enzyme for gluconate and ATP are 2 and 3 times, respectively, lower than those of GntK. Finally, the apparent K_m values of the reported enzyme for gluconate and ATP increased in the presence of 1 mM pyruvate, but pyruvate up to 10 mM showed no effect on GntK. The latter data seem to be consistent with the previous report that pyruvate did not affect the activity of the thermoresistant gluconokinase in the GntI system [3,8]. Therefore, we conclude that GntK purified here is different from the thermoresistant gluconokinase purified by Vivas et al., and it is likely that in addition to GntK and GntV, another thermoresistant gluconokinase may occur in *E. coli*.

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References

[1] Cohen, S.S. (1951) J. Biol. Chem. 189, 617–628.
[2] Eisenberg, R.C. and Dobrogosz, W.J. (1967) J. Bacteriol. 93, 941–949.
[3] Istúriz, T., Palmero, E. and Vitelli-Flores, J. (1986) J. Gen. Microbiol. 132, 3209–3219.
[4] Nagel de Zwaig, R., Zwaig, N., Istúriz, T. and Sánchez, R.S. (1973) J. Bacteriol. 114, 463–468.
[5] Faik, P. and Kornberg, H.L. (1973) FEBS Lett. 32, 260–264.
[6] Zwaig, N., Nagel de Zwaig, R., Istúriz, T. and Wecksler, M. (1973) J. Bacteriol. 114, 469–473.
[7] Hung, A., Orozco, A. and Zwaig, N. (1970) Bacteriological Proceedings, Abstract, p. 148.
[8] Bächi, B. and Kornberg, H.L. (1975) J. Gen. Microbiol. 90, 321–335.
[9] Tong S., Porco A., Istúriz, T. and Conway, T. (1996) J. Bacteriol. 178, 3260–3269.
[10] Vivas, E.I., Liendo, A., Dawidowicz, K. and Istúriz, T. (1994) J. Basic Microbiol. 16, 117–122.
[11] Klemm, P., Tong, S., Nielsen, H. and Conway, T. (1996) J. Bacteriol. 178, 61–87.
[12] Kayne, F.J. (1973) in: The Enzymes (Boyer, P.D., ed.), vol. 8, pp. 353–382, Academic Press, New York.
[13] Fraenkel, D.G. and Horecker, B.L. (1964) J. Biol. Chem. 239, 2765–2771.
[14] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.