Cloning of a Guanosine-Inosine Kinase Gene of Escherichia coli and Characterization of the Purified Gene Product

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We attempted to clone an inosine kinase gene of Escherichia coli. A mutant strain which grows slowly with inosine as the sole purine source was used as a host for cloning. A cloned 2.8-kbp DNA fragment can accelerate the growth of the mutant with inosine. The fragment was sequenced, and one protein of 434 amino acids long was found. This protein was overexpressed. The overexpressed protein was purified and characterized. The enzyme had both inosine and guanosine kinase activity. Therefore, we attempted to clone di- nosine kinase genes from E. coli. Weak inosine kinase activity in crude extracts was reported (1,15). However, it is difficult to correctly detect inosine kinase activity in crude extracts because of the other pathways that also produce 5'-IMP from inosine. One such pathway is hypoxanthine salvage, whereby nucleosidases in crude extracts break down inosine to hypoxanthine, which in turn can be used by 5'-IMP by hypoxanthine-guanine phosphoribosyltransferase (27, 30, 31). Nucleoside phosphotransferase (EC 2.7.1.77), and inosine kinase (EC 2.7.1.73). Of these enzymes, inosine kinase is the least studied (2, 8).

Some purine nucleotides and nucleosides are commercially produced by fermentation (7, 23, 32). However, fermentation is usually difficult because of poor permeability of nucleotides (32). The major industrial process of 5'-inosine monophosphate (5'-IMP) production is chemical phosphorylation of inosine (34). On the other hand, enzymatic production of 5'-IMP has been studied (18). Three enzymes are known to form 5'-IMP: hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8), nucleoside phosphoribosyltransferase (EC 2.7.1.77), and inosine kinase (EC 2.7.1.73). From these, inosine kinase is the least studied (2, 8).

Inosine kinase has been studied in both prokaryotes and eukaryotes (1–3, 6, 14, 28). In E. coli, weak inosine kinase activity in crude extracts was reported (1, 15). However, it is difficult to correctly detect inosine kinase activity in crude extracts because of the other pathways that also produce 5'-IMP from inosine. One such pathway is hypoxanthine salvage, whereby nucleosidases in crude extracts break down inosine to hypoxanthine, which in turn can be used by 5'-IMP by hypoxanthine-guanine phosphoribosyltransferase (27, 30, 31). Nucleoside phosphoribosyltransferase, which can phosphorylate nucleosides using various phosphate donors (4, 18), is yet another obstacle to detection of inosine kinase activity. Therefore, we attempted to clone directly a gene encoding inosine kinase from E. coli by complementation, using a mutant whose growth is dependent on 5'-IMP formed from inosine. We had already cloned a putative inosine kinase gene from E. coli and registered its nucleotide sequence (accession number D00798) in DDBJ in 1991. To confirm the activity of the cloned gene product, the overexpressed gene product was purified and studied. The enzyme is a guanosine kinase having low (Vmax/Km) activity with inosine.

Recently Harlow et al. (11) reported cloning of the gsk gene of E. coli by a strategy that was different from ours. They showed that the sequence of their clone was almost identical to the sequence of our clone, and a crude extract of a strain in which gsk was overexpressed had both guanosine and inosine kinase activities. However, detailed characterization of the enzyme was not done.

In this report, our cloning strategy for the guanosine-inosine kinase gene and the unique character of the purified guanosine-inosine kinase are described.

MATERIALS AND METHODS

Bacterial strains. The E. coli K-12 strains used are listed in Table 1.

Biochemicals and enzymes. The DNA sequencing kit was purchased from United States Biochemical (Cleveland, Ohio) and enzymes used for gene manipulation from Takara Shuzo (Kyoto, Japan). Other chemicals were obtained from Wako Chemicals (Osaka, Japan) unless otherwise mentioned.

Media and cell growth. Luria-Bertani broth (24) was used for cell cultivation unless otherwise mentioned. Minimum medium containing glucose was based on M9 salts (24), and supplemented with 1 mg of thiamine per liter, 80 mg of arginine per liter, 80 mg of proline per liter, and 5 mM nucleotide or nucleobase if necessary. The agar plate medium was prepared by adding 1.5% (wt/vol) agar.

Ampicillin was added if necessary.

Isolation of mutants with weak inosine-degrading activity. E. coli SB609 cells from the culture grown overnight were spread on MacConkey agar base plates (24) containing 5 g of inosine per liter, and the cells were irradiated with UV light. The killing rate was 99%. After incubation at 37°C for 1 day, white colonies on the plates were selected. Among the selected mutants, one which turned red on a MacConkey plate containing ribose were further selected and named HM70, HM80, and HM90.

Analysis of inosine, hypoxanthine, and 5'-IMP. The amounts of inosine, hypoxanthine, and 5'-IMP were determined by high-performance liquid chromatography (HPLC) system equipped with an Asahipak GS-320H column (Asahikasei Kogyo Co., Tokyo, Japan). HPLC conditions were as follows: mobile phase, 0.2 mM NaH2PO4 (pH 2.6); flow rate, 1 ml/min; column temperature, 50°C; and detection of peaks, A248. For further quantification of 5'-IMP, reac-

tions were analyzed by an HPLC equipped with an Inertisil ODS-2 column (Gaschro Kogyo Co., Ltd., Tokyo, Japan) to distinguish 5'-IMP from 3'-IMP under the same conditions used with the Asahipak GS-320H column. The amounts of guanosine and 5'-GMP were determined as described above.

Cellular hydrolysis of inosine to hypoxanthine. Strains were cultured in Luria-Bertani broth with shaking at 30°C. Cells from the stationary phase of culture were harvested and resuspended in the reaction mixture (100 g of cells [wet weight] per liter, 30 mM inosine, 100 mM sodium phosphate buffer, pH 7.0). The mixture was incubated at 37°C, and samples were withdrawn at intervals. The amounts of inosine and hypoxanthine in the supernatants of heat-inactivated samples were determined by HPLC.

Cloning and sequencing of a guanosine-inosine kinase gene. Chromosomal DNA isolated from HM70 was partially digested by Sau3A1 and ligated to the BamHI site of pUC19 (33). The recombinant plasmids were introduced into HM70 by the method of Hanahan (10). Of the recombinants that grew first, one grew 2 days before the control strain HM70 with pUC19. All tests were per-

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formed on M9 plates containing inosine. plasmid, pBM1, with a 2.8-kbp insert was obtained from this recombinant. A series of deletion mutants was constructed from pBM1 by using exonuclease III (12). The minimal region that allowed HM70 to grow rapidly on inosine was determined with these deletion plasmids (Fig. 1). The nucleotide sequence of the minimal region was determined by using a series of deletion mutants as sequencing templates.

**Construction of the expression plasmid.** The guanosine-inosine kinase structural gene was overexpressed in MC1000 (5) with an expression plasmid, pLK75, in which the structural gene is under the control of E. coli trp promoter on the vector plasmid pTRs30 (26). HI1006, a recA strain derived from MC1000 (13), was used for gene manipulation. We made several expression plasmids with different distances between the trp Shine-Dalgarno sequence and ATG codon. Of these plasmids, pLK75 overproduced the most guanosine-inosine kinase.

Details of the construction pathway will be described elsewhere.

**Enzyme assay.** Kinase activity was assayed at 30°C by measuring the formation of 5'-nucleotide from a nucleoside, namely, inosine or guanosine. The reaction mixture (0.1 ml) consisted of 100 mM HEPES (N-2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) (pH 7.2), 10 mM MgSO4, 300 mM KCl, 2 mM ATP or other phosphate donor, 5 mM inosine or guanosine, and an enzyme preparation. The reaction was terminated by dilution with the acid buffer used as the eluent in the HPLC system. The amount of 5'-IMP or 5'-GMP produced was measured. For determination of Km and Vmax, reactions were performed with varied substrate concentrations around Km, and each reaction was terminated at 30 s. Km's and Vmax's were determined by using Hoftree plots.

**Analytical methods for protein.** Protein concentration was assayed by the method of Lowry et al. (22). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli (21). The gels were stained with Coomassie brilliant blue R-250.

**Purification of guanosine-inosine kinase from a recombinant strain.** All the enzyme purification procedures were done at temperatures lower than 5°C. Buffer A consisting of 50 mM Tris-HCl, pH 8.0, 20% glycerol, and 5 mM 2-mercaptoethanol was used throughout purification. Six grams (wet weight) of MC1000 cells carrying pLK75 was suspended in 24 ml of buffer A. The cells were disrupted by sonication. Cell debris was removed by centrifugation at 14,000 × g for 20 min. After protamine sulfate precipitation, the supernatant was desalted with Sephadex G25 (Pharmacia, Uppsala, Sweden). The enzyme solution was put on a DEAE-Sephose (Pharmacia) column and eluted with a linear gradient of sodium chloride (0.1 to 0.6 M) in buffer A. Active fractions were put on a Sephacryl S-200 (Pharmacia) column and eluted with buffer A containing 0.1 M sodium chloride. Active fractions were combined and kept at −20°C until use.

**RESULTS**

### Inosine hydrolysis activity competes with inosine kinase activity. **E. coli** mutants with deficient de novo purine biosynthesis, such as S6069 (17) (Table 1), require exogenous nucleosides or nucleobases for their growth. The growth dependency of S6069 on several nucleosides was examined (Table 2). E. coli is known to have inosine kinase activity (1, 15) which converts inosine to 5'-IMP, the precursor molecule of other nucleotides needed for cell growth. However, S6069 did not salvage inosine (Table 2). At first, S6069 was thought to lack active inosine kinase. This strain was used as a host to clone an inosine-salvaging enzyme which would complement the growth of this host with inosine. However, only hpt and gpt genes were cloned (data not shown). This result indicated that added inosine was broken down to hypoxanthine by inosine-hydrolyzing activity of S6069 and that only genes of hypoxanthine-salvaging enzymes were cloned. Although S6069 is a deoD strain, apparent inosine-hydrolyzing activity of this strain was observed (Table 2). The inosine-hydrolyzing activity of S6069 had to be eliminated in order to clone an inosine kinase gene. Thus, we tried to isolate mutants deficient in inosine hydrolysis, S6069 on a MacConkey agar plate supplemented with inosine formed red colonies (Table 2). This phenotype is due to the utilization of ribose which was released from inosine by hydrolysis. It was expected that mutants deficient in inosine hydrolysis would form white colonies on the MacConkey plate with inosine. Three mutants which became red on a MacConkey plate with ribose but which became white on inosine were isolated (see Materials and Methods) and designated HM70, HM80, and HM90. The inosine-hydrolyzing activities of these mutants were one-third of S6069’s inosine-hydrolyzing activity (Table 2). Unexpectedly, all of these mutants were able to grow
withinosineasthesolepurinesource,althoughittook4days (Table 2). However, hypoxanthine did not serve as a purine sourceforallthemutantsaswellas S\textsuperscript{F}609 (Table 2). These results indicatethat S\textsuperscript{F}609doespossess weakinosine kinase activity but the hydrolyzing activity of inosine overcomes inosine kinase activity under normal conditions.

**Cloning of the gene encoding the inosine-salvaging enzyme.**

It was expected that if a gene encoding an inosine salvage enzymewereintroducedinto HM70 byamulticopy plasmid, the recombinants would grow normally because of the increased ability of utilizing inosine. Under this assumption, HM70 was used as a host for cloning an inosine kinase gene, and transformants growing first were selected. To avoid picking up hypoxanthine-salvaging genes, the chromosome DNA of HM70 itself was used for the DNAsource of this cloning, because HM70 showed the activity of salvaging inosine (Table 2) and lacks hypoxanthine phosphoribosyltransferase (Table 1). Of several selected transformants, one strain grew first reproducibly with inosine and did not grow with hypoxanthine. A plasmid, pBM1, was extracted from this recombinant (Table 2; Fig. 1). The minimal region of pBM1 responsible for rapid growth was determined by deletion analysis (Fig. 1) and sequenced (Fig. 2). Though this region contains one open reading frame (ORF) coding for a putative protein of 434 amino acids long, no obvious consensus sequence of either the promoter or the Shine-Dalgarno sequence was identified upstream of the ORF. In the sequence downstream of the ORF, a stem-loop structure followed by T-rich sequence (TTTG GCTT) exists (Fig. 2). Although the T stretch is short, this region seems to be a \(\rho\)-independent terminator (29). This sequence was submitted to DDBJ in 1991 and assigned accession number D00798. There are two other nucleotide sequences that are almost identical to the sequence we cloned. The first is ORF312 reported by Miyamoto et al. in 1991 (25). ORF312 is a frameshift derivative of the sequence we cloned and is shown in Fig. 3. The second is the gsk gene reported by Harlow et al. in 1995 (11) and is almost identical to the sequence we cloned and is shown in Fig. 3. It is clear that Harlow’s group and our group cloned the same gene, gsk, independently by different strategies. In Harlow’s paper, the product of their cloned gene was reported to have both guanosine and inosine kinase activities, but they did not complete the purification and characterization of the gene product.

**Overexpression, purification, and characterization of the guanosine-inosine kinase.** An overexpression plasmid, pIK75 (Fig. 4), was constructed as described in Materials and Methods. The ORF was placed 16 bp downstream of the E. coli trp Shine-Dalgarno sequence under the control of E. coli trp promoter of pTrS30 (26) as shown in Fig. 4. E. coli MC1000 carrying pIK75 overexpressed a protein with an apparent molecular mass of 43 kDa (Fig. 5). The expressed protein was purified about 3.4-fold from homogeneity from the cell extract of MC1000 with pIK75 by the procedures described in Materials and Methods. A summary of the purification is shown in Table 3.

The first 10 N-terminal amino acid residues of the purified

**FIG. 2. Nucleotide sequence of the cloned guanosine-inosine kinase gene.** The deduced amino acid sequence is indicated above the nucleotide sequence. The doubly underlined region downstream of the ORF could contain a stem-loop structure, which is supposed to be of a \(\rho\)-independent terminator.
protein was determined as MKFPGKRKSK by automated Edman digestion. The amino acid sequence of the C-terminal peptide fragment generated by digestion with lysyl endopeptidase of the purified protein was also determined as YANRVSYQVLNVQHSPRLTRGLPEREDSLEESYWDX (X was not determined). These sequences correspond to the deduced terminal amino acid sequences of the ORF (Fig. 2) and indicate that the determined nucleotide sequence of the gene is correct. The ORF encodes 434 amino acids, and the calculated molecular weight of the protein is about 48,400.

We then examined the enzymatic activity of the purified protein. As shown in Table 4, 5'-IMP was produced in the presence of both ATP and inosine. ATP served as a phosphoryl donor, but p-nitrophenyl phosphate, which is a good phosphoryl donor of nucleoside phosphotransferase (4, 18), was not accepted into the reaction. This result indicates that this enzyme is kinase but not nucleoside phosphotransferase. Guanosine served as a phosphoryl acceptor as well as inosine. Adenosine, uridine, and cytidine were not accepted as a phosphoryl acceptor. The $V_{\text{max}}$ for guanosine and inosine were 2.9 and 4.9 $\mu$mol/min/mg of protein, respectively. The $K_m$s for guanosine and inosine were 6.1 mM and 2.1 mM, respectively (Fig. 6). These results indicated that this enzyme is a guanosine kinase having low ($V_{\text{max}}/K_m$) activity with inosine. The unique property of this enzyme is that dATP can serve as a phosphoryl donor as well as ATP (Table 4). The $K_m$s for ATP and dATP as inosine kinase were 0.71 and 0.66 mM, respectively. The $K_m$s for ATP and dATP as guanosine kinase were 0.51 and 2.4 mM, respectively. UTP also served as a phosphoryl donor, but its efficiency as a phosphoryl donor was about 20% of that of ATP, ADP, AMP, GTP, acetylphosphate, triplyphosphate, tetrapolyphosphate, PP$_i$, and inorganic phosphate were not accepted as a phosphoryl donor of this guanosine-inosine kinase.

The optimum pHs for the reactions were 6.9 and 8.2 for inosine and guanosine kinase reactions, respectively. The optimum temperature was between 26 and 39°C for the inosine kinase reaction, and the optimum temperature was 38°C for guanosine kinase reaction. The purified protein required both potassium and magnesium ion for the activity. Addition of Cu$^{2+}$ or Zn$^{2+}$ inhibited both kinase reactions (Table 5).

**TABLE 3. Summary of purification of the protein**

| Purification procedure | Total amt (mg) of protein | Total activity (U$^a$) | Sp act (U/mg of protein) | Yield (%) |
|------------------------|--------------------------|------------------------|-------------------------|-----------|
| Crude extract          | 303                      | 332                    | 1.1                     | 100       |
| DEAE Sepharose         | 23.9                     | 68                     | 2.8                     | 21        |
| Sephacryl S-200        | 12.5                     | 46                     | 3.7                     | 14        |

$^a$ 1 U = 1 $\mu$mol of 5'-IMP per min.

**TABLE 4. Kinase activity of the purified protein**

| Phosphate donor     | Phosphate acceptor | 5'-Nucleotide formed (mM) |
|---------------------|--------------------|---------------------------|
| ATP                 | Inosine            | 1.6                       |
| ATP                 | Guanosine          | 1.4                       |
| ATP                 | Adenosine          | ND$^b$                    |
| dATP                | Inosine            | 1.2                       |
| dATP                | Guanosine          | 0.3                       |
| UTP                 | Inosine            | 0.3                       |
| UTP                 | Guanosine          | 0.3                       |
| p-NPP$^c$           | Inosine            | ND                        |
| p-NPP               | Guanosine          | ND                        |
| None                | Inosine            | ND                        |
| ATP                 | None               | ND                        |

$^a$ Kinase activity was measured by the methods described in Materials and Methods with the purified protein preparation at the concentration of 22 $\mu$g/ml. Reactions were carried out for 30 min.

$^b$ ND, not detected.

$^c$ p-NPP, p-nitrophenyl phosphate.
guanosine as a phosphate acceptor.

reaction in panel A was calculated by using the increased amounts of 5'9M, respectively. (B) Hufste plot of panel A. The initial velocity (V) of each reaction at inosine concentrations of 41, 86, 181, 470, 956, 1,911, and 4,105 μM, respectively.

S/Vmax for guanosine were determined in the same way, except for the use of 5'-nucleotide formed. The value of the reaction with no addition was set at 100%.

DISCUSSION

Although Sf609 lacks the major purine nucleoside phosphorylase encoded by deoD (16), the inosine-hydrolyzing activity of the strain was still strong. Only one other inosine-hydrolyzing gene is known, xapA (9, 20). Because xapA encodes a minor purine nucleoside phosphorylase and is not induced by inosine, the inosine-hydrolyzing activity of Sf609 could be due to new genes which might encode nucleosidases. These genes involved in nucleoside hydrolysis of Sf609 could complement the coloring phenotype of HM70 on MacConkey plates containing nucleosides. HM70 is expected to be a useful host for cloning nucleosidase genes.

Though Sf609 does possess a guanosine-inosine kinase gene, it could not grow on medium supplemented with both inosine and guanosine (Table 2). The E. coli guanosine-inosine kinase is phenotypically inactive under normal conditions, because interfering nucleoside-hydrolyzing activity depletes substrates of the kinase. However, this kinase has a low Km value for guanosine. It is possible that this enzyme could act as a guanosine kinase under some special conditions. Because this kinase has rather high Km s for phosphoryl donors, guanosine will be phosphorylated in vivo only when the concentration of ATP or dATP is sufficient. We speculate that the guanosine-inosine kinase of E. coli could act as a scavenger of excess ATP and dATP in vivo.

A deoD strain, HO1071, was used to clone gsk in the report of Harlow et al. (11). As far as related to purine hydrolysis, HO1071 has no other marker than deoD. It is possible that nucleoside-hydrolyzing activity other than deoD would also interfere with the complementation cloning of gsk using HO1071. We suppose that gsk was successfully cloned with Harlow’s strain because four λ clones (19) covering the region around gsk (15) were used instead of the entire E. coli chromosone.

The N-terminal amino acid sequence of the gsk product (11) is the same as the overproduced product of this report. This agreement suggests that the native start codon was used for overproduction in this study.

As shown in Fig. 3, there are six amino acid substitutions and one deletion between Harlow’s gsk sequence and the sequence we cloned. All of these seven sites exist in the 100-amino-acid-long C-terminal portion of the enzyme. Three of these seven sites are within a 14-amino-acid-long C-terminal region. On the other hand, inosine kinase activity was expressed by a mutant protein which has an abnormal C-terminal sequence, GMAQAGTELEFTGRRFTTS, instead of the native sequence EESYWDTR (data not shown). From these results, it is possible that the 14-amino-acid-long C-terminal region is unimportant for the activity of this enzyme. Excluding this C-terminal region, the two sequences are 99% identical. Because both sequences encode active kinases, we suppose that the 1% mismatch does not disable the active site.

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