Note

Functional Analysis of Type 1 Isopentenyl Diphosphate Isomerase from Halobacterium sp. NRC-1

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Received May 29, 2007; Accepted June 19, 2007; Online Publication, October 7, 2007 [doi:10.1271/bbb.70330]

Here we report the characterization of the type-1 isopentenyl diphosphate isomerase derived from Halobacterium sp. NRC-1. The expressed purified enzyme showed maximum isomerase activity in the presence of 1 M NaCl at 37 °C at pH 6.0. This type-1 enzyme appears to be the first for which the Co²⁺ ion is required for activity.

Key words: archaea; halophile; isopentenyl diphosphate isomerase

Isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2) is responsible for isomerization of the carbon–carbon double bond of isopentenyl diphosphate (IPP) to create the potent electrophilic dimethylallyl diphosphate (DMAPP). 1) IDI is categorized into two distinct types that show no sequence homology each other. 2) Type 2 IDI (IDI-2) has recently been reported to be produced by bacteria such as Streptomyces, 3) Bacillus, 4,5) and archaea. 6,7) Type 1 IDI (IDI-1) is known to be utilized by most eukaryotes and some bacteria, and requires Mg²⁺ or Mn²⁺ ion for its activity. The important catalytic residues of IDI-1 required for enzyme activity have been investigated. One of these residues is a cysteine residue (Cys139 in Saccaromyces and Cys67 in Escherichia coli). 8) This residue has been proposed to be a proton donor forming the carbocation intermediately in the IDI-1 reaction. In the case of IDI-1 genes from halophilic archaea, of which the whole genome has been fully sequenced, this cysteine residue is replaced by alanine, but this mechanistically attractive isomerase from halophilic archaea has not been enzymatically characterized until now. Here we report heterologous expression of IDI-1 gene from Halobacterium sp. NRC-1 in order to confirm the function of IDI-1 gene in halophilic archaea.

The IDI-1 gene was cloned from chromosomal DNA of Halobacterium sp. NRC-1 and expressed in E. coli as follows: The PCR primers were designed on the basis of the Halobacterium sp. NRC-1 genomic sequence. Two synthetic oligonucleotides, IDI-1f (AGGGTGCAATTCGTCGTACAG), and IDI-1r (CAGTACAGAATTCCGGTGGT), including NdeI site (solid underline) and EcoRI site (dashed underline), were used as primers for amplification of the IDI-1 gene. PCR reaction was carried out with Halobacterium sp. NRC-1 genome as a template using KOD-Plus-DNA polymerase (Toyobo, Tokyo). The initial denaturation step was 10 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 61 °C, and 2 min at 68 °C. PCR products were subcloned once into Litmus28 vector (New England BioLabs, Ipswich, MA), and its sequence was confirmed. The NdeI-EcoRI fragment was subcloned into the corresponding site of pET30b vector (Novagen, Madison, WI) to yield pHbIDI1.

E. coli BL21 (DE3) carrying pHbIDI1 was cultivated in 1.2-liter of LB medium containing 30 µg/ml of kanamycin at 37 °C until OD₆₀₀ reached 0.6. After the culture was cooled on ice, IPTG was added to a final concentration of 0.1 mM to induce expression, and the culture was allowed to grow for an additional 24 h at 15 °C. The cells were harvested by centrifugation and stored at −30 °C until use. The cells were thawed in 50 mM HEPES–NaOH buffer (pH 7.0) containing 0 or 4 mM NaCl, and disrupted by sonication. The supernatant was used as a cell-free extract, and the enzyme reaction was performed.

After addition of 10 mM IPP to the cell-free extract, the mixture was incubated for 12 h at 37 °C. To this solution, 0.1 ml of 0.1 M Tris–HCl buffer (pH 9.5) and alkaline phosphatase (1 U) were added, and the mixture was further incubated at 37 °C for 12 h in order to hydrolyze diphosphate group. The reaction products were extracted with diethyl ether and analyzed by gas chromatography as described previously. 9) While the expressed enzyme (HbIDI1) showed no activity without NaCl, in the presence of 4 M NaCl, which is the optimal culture condition for Halobacterium sp. NRC-1, HbIDI1

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Abbreviations: IDI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; IPTG, isopropyl β-thiogalactopyranoside
Halobacterium exhibited isomerase activity (Fig. 1a, b). This result indicates that HbIDI1 gene in Halobacterium sp. NRC-1 encodes isopentenyl diphosphate isomerase.

For further analysis, purification of HbIDI1 was performed. To the cell-free extract of HbIDI1, containing 0.5 M NaCl, 90% saturated ammonium sulfate in 50 mM HEPES–NaOH buffer (pH 7.0) was added at 4 °C to a final concentration of 25%. The precipitate was collected by centrifugation (10,000 × g, 20 min). The precipitate obtained was resuspended in 50 mM HEPES–NaOH buffer (pH 7.0) containing 0.5 M NaCl and then washed with the same buffer and then eluted with a 5-400 mM linear gradient of potassium phosphate. Fractions containing HbIDI1 (50–100 mM potassium phosphate fraction) were pooled and concentrated by ultrafiltration. SDS–PAGE analysis of the purified enzyme revealed a single band at 24.6 kDa (Fig. 1c), which corresponds to the calculated molecular mass of HbIDI1. The activity-guided purification procedure is summarized in Table 1.

To characterize the purified HbIDI1, the enzyme reactions were examined under various conditions. Maximum activity was observed in 50 mM MES–NaOH buffer (pH 6.0) in the presence of 1 M NaCl. The optimal temperature was estimated to be 37 °C. IDI-1 generally requires Mg2+ or Mn2+ for activity, and the diphosphate moiety of substrate is stabilized by interaction with a divalent metal ion.11) Recently, it was reported that Zn2+ ion exists in the active site of IDI-1 derived from E. coli.12) A divalent ion was assumed to activate the catalytic amino acid to form a cationic intermediate during isomerization reaction. Hence, metal requirement of purified HbIDI1 activity was examined. The enzyme reaction was performed with 10 μM HbIDI1 and 5 mM IPP in HEPES–NaOH (pH 7.0) containing 1 M NaCl in the presence of each metal ion (CaCl2, CoCl2, CuCl2, MgCl2, MnCl2, ZnCl2, 1 mM). The addition of Mg2+, Mn2+, Zn2+ ions to the enzyme reaction mixture did not enhance activity as shown in Fig. 2a. Hence, metal requirement of purified HbIDI1 was examined in detail. In the presence of 10 mM EDTA, HbIDI1 showed no enzyme activity. After removal of EDTA by dialysis, the divalent ions (CaCl2, CoCl2, CuCl2, MgCl2, MnCl2, ZnCl2, 1 mM) were added in turn. Surprisingly, HbIDI1 showed enzyme activity only in the presence of Co2+ ion, while no enzyme activity was observed in the presence of other divalent ions as shown in Fig. 2b. Maximum activity was observed in the presence of more than 5 mM CoCl2 under these conditions, as shown in Fig. 2c. These results indicate that HbIDI1 utilizes only Co2+ ion for enzyme activity. To our knowledge, this is the first example in which IDI-1 utilizes Co2+ ion for its activity.

Under these conditions, the kinetic constants of HbIDI1 were determined as follows: To a solution of a CHT hydroxyapatite column (Bio-Rad, Hercules, CA) equilibrated with 5 mM potassium phosphate buffer (pH 7.0) containing 0.5 M NaCl, the column was washed with the same buffer and then eluted with a 5–400 mM linear gradient of potassium phosphate. Fractions containing HbIDI1 (50–100 mM potassium phosphate fraction) were pooled and concentrated by ultrafiltration. SDS–PAGE analysis of the purified enzyme revealed a single band at 24.6 kDa (Fig. 1c), which corresponds to the calculated molecular mass of HbIDI1. The activity-guided purification procedure is summarized in Table 1.

### Table 1. Purification of Recombinant HbIDI-1 with Enzyme Activity at 37 °C in 50 mM HEPES–NaOH Buffer (pH 7.0) Containing 1 M NaCl

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>8430</td>
<td>25.4</td>
<td>3.01 × 10⁻³</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Precipitation</td>
<td>563</td>
<td>10.2</td>
<td>18.1 × 10⁻³</td>
<td>6.01</td>
<td>40</td>
</tr>
<tr>
<td>CHT Hydroxyapatite</td>
<td>387</td>
<td>8.12</td>
<td>21.0 × 10⁻³</td>
<td>6.98</td>
<td>32</td>
</tr>
</tbody>
</table>

*Units in μmol min⁻¹
(total volume, 100 μl) of 10 μM HbIDI1 in MES–NaOH buffer (pH 6.0) containing 1 M NaCl, 5 mM CoCl₂, and 0.5–10 mM IPP was added, and the resulting mixture was incubated for 3 h at 37 °C. The reaction was terminated by heat treatment for 5 min at 100 °C. After treatment with alkaline phosphatase, the reaction products were extracted with diethyl ether and analyzed as described above. The obtained initial velocities of the reactions were fitted to the Michaelis–Menten equation. The kinetic constants were estimated to be $K_m = 2.93$ mM and $k_{cat} = 0.11$ s⁻¹. The $k_{cat}$ value was smaller than other IDIs from eucaryotes (1.1–13 s⁻¹), but similar to that of prokaryotes (E. coli, 0.33 s⁻¹) (13).

In halophilic archaeon Halobacterium sp. NRC-1, IDI-1 gene presents in the chromosomal DNA, and a total of four copies of IDI-2 gene exist in the two microchromosomes. We also expressed the IDI-2 gene from Halobacterium sp. NRC-1 in E. coli. The expressed enzyme showed isomerase activity in the presence of NADH, FMN, and 4 M NaCl. Unfortunately, several attempts at purification of IDI-2 have not been successful because of its instability, but the present results suggest that both genes were operative in the cells.

In summary, we successfully expressed IDI-1 from Halobacterium sp. NRC-1. This enzyme appeared to be the first for which the Co²⁺ ion is required for its activity. Further studies are necessary to clarify the detailed enzyme mechanisms, especially roles of the divalent metal ion and the catalytic residues.

References
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