Note
Two Proteins with Diaphorase Activity from *Clostridium thermocellum* and *Moorella thermoacetica*

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Two high-molecular-weight rubredoxin genes (*hrb*) from *Clostridium thermocellum* and *Moorella thermoacetica* were expressed in *Escherichia coli* and their translated products (*Hrb*) were characterized. *M. thermoacetica* Hrb showed strong diaphorase activity. In contrast, *C. thermocellum* Hrb, containing neither FMN nor FAD in the molecule, showed flavin reductase activity with moderate diaphorase activity. Both enzymes were optimally active at about 50°C.

Key words: diaphorase; *Clostridium thermocellum*; *Moorella thermoacetica*; rubredoxin; flavin reductase

The term “diaphorase” refers to a ubiquitous class of flavin-bound enzymes that show NADH dehydrogenase activity, where the electron acceptor is generically an oxidized synthetic dye such as 2,6-dichlorophenolindophenol (DCPIP) or nitro blue tetrazolium (NBT).1) Diaphorases are applicable in the colorimetric determination of NAD(P)H, and hence various dehydrogenase activities can be assayed by taking advantage of diaphorases and synthetic dyes. In fact, various diaphorases are commercially available on the market, such as diaphorase enzyme from *Clostridium kluyveri*. A diaphorase enzyme from *M. thermoacetica* was expected to be a proteolysis product of DLD (this result will be published elsewhere). We found several *hrb* genes from bacteria, including two thermophiles, in DDBJ/GenBank/EMBL DNA databases, and cloned and expressed the *hrb* genes from thermophilic bacteria, *Clostridium thermocellum* ATCC 27405T and *Moorella thermoacetica* ATCC 39073, since thermostable enzymes might be desirable in practical applications of diaphorase due to expected high stability during storage. Although Hrb of *M. thermoacetica* was identified as a component of nitric acid oxidase, its diaphorase activity has not been characterized.3,4) Two sets of primers were used in amplification of the *hrb* genes: 5′-GGATCCAT-GAATC(AAAAGCTTTATGG)GTTTATGG-3′ and 5′-GTCGAC-TATTCTTTCTTTTCAAATAC-3′ for *C. thermocellum* ATCC 27405T *hrb* (DDBJ accession no. CP000568) and 5′-GGATCCATGGACACAAAGCCTGAC-3′ and 5′-GTCGAC(TTTAAATTTGCTCAAAGGCGTC-3′ for *M. thermoacetica* ATCC 39073 *hrb* (AF202316). Additional BamHI and SalI sites are underlined. The amplified DNA fragments were purified from agarose gels with GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Buckinghamshire, UK), ligated into TA cloning vector pCR 2.1 (Invitrogen, Carlsbad, CA), and introduced into *Escherichia coli* DH5α by standard transformation protocol. The insert DNA was recovered from the plasmid by double digestion with BamHI and SalI and ligated into pET-28a(+) (Novagen, Madison, WI), yielding pET-Hrbct and pET-Hrbcmt constructs, which produced recombinant Hrbs from *C. thermocellum* (rHrbct) and *M. thermoacetica* (rHrbcmt) respectively. Recombinant enzymes having an N-terminal 6xHis-tag were produced by *E. coli* BL21(DE3) carrying one or the other of these plasmid constructs, and purified with a Hitrap Chelating HP column (1 ml; Amersham Bioscience, UK) according to the supplier’s protocol. Active fractions were collected and desalted by dialysis against 50 mM sodium phosphate buffer (pH 7.4). Both the purified proteins

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**Abbreviations:** DCPIP, 2,6-dichlorophenolindophenol; DLD, dihydrolipoyl dehydrogenase; hrb, high-molecular weight rubredoxin; NBT, nitro blue tetrazolium; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; rDiaA, recombinant DiaA; TLC, thin layer chromatography; Tris, Tris(hydroxymethyl)aminomethane
against DCPIP was determined at 50 /C14 results). Hence Hrb proteins might require FMN but not a tightly bound FMN in the molecule (unpublished values for DCPIP and NADH were comparable with diaphorase activity, and these apparent NADH. As shown in Table 1, rHrbmt showed strong diaphorase activity with 0.4 m M NADH, and the apparent V_max values for NADH were determined with 0.2 m M FMN. As shown in Table 1, rHrbct was active on FMN, FAD, and riboflavin without any preference, similarly to an E. coli enzyme, which has been reported to be active on NADH and NADPH as electron donors and FMN, FAD, and riboflavin as electron acceptors. In contrast, some flavin reductases have narrow substrate specificity; for example, for a Rhodococcus erythropolis enzyme, FAD was a poor substrate, and NADPH was inert. In the case of the Streptomyces pristinae spiralis enzyme, it was active on FAD and riboflavin in addition to FMN although its specificity for the pyrimidinic substrate was restricted to NADH. Flavin reductases are usually components of enzyme systems. For example, the S. pristinae spiralis enzyme is a component of a two-enzyme system catalyzing the oxidation of the D-proline residue of pristinamycin II7) and the R. erythropolis enzyme is involved in dibenzothiophene desulfurization. Although rHrbct was specified as diaphorase and flavin reductase in this study, it may be involved in pyruvate metabolism, since a gene encoding a pyruvate ferredoxin oxidoreductase has been identified downstream of the hrb gene (accession no. CP0000568). On the other hand, rHrbmt is known to be a component of nitric acid oxidase and its gene resides in a gene cluster including A-type flavoprotein and ruberythrin genes.  

Table 1. Kinetic Constants of rHrbmt and rHrbct

<table>
<thead>
<tr>
<th>Enzyme (type of activity)</th>
<th>Substrate</th>
<th>Apparent K_m (mM)</th>
<th>Apparent V_max (μmoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rHrbmt (diaphorase)</td>
<td>DCPIP</td>
<td>0.030</td>
<td>1434</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>0.50</td>
<td>2540</td>
</tr>
<tr>
<td>rHrbct (diaphorase)</td>
<td>DCPIP</td>
<td>0.015</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>0.32</td>
<td>201</td>
</tr>
<tr>
<td>rHrbct (flavin reductase)</td>
<td>FMN</td>
<td>0.075</td>
<td>0.092</td>
</tr>
<tr>
<td></td>
<td>FAD</td>
<td>0.10</td>
<td>0.075</td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
<td>0.17</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>NADH</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>

* NADH was used as an electron donor.
* DCPIP was used as an electron acceptor.
* FMN was used as an electron acceptor.

showed a single band on SDS–PAGE (data not shown), and their molecular sizes were in good agreement with the calculated molecular weights of rHrbct (28,206) and rHrbmt (28,430) respectively. Purified rHrbmt was yellowish in color, but surprisingly, rHrbct was colorless, indicating that the former protein included a tightly bound FMN molecule whereas the latter did not. These observations were further validated by UV-visible absorption spectra (Fig. 1). Although rHrbmt showed absorption peaks at 267, 377, and 447 nm due to the presence of tightly bound FMN, rHrbct did not show any visible absorption peak between 350 and 500 nm. It is interesting that one protein has a noncovalently bound FMN in the molecule while the other does not, although the two proteins share 39% sequence identity between them. Recently, we characterized a recombinant Hrb of C. kluyveri and discovered the presence of a tightly bound FMN in the molecule (unpublished results). Hence Hrb proteins might require FMN but not FAD as a prosthetic group when necessary.

The diaphorase activity of these purified enzymes against DCPIP was determined at 50 °C by measuring the decrease in absorbance at 600 nm in a reaction mixture containing 50 mM Tris–HCl (pH 8.0) and NADH as an electron donor. The apparent V_max and K_m values for NADH were determined by assaying catalytic activity with different concentrations of NADH and 0.05 mM DCPIP. The apparent V_max and K_m values for DCPIP as an electron acceptor were determined using different concentrations of DCPIP and 0.2 mM NADH. As shown in Table 1, rHrbmt showed strong diaphorase activity, and these apparent V_max and K_m values for DCPIP and NADH were comparable with those of the enzyme from C. kluyveri (unpublished results). rHrbmt showed strong diaphorase activity with NBT as an electron acceptor, although quantitative analysis was not done because of insoluble precipitate (formazan) formation due to the enzyme reaction. Although rHrbct also showed diaphorase activity against DCPIP in the presence of 0.01 mM FMN in the reaction mixture, its activity was much lower than that of rHrbmt. Both enzymes oxidized NADPH as efficiently as NADH.

Since rHrbct did not include FMN as a prosthetic group, its flavin reductase activity was determined by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH. The apparent K_m and V_max values for various flavin substrates were determined with 0.4 mM NADH, and the apparent K_m and V_max values for NADH were determined with 0.2 mM FMN. As shown in Table 1, rHrbct was active on FMN, FAD, and riboflavin without any preference, similarly to an E. coli enzyme, which has been reported to be active on NADH and NADPH as electron donors and FMN, FAD, and riboflavin as electron acceptors. In contrast, some flavin reductases have narrow substrate specificity; for example, for a Rhodococcus erythropolis enzyme, FAD was a poor substrate, and NADPH was inert. In the case of the Streptomyces pristinae spiralis enzyme, it was active on FAD and riboflavin in addition to FMN although its specificity for the pyrimidinic substrate was restricted to NADH. Flavin reductases are usually components of enzyme systems. For example, the S. pristinae spiralis enzyme is a component of a two-enzyme system catalyzing the oxidation of the D-proline residue of pristinamycin II7) and the R. erythropolis enzyme is involved in dibenzothiophene desulfurization. Although rHrbct was specified as diaphorase and flavin reductase in this study, it may be involved in pyruvate metabolism, since a gene encoding a pyruvate ferredoxin oxidoreductase has been identified downstream of the hrb gene (accession no. CP0000568). On the other hand, rHrbmt is known to be a component of nitric acid oxidase and its gene resides in a gene cluster including A-type flavoprotein and ruberythrin genes.

Fig. 1. UV-Visible Absorption Spectra of rHrbct (A) and rHrbmt (B). Measurement was carried out at room temperature in 50 mM sodium phosphate buffer (pH 7.4).
indicating that the homologous Hrb proteins in \textit{C. thermocellum} and \textit{M. thermoacetica} play completely different physiological roles.

In this study, we confirmed that two Hrb proteins comprising an N-terminal flavin reductase-like domain and a C-terminal rubredoxin-like domain from \textit{C. thermocellum} and \textit{M. thermoacetica} had diaphorase activity. Hrb proteins might be good candidates for the screening of novel diaphorases.

References


