Structure and Properties of the Recombinant NADH–Cytochrome b5 Reductase of Physarum polycephalum

Terumi IKEGAMI, Eiji KAMEYAMA, Shin-ya YAMAMOTO, Yoshiko MINAMI, and Toshitsugu YUBISUI†

Department of Biochemistry, Faculty of Science, Okayama University of Science, Ridaï-cho, Okayama 700-0005, Japan

Received November 6, 2006; Accepted December 7, 2006; Online Publication, March 7, 2007 [doi:10.1271/bbb.60625]

A cDNA for NADH–cytochrome b5 reductase of Physarum polycephalum was cloned from a cDNA library, and the nucleotide sequence of the cDNA was determined (accession no. AB259870). The DNA of 943 base pairs contains 5′- and 3′-noncoding sequences, including a polyadenylation sequence, and a coding sequence of 843 base pairs. The amino acid sequence (281 residues) deduced from the nucleotide sequence was 25 residues shorter than those of vertebrate enzymes. Nevertheless, the recombinant Physarum enzyme showed enzyme activity comparable to that of the human enzyme. The recombinant Physarum enzyme showed a pH optimum of around 6.0, and apparent \( K_m \) values of \( 2 \mu M \) and \( 14 \mu M \) for NADH and cytochrome b5 respectively. The purified recombinant enzyme showed a typical FAD-derived absorption peak of cytochrome b5 reductase at around 460 nm, with a shoulder at 480 nm. These results suggest that the Physarum enzyme plays an important role in the organism.

Key words: NADH–cytochrome b5 reductase; Physarum polycephalum; nucleotide sequence; amino acid sequence; recombinant enzyme

NADH–cytochrome b5 reductase (b5R) of vertebrates is a FAD-containing flavoprotein that mainly binds to the endoplasmic reticulum membrane of cells, accepts two electrons from NADH, and transfers one electron to cytochrome b5. Thus cytochrome b5 (b5) participates in many functions, such as fatty acid metabolism, cholesterol biosynthesis, steroid hormone biosynthesis, and so on. The properties of the enzyme in vertebrates have been well characterized, and much information has been accumulated. Especially, those of humans have been in the lead in this area. The amino acid sequences, nucleotide sequences, and genome structures have been determined. Analyses of hereditary methemoglobinemia due to a deficiency of b5R have also been determined. Various kinetic, functional, and protein chemical studies on steer-liver microsomal b5R were reported by Strittmatter et al. In contrast to these detailed studies on vertebrate enzymes, the enzymes of few plants or other organisms have been characterized, although many sequence data have recently been accumulated in the databases.

Physarum polycephalum is a slime mold that shows dynamic morphological changes during a unique life cycle, which includes plasmodium, microplasmodium, sclerotium, spore, and amoeba stages. The organism excretes large amounts of slime from the plasmodium and microplasmodium. The plasmodium is an extremely large single cell with a multinucleus. We have studied the DNAs and proteins of P. polycephalum relating to the unique life cycle of the organism. During systematic analysis of the DNAs of Physarum, we cloned a cDNA for b5R. This study was done as an approach to analyze the role of b5R in the unique life cycle of the organism, since the b5R/b5 system of vertebrates is known to participate in fatty acid metabolism, and hence production of the cell membrane. The amino acid sequence deduced from the nucleotide sequence of the Physarum b5R DNA was similar to those of Dictyostelium (Q54NC1|DICDI) and Aspergillus (Q5AZB4|EMENI) b5Rs, rather than to those of vertebrate b5Rs, and the properties were different from those of vertebrate b5Rs in terms of molecular weights, amino acid sequences, and isoelectric points. The properties of the recombinent enzyme of Physarum b5R compared with those of vertebrate b5Rs is an interesting problem to be solved.

Materials and Methods

Reagents. β-NADH and β-NADPH were purchased from Wako Pure Chemicals (Osaka, Japan). Endonucleases EcoRI, BamHI, PstI, SmaI, and XhoI were...
obtained from New England Biolabs (Boston, MA). Toyopearl HW-55 for gel filtration was from Toyo Soda (Tokyo), and 5′-AMP-Sepharose 4B was from Amersham Bioscience (Uppsala, Sweden). All other reagents used in this study were reagent grade.

Culture of *P. polycephalum* and extraction of RNA. Microplasmodia of *P. polycephalum* was cultured on corn meal agar at room temperature. Total RNA was extracted from the lyophilized microplasmodia as described previously, and mRNA was purified with mAP, a poly U-conjugated affinity filter paper (Takara, Kyoto, Japan).

cDNA library. A cDNA library of *P. polycephalum* was constructed using mRNAs extracted from lyophilized microplasmodia, as described previously, using a double-stranded Uni-Zap XR vector. DNA from *Physarum* was ligated with the vector at the *XhoI* and *SmaI* sites.

Nucleotide sequence analysis. Nucleotide sequences were determined using the Big Dye Polymerase Sequencing Kit (Applied Biosystems, Foster City, CA) with the automatic DNA sequencer of ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Construction of the expression system for *Physarum* b5R. Insert DNA for b5R was amplified using T7 promoter and T3 primers of the vector by polymerase chain reaction (PCR) with Taq Gold DNA polymerase (Applied Biosystems), and the PCR product was ligated once to TA-vector (Invitrogen, San Diego, CA) for storage. Then the insert DNA for b5R was again amplified with b5R-specific primers having restriction enzyme sites (*XhoI* and *SmaI*) for ligation to the expression vector, pUC 13. The forward primer was 5′-CCGGCTCGAGGGGTCCAGCTCGTCTAAG-3′, and the reverse primer was 5′-TCCCCCGGTTAAAAAGATAAACCCTGC-3′. The expression vector pUC13 was prepared from the human b5R expression vector, and digested with *XhoI* and *SmaI* for ligation with the insert DNA for b5R. Expression vector pUC13 digested with *XhoI* and *SmaI* was then ligated with insert DNA for *Physarum* b5R to construct the expression plasmid of *Physarum* b5R.

Determination of enzyme activity. The enzyme activity of b5R was determined with *Physarum* recombinant b5 as the substrate, as described previously, by following the absorbance increase of reduced b5 at 424 nm. The activity was calculated using the molecular extinction coefficient of 123 mM⁻¹ cm⁻¹ at 424 nm for the difference between the reduced and oxidized forms, which was determined by the pyridine–hemochrome method. Spectrophotometric determinations were carried out with a Hitachi double beam spectrophotometer U-2800 (Hitachi, Tokyo).

Cytochrome b5. A cDNA for *Physarum* b5 was cloned from the cDNA library of *P. polycephalum* using digoxigenin (DIG)-labeled DNA as a probe by the plaque hybridization method. As the probe, a DNA fragment for b5 from *Polygonum tinctorium* was used. *Physarum* b5 DNA, which encodes from initial Met to Pro99, was ligated with pUC13 vector to construct the expression plasmid for soluble b5, as described above. Recombinant soluble *Physarum* b5 was expressed in *E. coli* (RB791), and recombinant b5 was purified by ion-exchange chromatography and gel filtration.

Purification of b5. The purity of the enzyme was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE). Protein was determined with Advanced protein assay reagent (Cytoskeleton, Denver, CO), using bovine serum albumin as a standard.

Results and Discussion

Sequence analysis
During EST analysis of DNAs of *P. polycephalum*, we cloned a cDNA for b5R. The nucleotide thus obtained consisted of 943 base pairs, including 5′- and 3′-noncoding sequences and a coding sequence of 843 base pairs for 281 amino acid residues, as shown in Fig. 1. The 3′-noncoding sequence contained about 20 base pairs of poly (A), but the typical poly (A) addition signal (AATAAA) for the vertebrate DNA was not found. Another sequence might have functioned as the polyadenylation signal, but that was not clear. The amino acid sequence deduced from the nucleotide sequence was most closely homologous to that of *Dictyostelium discoideum* b5R (63% identity and 82% homologous), similar to *Aspergillus nidulans* (55% identity and 74% homologous), and resembled plant b5Rs rather than to those of vertebrate b5Rs. In the amino acid sequence deduced from the nucleotide sequence, the FAD-binding motif (RxGxxP) common to the enzymes of the Ferredoxin NADP⁺ reductase family were contained, implying that the cDNA we cloned was apparently for the b5R of *P. polycephalum*, as shown in Fig. 1.

Characterization of recombinant *Physarum* b5R
The peptide from the putative initial Met to the 33rd residue (Phe) contained many hydrophobic residues, and was considered to be the membrane-binding domain from the hydropathy plot, as shown in Fig. 2. Furthermore, this domain contained the putative signal sequence, as predicted by a program (Software IP3.0 Server), and is shown by the underline in Fig. 1. Very interestingly, the absence of two big peptides of 9 and 16 residues was found between the FAD- and NADH-binding motifs in the amino acid sequence, as compared with those of vertebrate b5Rs, as shown in Fig. 3. When
the structure of the *Physarum* b5R was compared with that of the human enzyme, the former peptide corresponded to the peptide 115–123 (DTHPKFPAG), and the latter corresponded to that of 154–169 (GKFAIRPDKKSNPIIR) in the human enzyme, and both resided on the outer surface of the enzyme. The

---

**Fig. 1.** Nucleotide Sequence of the cDNA of *Physarum* b5R and Amino Acid Sequence Deduced from the Nucleotide Sequence.

The nucleotide sequence of the *Physarum* b5R DNA was determined as described in “Materials and Methods,” and the putative initial methionine was predicted by comparing the deduced amino acid sequence of the *Physarum* b5R with those of other organisms. The initial methionine codon is double underlined, bold letters show the putative N-terminal membrane-binding domain, and the signal sequence (see text) is indicated by the underline. FAD- and NADH-binding motifs (RxYTxxS, and GxGxxP) are boxed.
The former peptide is in the outside loop of the N-terminal domain of the human enzyme, which covers FAD from solvent, and the aspartic acid in the peptide has been found to be important to stabilize the binding of FAD by forming a hydrogen bond.19) This outside loop in the human enzyme also appears to restrict the specificity of the binding to NADH, as described below. The latter peptide was also in the outside loop, which connects the N- and C-terminal domains. This peptide is characteristic, since it contains many of basic residues. Indeed, K162 and K163 in the human b5R have been found to play important roles in interacting with b5, which has a negative charge cluster around the heme.25)

Although human b5R contains four cysteine residues (C203, 273, 283, and 297),6,7,26) Physarum b5R contains only one cysteine residue, corresponding to C273 of the human b5R, as shown in Fig. 3. Since only C273 in human b5R affects the efficiency of the electron transfer from NADH to b5,26) the cysteine in the Physarum b5R might also participate in the function.

### Purification of recombinant Physarum b5R

Based on these results, recombinant Physarum b5R was expressed to characterize the Physarum enzyme. Removing the DNA for the membrane-binding domain, the DNA encoding residues 34–281 (248 residues) was used to construct the expression plasmid for the soluble catalytic domain, as described in “Materials and Methods.” Due to absence of big peptides, the catalytic domain of soluble recombinant Physarum b5R consisted
of 248 residues, the calculated molecular weight from the amino acid sequence was 28,151, and the estimated isoelectric point (pI) was 8.88, in contrast to the neutral pI (6.9) of the human enzyme.

Probably due to the basic nature of the *Physarum* b5R protein, the purification method for human b5R\(^{20}\) was not applicable to *Physarum* b5R. Therefore, the *Physarum* enzyme was purified by fractionation with ammonium sulfate (35–65% saturation), gel filtration with Toyopearl HW-55, and affinity chromatography on a 5'-AMP-Sepharose, as summarized in Table 1. Before affinity chromatography, the b5R expressed as a fusion protein was digested with α-thrombin to remove the β-galactosidase peptide. By these purification methods, the *Physarum* enzyme was purified about 30-fold from the crude extract, with 30% yield as shown in Table 1. The purity of the *Physarum* b5R was examined by SDS-PAGE, and a single band of about 29 kD was observed, as shown in Fig. 4.

**Characterization of recombinant *Physarum* b5R**

The absorption spectra of the purified *Physarum* b5R were determined, and are shown in Fig. 5. The enzyme showed the typical spectra of b5R, with a peak at 462 nm of FAD bound to the enzyme protein, with a shoulder at 480 nm. The spectrum of flavin released from the enzyme by adding 0.1% SDS to it showed absorption peaks at 450 nm with a shoulder at 480 nm, which coincides with that of free FAD (Fig. 5). Based on the absorbance of the FAD released from the enzyme, the molecular extinction coefficient of *Physarum* b5R at 462 nm was determined to be 10.34 m\(^{-1}\) cm\(^{-1}\).

In spite of the absence of two big peptides (a total 25 residues) as compared with that of human b5R, the recombinant *Physarum* b5R in the crude extract of *E. coli* showed enzyme activity comparable with human b5R. This is surprising, when we recall that some single mutations (point mutations, or a single residue deletion) in human b5R caused severe impairment of function\(^{9–11}\) leading to hereditary methemoglobinemia. Therefore, the kinetic properties of the purified enzyme were determined, with *Physarum* recombinant b5 as the substrate, to analyze the function of the *Physarum* enzyme. Since the pH optimum of the purified enzyme was determined to be about 6.0, the enzyme activities described below were all determined at pH 6.0. This pH optimum of the enzyme was a reasonable value to display the function, since the intracellular pH of the microplasmoium of *Physarum* was estimated to be 6.2 by determining the pH of the water homogenates of the *Physarum*. The apparent \(K_m\) values determined were 2 μM and 14 μM for NADH and b5, respectively. The slightly larger \(K_m\) value for NADH (2 μM) than that of the human enzyme (0.6 μM) coincided well with the fact that the binding of the *Physarum* enzyme to 5'-AMP-Sepharose was slightly loose. This result apparently relates to the absence of the former peptide (9 residues) as compared with human b5R as described above. The apparent \(V_{max}\) was calculated to be 105 μmol/min/mg, and this value is comparable to that of human b5R.\(^{9–11}\)

### Table 1. Summary of Purification of *Physarum* b5R

<table>
<thead>
<tr>
<th>Step</th>
<th>TP(^{\dagger})</th>
<th>TA(^{\dagger})</th>
<th>SA(^{\dagger})</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>701.2</td>
<td>1077</td>
<td>1.5</td>
<td>100</td>
</tr>
<tr>
<td>(NH(_4))(_2)SO(_4)</td>
<td>363.6</td>
<td>583</td>
<td>1.6</td>
<td>54.1</td>
</tr>
<tr>
<td>Toyopearl HW55</td>
<td>255.5</td>
<td>421</td>
<td>1.7</td>
<td>39.1</td>
</tr>
<tr>
<td>5'-AMP-Sepharose</td>
<td>6.8</td>
<td>329</td>
<td>48.4</td>
<td>30.6</td>
</tr>
</tbody>
</table>

\(^{\dagger}\)TP = Total protein, TA = total activity (μmol/min), SA = specific activity (μmol/min/mg)

---

**Fig. 4.** SDS–PAGE of Recombinant *Physarum* b5R.

Lane 1, molecular weight markers (bovine serum albumin, 36 kD; ovalbumin, 45 kD; carbonic anhydrase, 21 kD; and lysozyme, 11 kD); lane 2, crude extract (211 μg); lane 3, ammonium sulfate 35–65% saturation (217 μg); lane 4, gel filtration (30 μg); lane 5, fraction after α-thrombin-digestion (27 μg); lane 6, 5'-AMP-Sepharose (6 μg).

**Fig. 5.** Absorption Spectrum of Purified Recombinant *Physarum* b5R.

The absorption spectrum of recombinant *Physarum* b5R (170 μg) in 0.05 M Tris–HCl buffer (pH 7.5) was determined. The oxidized and NADH-reduced forms are shown by solid and dotted lines respectively. The inset shows the spectrum of the oxidized form by the solid line, and the broken line shows the spectrum of free-FAD released from the enzyme protein by the addition of 0.1% SDS (final concentration).
With NADPH, the Physarum enzyme showed about 20% of the activity with NADH. This might be related to the absence of the peptide of 9 residues as compared with the human enzyme, which corresponds to peptide 115–123 (DTHPKFPAG) in the human enzyme. The peptide in the human enzyme covers the crevice between the two domains of the enzyme, and maintains NADH-specificity. The absence of the covering peptide in the Physarum enzyme might cause the lack of specificity for NADH.

Next, the heat stability of Physarum b5R was examined, as shown in Fig. 6, to assess structural stability. In Fig. 6, those of the human enzyme determined under the same conditions are shown for comparison. When the Physarum enzyme (20 µM, 0.56 mg/ml) was incubated for 10 min at the temperatures indicated in Fig. 6, the enzyme activity decreased rapidly at above 50°C. When incubated at 50°C, the human enzyme retained more than 80% of its original activity after 10 min, but the Physarum enzyme lost almost all of its activity. Thus the Physarum enzyme was more unstable than the human enzyme. 9,10)

The vulnerability of the enzyme was also examined by treatment with trypsin. The results were compared with that of the human enzyme, as shown in Fig. 7. Interestingly, the resistance against trypsin treatment of the Physarum b5R was similar to but slightly higher than that of human b5R. 11) When both enzymes were incubated with trypsin, the human enzyme lost about 15% of its original activity after 30 min, but the Physarum enzyme retained about 90% activity, as shown in Fig. 7. This might be related to the absence of the peptide containing Lys and Arg (GKFAIRPDKKSNPIIR) in Physarum b5R (Fig. 3), which are known as the cleavage sites of trypsin. The difference in observed vulnerability against trypsin as between the two enzymes was, however, not very significant compared with that in the heat stabilities of the two enzymes.

Physarum b5

A cDNA for Physarum b5 was cloned from the cDNA library of Physarum plasmoid, as described in "Materials and Methods." A DNA fragment for b5 from Polygonum tinctorium was DIG-labeled, and the labeled probe was used to clone Physarum b5 DNA. By screening about 200,000 plaques, a few positive clones were obtained by the first screening, and then one positive clone for b5 was obtained by the second screening. Physarum b5 DNA was consisted of 467 base
pairs, including 5'- and 3'-noncoding sequences, and a coding sequence of 396 base pairs for 132 amino acid residues. The 3'-noncoding sequence contained the poly(A) additional signal, AATAAA, 35 base pairs upstream of the poly(A) sequence (see accession no. AB259833 for the nucleotide sequence). The amino acid sequence deduced from the nucleotide sequence contained two heme-binding histidines, and the sequences around the heme-binding histidines were very similar to b5s from various organisms as shown in Fig. 8.

Using this DNA for b5, recombinant soluble Physarum b5R was expressed, as described in “Materials and Methods,” to be used as the substrate for Physarum b5R. The reduced Physarum b5 showed the typical spectrum of b5, with the absorption peak at 556 nm and a shoulder at 560 nm, as shown in Fig. 9. The molecular extinction coefficient of Physarum b5 for the reduced minus oxidized forms was determined to be 123 mM⁻¹ cm⁻¹ at 423 nm by the pyridine–hemochrome method.²² This value is very similar to those of human²⁷ and chicken b5s.²⁸ In contrast to Physarum b5R, the primary structure of Physarum b5 does not have any significant peptide deletion as compared with various b5s. This suggests that the structures of b5s satisfy the required minimum for the electron carrier protein.

Based on the results described above, the whole peptides of Physarum b5R appear to be folded properly to bind NADH and b5 to maintain effective electron transfer, in spite of the absence of big peptides as compared with vertebrate enzymes. These results suggest that the Physarum enzyme as well as the Dictyostelium and Aspergillus enzymes had the mature structure for the required functions in those organisms. The vertebrate have come to have stable structure against heat stress by insertion of the extra peptides during evolution, but in turn, it incurred vulnerability against trypsin. Based on these points, the analysis of the structure of the Physarum enzyme is an interesting and important problem to be solved. Analysis of the crystal structure of the Physarum enzyme is now proceeding to determine the nature of the enzyme by comparing it with those of vertebrate enzymes.¹⁷–¹⁹

References


