Mitochondrial NADH- or NADPH-Linked Aquacobalamin Reductase Activity Is Low in Human Skin Fibroblasts with Defects in Synthesis of Cobalamin Coenzymes

FUMIO WATANABE,2 HISAKO SAIDO,* RYOICHI YAMAJI,* KAZUTAKA MIYATAKE,* YUJI ISEGAWA,† AKIO ITO,** TOSHITSUGU YUBISUI,† DAVID S. ROSENBLATT†† AND YOSHIHISA NAKANO*  

Department of Food and Nutrition, Kochi Women’s University, Kochi 780, Japan; *Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai, Osaka 593, Japan; †Department of Preventive Medicine, Osaka University, Toyonaka, Osaka 560, Japan; **Department of Biology, Kyushyu University, Fukuoka 812, Japan; †Department of Biology, Kochi University, Kochi 780, Japan; and ††MRC Genetics Group, Department of Medicine and Human Genetics, McGill University, Montreal, Quebec H3A 1A1, Canada

ABSTRACT Mammalian livers have been reported to contain NADH- and NADPH-linked aquacobalamin reductases, which are distributed in both mitochondria and microsomes. The four aquacobalamin reductase isozymes have been purified and characterized from rat liver. It is unclear which aquacobalamin reductase among the four reductase isozymes participates in the synthesis of cobalamin coenzymes. To clarify the physiological roles of the aquacobalamin reductase isozymes, human mutant fibroblasts (cblC and cblA cells) with defects in cobalamin reductases involved in the coenzyme synthesis were used. In the cblC cells, the activity of the mitochondrial NADH-linked aquacobalamin reductase was reduced significantly, compared with normal human fibroblasts but the mitochondrial NADPH-linked enzyme was not. The reduced specific activity of the NADH-linked enzyme was not due to reduction in levels of the enzyme, but in its affinity for NADH. Although there was not a significant difference in the mitochondrial NADH-linked enzyme activity between normal and cblA cells, the activity of the mitochondrial NADPH-linked enzyme was not detectable in the mutant cells. These results indicate that the defects in the mitochondrial NADH- and NADPH-linked aquacobalamin reductases underlie cblC and cblA disorders, respectively. J. Nutr. 126: 2947–2951, 1996.

INDEXING KEY WORDS:  
• vitamin B-12 • cobalamin  
• aquacobalamin reductase • human mutant fibroblasts • mitochondria

Rat and human livers contain both NADH- and NADPH-linked aquacobalamin reductases [AR]3 a cob-  

[III]alamin reductase], catalyzing the one-electron reduction of hydroxocobalamin or aquacobalamin (H2O-Cbl) to cob[II]alamin in the synthesis of cobalamin (Cbl) coenzymes. The mammalian enzymes are distributed in both mitochondria and microsomes [Watanabe et al. 1989 and 1991a]. Activities of the microsomal NADH- and NADPH-linked AR are derived from cytochrome b5/cytochrome b5 reductase complex and cytochrome P-450 reductase, respectively [Watanabe et al. 1992a and 1992b]. The mitochondrial NADH- and NADPH-linked AR are, respectively, an outer membrane cytochrome b5-like hemoprotein (OM-cytochrome b5/mitochondrial cytochrome b5 reductase complex and a flavoprotein immunoreacted with the microsomal cytochrome P-450 reductase (Saido et al. 1993 and 1994). It is, unclear however, which AR among four AR isozymes in mammalian cells participates in the synthesis of 5'-deoxyadenosylcobalamin (AdoCbl).

Seven inherited disorders in intracellular Cbl utilization, designated cblA–G, have been identified (Shevell and Rosenblatt 1992). The defects in cblC and cblA are presumed to be defects in cytosolic cob[III]alamin reductase(s) and mitochondrial cob[II]alamin reductase,
respectively, which are involved in the synthesis of coenzyme Cbl [Fenton and Rosenberg 1989]. There has been little on characterization of AR deficiency in these Cbl mutant cells. To clarify the physiological roles of these AR isozymes in the synthesis of AdoCbl, human mutant fibroblasts, cblC and cblA, were used. We characterized the four AR isozymes in the human mutant fibroblasts and also discuss mechanisms for the synthesis of AdoCbl in human cells.

MATERIALS AND METHODS

Cell culture. Human normal and mutant skin fibroblasts were grown in Dulbecco's modified Eagle medium (Nissui Pharmaceutical, Tokyo, Japan) containing 100 g/L fetal bovine serum (GIBCO BRL, Gaithersburg, MD), 62 mg/L gentamycin sulfate [Nacalai Tesque, Kyoto, Japan], 0.1 g/L fungizone (Sigma Chemical, St. Louis, MO), and 0.88 g/L NaHCO₃ [Nacalai Tesque] at 37°C in an air-CO₂ humidified atmosphere. The cells were harvested at the late logarithmic growth phase. The cells cultured were washed twice with PBS [8.0 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄] and then treated with PBS containing 2.5 g/L trypsin [Sigma Chemical] and 0.2 g/L EDTA. The trypsin-digested cells were diluted with the medium and collected by centrifuging at 300 × g for 5 min.

Human normal skin fibroblasts (OT-40 and WI-38) were kindly provided by Dr. S. Mattuyama of Research Institute, Osaka Medical Center for Maternal and Children Health. Human mutant skin fibroblasts (cblC, WG1998 and WG2161, and cblA, WG2230) were obtained from the laboratory of Dr. D. S. Rosenblatt, McGill University.

Subcellular fractionation. The cells collected were washed twice with PBS and homogenized in about 4 volumes of 10 mmol/L Tris-acetate buffer, pH 7.4, containing 0.25 mol/L sucrose using a Potter-Elvehjem-type glass homogenizer with a Teflon pestle (20–30 strokes at 1000 rpm). The homogenate was centrifuged at 500 × g for 10 min to remove unbroken cells. The supernatant fraction was centrifuged at 10,000 × g for 20 min. The supernatant was further centrifuged at 100,000 × g for 60 min. The precipitates from the 10,000 × g and 100,000 × g centrifugations were suspended in 10 mmol/L Tris-acetate buffer, pH 7.5, containing 1 mmol/L EDTA and 1 μmol/L dithiothreitol (Nacalai Tesque), and used as mitochondrial and microsomal fractions, respectively. All procedures were done at 4°C.

Specific activities of NADH- and NADPH-linked AR in the subcellular fraction of the human normal and mutant fibroblasts were corrected by cross-contamination of the activities of glucose-6-phosphatase, a marker enzyme of microsomes, and succinate dehydrogenase, a marker enzyme of mitochondria.

Assays. The activities of NADH- and NADPH-linked AR were spectrophotometrically assayed at 40°C as described previously [Watanabe et al. 1989]. The reaction mixture (1.0 mL) contained 50 mmol/L Tris-acetate buffer (pH 7.1, 8.2, 6.6 and 7.5 were used for mitochondrial NADH- and NADPH-linked, and microsomal NADH- and NADPH-linked AR, respectively), 0.1 mmol/L hydroxocobalamin [Sigma Chemical], 0.2 mmol/L NAD[P]H (Wako Chemical Industry, Osaka, Japan) and enzyme. The cob(II)alamin formed was assayed by measuring the decrease in absorbance of H₂O-Cbl at 525 nm [E₅₂₅ = 5.57 × 10³ (mol/L)⁻¹·cm⁻¹]. The activities of NADH-cytochrome c and potassium ferricyanide reductases were assayed by the method of Strobel and Digman [1978]. Succinate dehydrogenase [EC 1.3.99.1] [Ackrell et al. 1978], a mitochondrial marker enzyme, and glucose-6-phosphatase [EC 3.1.3.9] [De Duve et al. 1955], a microsomal marker enzyme, were assayed by the methods described in the cited references. Protein was assayed by the method of Bradford [1976] with bovine serum albumin as a standard.

Western blotting. To determine protein levels of the mitochondrial NADH-AR (OM-cytochrome b₅/cytochrome b₅ reductase complex) in the cblC (WG1998 and WG2161) and cblA (WG2230) cells, Western blotting was done with antibodies against rat liver OM-cytochrome b₅ and human erythrocyte cytochrome b₅ reductase. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done in 125 g/L polyacrylamide slab gel as described by Laemmli [1970]. Electrophoresis was done at a constant current [10 mA/gel], with Bromophenol blue as a migration marker. The electrophoretic transfer with a semi-dry transfer cell (Trans-Brot SD, Bio-Rad Laboratories, Richmond, CA) was performed as instructed by the manufacturer. A polyvinylidene difluoride membrane filter (Immobilon™, Millipore, Bedford, MA) was used as a transfer sheet. Cytochrome b₅ reductase and OM-cytochrome b were detected immunologically as described by Towbin et al. [1979]. Human erythrocyte NADH-cytochrome b₅ reductase and its antibody were prepared as described previously [Yubisui et al. 1987]. Rat liver mitochondrial OM-cytochrome b and its antibody were prepared as described previously [Ito 1980].

Statistics. Statistical significance was determined using Student's t test; P < 0.05 was considered significant. All values are presented as means ± SD.

RESULTS

Specific activities of NADH- and NADPH-linked AR in human normal and mutant fibroblasts. Activities of both NADH- and NADPH-linked AR were
activity of the mitochondrial NADH-AR in the cblC
(WG1998 and WG2161) cells, $K_m$ values of the enzyme
were studied. The $K_m$ of the mutant enzyme for $H_2$O-Cbl was 39–72% higher than that of the normal en-
zyme [Table 2]. In addition, when cytochrome $c$ instead
of $H_2$O-Cbl was the electron acceptor, the $K_m$ was 56–
70% higher than that of the normal enzyme. The $K_m$
of the mutant enzyme for NADH was 130–206% higher
than that of the normal enzyme in the presence
of 0.1 mmol/L $H_2$O-Cbl.

The $K_m$ of the mutant mitochondrial cytochrome $b_5$
reductase for potassium ferricyanide and NADH were,
50–153% and 157–209%, respectively higher than those
of the normal enzyme.

**DISCUSSION**

The defect in the cblC cells is presumed to be a
defect in cytosolic reductase(s) involved in the one-electron
reduction of cob(III)alamin to cob(II)alamin in the
Ado- and methylcobalamin (Me-Cbl) synthesis (Shevell and Rosenblatt 1992). However, the AR catal-
izing the reduction of $H_2$O-Cbl to cob(II)alamin has

---

**TABLE 1**

| Specific activities of NADH- and NADPH-linked aquacobalamin reductases in human normal and mutant fibroblasts
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aquacobalamin reductase activity</strong></td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
</tr>
<tr>
<td><strong>Cells</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>OT-40</td>
</tr>
<tr>
<td>WI-38</td>
</tr>
<tr>
<td>WG1998</td>
</tr>
<tr>
<td>WG2161</td>
</tr>
</tbody>
</table>

---

All values represent means ± SD from six experiments. Signifi-
cantly different from the control cells (*OT-40 and **WI-38), $P <$
0.05. ND, not detectable.

found in both mitochondrial and microsomal fractions
of human normal fibroblasts (control) [Table 1]. The
activity ratio of NADH-AR/NADPH-AR was approxi-
mately 8 and 6 in the mitochondria and microsomes
of the control cells, respectively. In cblC and cblA cells,
the microsomal NADH- and NADPH-linked AR had
specific activities that were not different than the con-
trol enzymes, indicating that both microsomal NADH-
and NADPH-linked AR are not affected in the mutant
cells.

In the cblC (WG1998 and WG2161) cells, activity of
the mitochondrial NADH-linked AR was reduced signifi-
cantly (55–57% and 60–63% of controls, respectively),
but the mitochondrial NADPH-linked AR was not.
In the cblA (WG2230) cells, activity of the mitochondrial NADH-linked AR was reduced slightly
but not significantly (77–81% of controls), and the
mitochondrial NADPH-linked AR activity was not
detectable.

**Western blotting analysis of the mitochondrial NADH-AR (OM-cytochrome b/cytochrome b$_5$ reductase complex) in human normal and mutant fibroblasts.** Figure 1 shows immunoblotting patterns of proteins of the mitochondria fractionated from the
normal and mutant cells. Mitochondria of both normal
and mutant cells contained immunoreactive bands
with $M_i$ of 16,000 [Fig. 1A] and 33,000 [Fig. 1B],
which were identical to those of native OM-cytochrome b [Ito
1980] and cytochrome b$_5$ reductase [Mihara and Sato
1978], respectively. These results indicate that the pro-
tein levels of the mitochondrial NADH-AR (OM-cyto-
chrome b/cytochrome b$_5$ reductase complex) were not
reduced in the cblC or cblA cells.

**$K_m$ of the mitochondrial NADH-AR (OM-cyto-
chrome b/cytochrome b$_5$ reductase complex) in human
normal and mutant fibroblasts.** To clarify the reduced
been located in the outer mitochondrial and microsomal membranes of rat and human livers [Watanabe et al. 1989 and 1991a], but not in the cytosol. In this study, no activities of either NADH- or NADPH-linked AR were found in the cytosolic fraction of the human normal and mutant fibroblasts. The subcellular distribution of the mitochondrial AR suggests that the enzymes can supply cob(II)alamin to both mitochondrial AdoCbl and cytosolic MeCbl synthetic systems. In the cblC [WG1998 and WG2161] cells, activity of the mitochondrial NADH-linked AR was reduced significantly (55–57% and 60–63%, respectively, of controls), but is not abolished completely. If the reduction of H2O-Cbl to cob(II)alamin is a rate-limiting step in AdoCbl synthesis, the reduction in the mitochondrial NADH-linked AR activity could lead to the severe metabolic disorder. These results suggest that the defect in the mitochondrial NADH-linked AR underlies the cblC disorder, and that the enzyme is essential for synthesis of Ado- and Me-Cbl.

Although a physiological role of rat liver OM-cytochrome b/mitochondrial cytochrome b5 reductase complex is unclear, we have demonstrated that the OM-cytochrome b complex has the activity of NADH-linked AR [Saido et al. 1994]. To determine protein levels of the mitochondrial NADH-AR (OM-cytochrome b/cytochrome b5 reductase complex) in the cblC cells, Western blotting was conducted using antibodies to rat liver OM-cytochrome b and human erythrocyte cytochrome b5 reductase. The protein levels of the components of the mitochondrial NADH-AR were not reduced in the cblC and cblA cells [Fig. 1 A and B], indicating that the reduced activity of the mitochondrial AR of the mutant fibroblasts is not due to reduction in the levels of enzyme protein.

To clarify the reduced activity of the mitochondrial NADH-AR in the cblC [WG1998 and WG2161] cells, we studied the NADH-linked AR in human normal and mutant fibroblasts1,2.

### Table 2

<table>
<thead>
<tr>
<th>OM-cytochrome b/cytochrome b5 reductase complex</th>
<th>Cytochrome b5 reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Electron Donor</td>
</tr>
<tr>
<td></td>
<td>Acceptor</td>
</tr>
<tr>
<td></td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>OT-40</td>
<td>Aquacobalamin</td>
</tr>
<tr>
<td>cblC</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>WG1998</td>
<td>98.6 ± 9.2*</td>
</tr>
<tr>
<td>WG2161</td>
<td>79.5 ± 8.5*</td>
</tr>
</tbody>
</table>

1 The mitochondria fractionated from human normal and mutant fibroblasts were used in the experiments. The KM values were calculated from double-reciprocal plots of the activity of OM-cytochrome b/cytochrome b5 reductase complex or cytochrome b5 reductase and the concentration of the substrate indicated. KM value of OM-cytochrome b/cytochrome b5 reductase complex (as the activity of NADH-linked AR) for NADH was determined in the presence of 0.1 mmol/L H2O-Cbl. All values represent means ± SD from four experiments.

2 Abbreviations used: OM cytochrome b, outer membrane cytochrome b5-like hemoprotein; AR, aquacobalamin reductase.

* Significantly different from the control cells, P < 0.05.
In the human mutant fibroblasts used in this study, activities of the microsomal NADH- and NADPH-linked AR (cytochrome b5/cytosochrome b5 reductase complex and cytochrome P-450 reductase, respectively) were not reduced significantly, indicating that both microsomal AR are normal in the mutant cells. Dietary Cbl deficiency, however, significantly increases specific activity of rat liver microsomal NADPH-linked AR [Watanabe et al. 1991b]. The cytochrome b5/cytosohrome b5 reductase complex and cytochrome P-450 reductase function in the microsomal electron-transfer chain which has several functions in mammalian cells [Gennis 1989]. The reduction of H2O-Cbl to cob(II)alamin by the enzymes may be one of the functions. Although there is a possibility that the microsomal NADH- and NADPH-linked AR may function in the synthesis of MeCbl or in other aspects of Cbl metabolism, we have no evidence of the physiological role of the enzymes.

LITERATURE CITED


