Analysis of Mutant NADH-Cytochrome b5 Reductase: Apparent "Type III" Methemoglobinemia Can Be Explained as Type I With an Unstable Reductase

By Takushi Nagai, Komei Shirabe, Toshitsugu Yubisui, and Masazumi Takeshita

A patient in Kurobe, Japan, was previously reported to have a new class of hereditary methemoglobinemia, type III. In this patient, NADH cytochrome b5 reductase deficiency was observed in lymphocytes and platelets as well as in erythrocytes, but this was not associated with mental retardation. A base change was identified in the gene for NADH cytochrome b5 reductase, causing an amino acid substitution from Leu-148 to Pro. In the present study, the mutant enzyme (Leu-148 → Pro) was expressed in Escherichia coli, purified, and characterized. The mutant enzyme retained about 60% of the catalytic activity of the wild type, but was remarkably heat unstable. By incubating the mutant enzyme at 42°C for 10 minutes, 80% of the enzyme activity was lost, whereas the wild-type enzyme lost <20% activity after incubation at 50°C for 30 minutes. Another mutant in which Leu-148 was replaced by Ala was prepared to establish the role of the residue. This mutant was apparently less heat stable than the wild type, implying a structural role for Leu-148. Reinvestigation of the enzyme activity in the blood cells and fibroblasts of the type III Kurobe patient, revealed that about 40% of the normal activity was detected in these cells, in contrast to the previous report. Thus, this patient reported previously as having hereditary methemoglobinemia type III was shown to have type I.

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genetic defect of the single gene can cause various types of the disease, and to clarify the role of Leu-148 in the enzyme. We have also examined the enzyme activity in the blood cells and fibroblasts from the patient with apparent type III to clarify the difference among type I, type II, and type III. We conclude that the patient reported previously to be type III is actually a type I.

MATERIALS AND METHODS

Materials. Restriction endonucleases and T4 polynucleotide kinase were purchased from Takara Shuzo Co (Kyoto, Japan). [35S]Deoxyctydine triphosphate (dCTP) (>1,000 Ci/mmol) and an in vitro oligonucleotide-directed mutagenesis system were products of Amersham (UK). Sequenase DNA sequencing kit was obtained from US Biochemical (Cleveland, OH). Oligonucleotides for mutagenesis were synthesized with a Model 8600 DNA synthesizer (Biosearch, Sun Rafael, CA). Diethyl aminomethyl (DEAE)–Tosyipel used for enzyme purification was the product of Toso (Tokyo, Japan) and 5'-AMP-Sepharose 4B and Sephacryl S-200 were products of Pharmacia (Uppsala, Sweden). Amphotericin B and (p-amidinophenyl)-nol fluoride hydrochloride (A-PPMSF) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Prestained molecular weight markers were obtained from Bio-Rad (Richmond, CA). All other chemicals were of reagent grade.

Patient description. Studies were performed on the patient, M.N., who was described by Tanishima et al as a case of hereditary methemoglobinemia type III. In brief, he is a 29-year-old Japanese man with a grayish cyanosis but without mental or neurologic abnormalities. The clinical presentations and hematologic data have been described previously.

Blood samples. Heparinized venous blood samples obtained from the patient and normal subjects were fractionated within 48 hours. Blood samples were at first centrifuged at 300g for 5 minutes. Supernatant fractions of the blood samples were carefully collected to prevent contamination by red blood cells, diluted two-fold with phosphate-buffered saline (PBS), and overlayed on a leukocyte separation medium. Histoplaques, obtained from the Sigma Chemical Co (St Louis, MO) consisting of 10 mL each of Histopaque-1077 (upper layer) and Histopaque-1119 (lower layer). Platelet-rich layers and lymphocyte-rich layers were obtained after centrifugation at 600g for 30 minutes (at 4°C), and 3 vol of PBS were added to each layer. After centrifugation at 500g for 10 minutes, pellets were washed with PBS and centrifuged at 500g for 10 minutes. The resulting pellets were suspended in 0.28% (wt/vol) NaCl to disrupt the small number of contaminating red blood cells and were washed again. Sedimented platelets and lymphocytes were stored at −80°C for later use.

Fibroblast preparation. Skin samples obtained from the patient’s arm and from healthy males were cut into small pieces (1 × 1 mm) by surgical knives and cultured at 37°C for about 4 weeks in Dulbecco’s Modified Eagle’s Minimum Essential Medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (PBS; Gibco, Grand Island, NY), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, and 2.5 μg/mL of Amphotericin B in a humidified atmosphere containing 5% CO2. Skin fibroblasts thus cultivated were treated with 0.25% (wt/vol) trypsin in PBS to remove them from the culture flask. Cells were harvested by centrifugation at 250g for 5 minutes. The resulting pellets were washed twice with PBS containing 1 mmol/L CaCl2 and 1 mmol/L MgCl2 and were stored at −80°C for later use.

Enzyme activity. Enzyme activity was assayed by following the increase in absorbance at 424 nm caused by the reduction of recombinant human erythrocyte cytochrome b5 in the presence of NADH as an electron donor as described previously.8 In this assay system, electrons are transferred from NADH to cytochrome b5 via the enzyme. The rate of the reaction was calculated on the basis of the difference of the absorbance of the reduced and the oxidized forms of cytochrome b5 at 424 nm (124 [mmol/L]−1 cm−1).23 The standard assay contained 100 μmol/L NADH, 2 μmol/L cytochrome b5, and an appropriate amount of enzyme in 5 mmol/L TRIS-HCl (pH 7.5). Apparent Vmax values were first obtained as intercepts of double reciprocal plot at various concentrations of cytochrome b5. Km for cytochrome b5 and Vmax values were determined by secondary plots of the apparent Vmax values as a function of the reciprocal of cytochrome b5 concentration. Km value for NADH was determined by the same process. The value of Vmax (μmol/min) of enzyme was calculated using the molecular weight of 32,000. Blood cells and fibroblasts were suspended in 20 to 30 μL of 50 mmol/L TRIS HCl (pH 8) containing 1 mmol/L EDTA, 0.1 mmol/L diethiothreitol (DTT), 0.5% Triton X-100, 10 μmol/L A-PPMSF, and 10 μmol/L pepstatin and were then placed on ice for 20 minutes. After centrifugation at 20,000g for 2 minutes, the supernatant fraction was used as the enzyme solution. NADH cytochrome b5 reductase activity was measured in a reaction mixture (2 mL) containing 5 mmol/L TRIS HCl (pH 7.5), 2 μmol/L human erythrocyte cytochrome b5, 100 μmol/L NADH, and 0.05% Triton X-100. The reaction was started by the addition of 5 to 10 μL of enzyme solution.

Site-directed mutagenesis. The HindIII–PstI fragment (503 bp), which includes the NH2-terminal portion of b5R cDNA, was excised from expression plasmid pM227-APR-b-R15 and cloned into HindIII–PstI site of M13mp19. Synthetic antisense oligonucleotides were used to alter the Leu-148 codon (CTG) to one coding for proline (CCG): 5′-CTGTTAGACGGCCGACCCCCT-3′ and alanine (GCC): 5′-CTGTTAGACGGCCGACCCCCT-3′ (the underlined bases were exchanged). Mutants generated with these oligonucleotides were named L148F and L148A, respectively. Site-directed mutagenesis was performed by the strand selection method of Taylor et al., using the Amersham mutagenesis system. M13 phage single-strand DNA was prepared from four independent plaques and mutant clones were selected by dideoxy sequencing.22 Nucleotide sequences of the HindIII–PstI fragments were verified. The mutated HindIII–PstI fragments were excised from the mutant M133 phage DNA and cloned into pUC-b-R/B to construct the mutant expression plasmid 13,15

Preparation of the recombinant mutant enzymes. Mutant forms of b5R were expressed in E. coli RB791 and purified as described previously as an α-thrombin–cleavable fusion protein.13 For the wild type and L148A, α-thrombin treatment to obtain the authentic soluble form of the enzymes was carried out as described previously.5 Because L148F showed a significant decrease in the catalytic activity by the treatment, α-thrombin digestion was not performed for the mutant. Therefore, L148F was purified as a fused protein having extra 11 amino acid residues at the NH2-terminus.13 The kinetic values of L148F can be compared with that of the wild type because kinetic properties of the wild type do not change before and after the α-thrombin treatment (data not shown). Enzyme activity of b-R during purification was monitored by assaying NADH-ferricyanide reductase activity. The activity was measured by following the absorbance change at 420 nm at 25°C using a difference in absorption coefficient of the oxidized and reduced compounds, 1 (mmol/L)−1 cm−1. The standard reaction contains 100 μmol/L NADH, 0.5 mmol/L potassium ferricyanide, an appropriate amount of enzyme, and 10 mmol/L potassium phosphate, pH 7.5. The enzyme concentration was determined on the basis of the absorption coefficient of FAD at 450 nm (1.3 [mmol/L]−1 cm−1) released from the enzyme by adding 0.1% of sodium dodecyl sulfate (SDS) or by the method of Lowry et al. with bovine serum albumin as a standard.25

Preparation of the recombinant human erythrocyte cytochrome (HE) cytochrome b5. Expression vector pKK223-3 containing the HE cytochrome b5 cDNA was a generous gift from Dr Alan W. Steggs. Because
several codons downstream to an initiation codon have been reported to be important;\textsuperscript{29} we have changed the sequence of the expression vector from 5'-ATGGCAGAGCAGTCG-3' (first ATG is an initiation codon) to 5'-ATGGCTGAAACAAGC-3' (the underlined bases were exchanged) by means of site-directed mutagenesis referring to the reported sequence for a synthesis of rat cytochrome b\textsubscript{5}.\textsuperscript{29} This modification did not change the amino acid sequence of recombinant HE cytochrome b\textsubscript{5} but increased the yield twofold to threefold (data not shown). The recombinant HE cytochrome b\textsubscript{5} was expressed in E\textit{coli} RB791 and purified by chromatography on DEAE-Toyopearl and gel filtration on a Sephacryl S-200 column as described previously.\textsuperscript{13} The purified HE cytochrome b\textsubscript{5} was dialyzed against 10 mmol/L TRIS HCl (pH 8). The concentration of cytochrome b\textsubscript{5} was determined from the absorbance difference at 424 nm using an extinction difference of 124 (mmol/L)\textsuperscript{-1} cm\textsuperscript{-1}.\textsuperscript{23} From a 1-L culture of E\textit{coli}, about 20 mg of the purified HE cytochromes b\textsubscript{5} were obtained. The amino acid sequence of NH\textsubscript{2}-terminus was verified by Edman degradation with an Applied Biosystems (Foster City, CA) model 473 A.\textsuperscript{30}

\textit{Western-blotting analysis.} Western-blotting analysis of b\textsubscript{5}Rs in crude enzyme solutions of blood cells and fibroblasts was performed as described by Hawkes et al.\textsuperscript{31} using rabbit anti-serum against HE cytochrome b\textsubscript{5} and peroxidase-conjugated anti-rabbit IgG. The b\textsubscript{5}Rs was detected on the membrane by color development using 4-chloro-I-naphthol as substrates of the peroxidase conjugated to the second antibody.

\section*{RESULTS}

\textit{Preparation of the recombinant mutant enzymes.} L148P mutant was prepared and characterized using the expression system in E\textit{coli} and site-directed mutagenesis to examine whether the phenomena occurring in the patient could be attributed to the base change identified before.\textsuperscript{22} Because we have noticed a heat instability of L148P mutant, \textalpha-thrombin treatment was not performed for the mutant. Preparation of L148A was also achieved to clarify the role of the residue in enzyme function. Yield of the mutant enzymes during purification was 3.4\% for L148P and 48.2\% for L148A. From a 1-L culture of E\textit{coli}, about 80, 6, and 59 mg of the purified b\textsubscript{5}Rs were obtained for the wild type, L148P, L148A, respectively. The wide-type and L148A mutant enzymes were purified to homogeneity as judged by SDS-polyacrylamide gel (12.5\%) electrophoresis,\textsuperscript{32} whereas the purity of L148P was 83\% as determined by the difference between the amount of the enzyme estimated by FAD content and that of protein determined by the method of Lowry et al.\textsuperscript{27}

\textit{Kinetic properties of the enzymes.} The kinetic properties of the mutant enzymes are summarized in Table 1. The Km value of L148P for cytochrome b\textsubscript{5} was about 1.5-fold higher than that of the wild type, whereas the value for NADH was unchanged. The k\textsubscript{cat} value of L148P was about 60\% of the value of the wild type. Values of k\textsubscript{cat}/Km (NADH) and k\textsubscript{cat}/Km (cytochrome b\textsubscript{5}) for L148P were 47\% and 38\% of the value of the wild type, respectively. Kinetic properties of L148A were almost the same as those of the wild type except that k\textsubscript{cat} value of the mutant was about 90\% of that of the wild type.

\textit{Heat stability.} When the diluted b\textsubscript{5}Rs (10 \textmu mol/L, 0.32 mg/mL) were incubated for 10 minutes at various temperatures, the distinct differences in heat stability of mutant enzymes and the wild type were observed (Fig 1A). Residual activities of L148P after a 10-minute incubation at 37°C and 42°C were about 70\% and <20\% of the initial activity, respectively. In contrast, the wild type retained >90\% of the initial activity at 50°C. The heat stability of L148A was intermediate between the wild type and L148P. Although the wild type was very stable at 42°C (almost 100\% activity after

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|c|}
\hline
Enzymes & $K_m$ (NADH) (\textmu mol/L) & $K_m$ (Cytochrome b\textsubscript{5}) (\textmu mol/L) & $k_{cat}$ (NADH) (s\textsuperscript{-1}) & $k_{cat}$ (Cytochrome b\textsubscript{5}) (s\textsuperscript{-1} \textmu mol/L) \\
\hline
Wild & 0.4 & 8.4 & 731 & 182.8 \times 10\textsuperscript{7} & 8.7 \times 10\textsuperscript{7} \\
L148P & 0.5 & 12.9 & 427 & 85.4 \times 10\textsuperscript{7} & 3.3 \times 10\textsuperscript{7} \\
L148A & 0.5 & 8.1 & 661 & 132.2 \times 10\textsuperscript{7} & 8.2 \times 10\textsuperscript{7} \\
\hline
\end{tabular}
\caption{Kinetic Properties of Mutant NADH Cytochrome b\textsubscript{5} Reductases}
\end{table}

\begin{figure}[h]
\centering
\begin{subfigure}{0.45\textwidth}
\includegraphics[width=\textwidth]{A}
\caption{Heat stability of the wild-type and mutant b\textsubscript{5}Rs. b\textsubscript{5}Rs were diluted to 10 \textmu mol/L (0.32 mg/mL) with 50 mmol/L TRIS HCl (pH 8) containing 1 mmol/L EDTA and 0.1 mmol/L DTT. Samples (150 to 300 \textmu L) were incubated for various times and 10 \textmu L of aliquots of the incubated b\textsubscript{5}Rs were then diluted 10-fold with an ice-cold buffer and assayed for activity. (\textbullet) Wild type; (\textbullet) L148P; (\textbullet) L148A. (A) Incubation for 10 minutes at various temperatures. (B) Incubation at 42°C for times as indicated.}
\end{subfigure}
\end{figure}
In addition to the classic types I and II, hereditary methemoglobinemia caused by \(b_5R\) deficiency was classified as three types\(^3\) after Tanishima et al reported that the Kurobe patients with no mental retardation had no \(b_5R\) activity in any blood cells.\(^9\) The base change causing amino acid substitution from Leu-148 to Pro was identified in Kurobe patients with type III by Katsube et al.\(^2\)

In the present study, we have purified mutant proteins in which Leu-148 is replaced by Pro or Ala by means of bacterial expression system and site-directed mutagenesis in order to test if the enzymatic properties of L148P can explain the pattern of deficiency in the patient with type III and to study the role of Leu-148 in \(b_5R\). The \(K_m\) values for both NADH and cytochrome \(b_5\) of L148P were not significantly different from those of the wild type. The \(k_{cat}\) value of L148P retains about 60% of that of the wild type (Table I). A remarkable characteristic of L148P determined in this study is its heat instability. More than 80% of activity was lost after incubation at 42°C for 10 minutes (Fig IA and B), whereas the wild type loses less than 20% activity after incubation at 50°C for 30 minutes.\(^{2,1}\)

One of the important purposes of this study is to clarify the puzzling reported observation that this mutation resulted in a specific deficiency of the enzyme in blood cells (white blood cells and platelets as well as erythrocytes).\(^{33}\) Because this pattern of enzyme deficiency will not be able to be explained by the recent identification of the mutant in the coding region,\(^{23}\) we reinvestigated carefully the enzyme activities in the lymphocytes, platelets, and fibroblasts of the Kurobe patient with type III hereditary methemoglobinemia. In con-
trast to the previous report, significant activities, about 40% of the normal, were detected in all these cells (Table 2). Furthermore, by the Western blotting analysis, the amounts of \( b_5 \)R protein detected in the blood cells and fibroblasts of the patient were comparable to those detected in a normal control (Fig 2). These results suggest that the failure in detecting the enzyme activity in blood cells in the previous report was caused by the inactivation of the enzyme during the sample processing. The fact that the mutant enzyme was equally expressed in lymphocytes, platelets, and fibroblasts is consistent with the finding of missense mutation in the coding region, not in the regulatory region, of the gene in this patient.

We have recently analyzed the base sequences of the gene in three independent probands of type I and identified two different mutant alleles in the patients that result in single amino acid substitutions, Arg-57→Gln and Val-105→Met. As discussed previously, the \( K_m/K_c \) value is a good indicator of catalytic efficiency in vivo and the value of \( K_{Leu-148} \) is 47% of the normal. This is rather higher than those of type I mutant enzymes (Table 3). In contrast, the value of type II mutant enzyme, Ser-127→Pro, is 3.4% of the normal, which is distinctly lower than those of type I enzymes and may not be sufficient for cell functions.

Because in the earlier stage of the studies on hereditary methemoglobinemia the activities in the patients’ tissues are based on NADH-diaphorase activity using artificial dyes, such as 2,6-dichloroindophenol as an electron acceptor that covers activities of various reductases in addition to \( b_5 \)R, it is likely that a partial decrease in \( b_5 \)R activity in the tissues other than erythrocytes in patients with type I had been overlooked. The recent measurements of the enzyme activity using cytochrome \( b_5 \) performed by ourselves, as summarized in Table 3 and by other groups, showed that a decrease in the activity does occur in blood cells other than erythrocytes in patients with type I. These results are consistent with our finding that type I disease is caused by missense mutations that result in instability and somewhat impaired activity of the enzyme. Therefore, the patient would be associated with general symptoms only when the enzyme activity in somatic cells is reduced to a level that is not sufficient for the various metabolic pathways in which the enzyme is involved. In contrast, in patients with type I, the functional enzyme deficiency occurs only in erythrocytes where there can be no compensation by new protein synthesis during its long life span because these patients have unstable variant enzymes with relatively high catalytic efficiency. As shown in Table 3, the enzyme activity of various cells of the Kurobe patient is in the range of type I rather than type II.

Consequently, the patient analyzed in the present study should be classified into type I, considering his symptoms, the similar kinetic properties of Leu-148→Pro mutant enzyme to those of the type I mutants analyzed in the former study, and activities in blood cells and fibroblasts. Though Arnold et al reported in abstract form a German family of type III, the data have not been published and the details are unknown. Because, at present, a detailed report on type III hereditary methemoglobinemia is only available for the family in Kurobe, Japan, it seems now unnecessary to have a “type III” classification in this disease.

The majority of the patients with hereditary methemoglobinemia have only asymptomatic cyanosis, whereas about 10% of these patients have associated severe general symptoms, including mental and growth retardation in addition to cyanosis. For most of these patients, it may be possible to categorize them into either erythrocyte-restricted type (type I) or generalized type (type II) on the basis of mental development. When methemoglobinemic cyanosis is noted in the neonatal stage, it would be feasible to predict if the patient will undergo normal mental development by determining whether or not the enzyme activity is severely impaired (to around 10% of normal) in the tissues other than erythrocytes. The activity of lymphocytes and platelets can be used to determine the prognosis of the methemoglobinemic newborns because the low activity of the enzyme in those cells would be linked to general symptoms including neurological disorders as shown in this study. Thus, also from clinical aspects, hereditary methemoglobinemia caused by \( b_5 \)R deficiency

### Table 3. Summary of NADH Cytochrome \( b_5 \) Reductase Activities of Type I, Type II, and 'Type III' Patients and Respective Mutant Enzymes

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Mutations</th>
<th>Erythrocytes</th>
<th>Lymphocytes</th>
<th>Platelets</th>
<th>( K_m/K_c ) (NADH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Type I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kagoshima, Japan†</td>
<td>Arg-57→Gln</td>
<td>7.7</td>
<td>40.1</td>
<td>30.3</td>
<td>24.6</td>
</tr>
<tr>
<td>Italy</td>
<td>Val-105→Met</td>
<td>7.5</td>
<td>NT</td>
<td>NT</td>
<td>14.5</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hiroshima, Japan</td>
<td>Ser-127→Pro</td>
<td>2.1</td>
<td>10.6</td>
<td>13.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Type III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kurobe, Japan</td>
<td>Leu-148→Pro</td>
<td>1.1†</td>
<td>36.1§</td>
<td>36.0§</td>
<td>46.7§</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.

* \( K_m/K_c \) values were determined for the recombinant enzymes.
† Data are taken from the report of Shirabe et al.
‡ Data are from the reports of Kobayashi et al and Yubisui et al.
§ Data calculated from those in Table 1 and 2 in the present study.

† \( b_5 \)R activity in erythrocyte hemolysate was determined in this study as described before.
seems enough to be classified as only two types: an erythrocyte type (type I) and a generalized type (type II).

Sequence similarities among \( b_5R \), ferredoxin-NADP+ reductase (FNR), and NADPH-cytochrome P-450 reductase (CPR) were first pointed out by Porter and Kasper.\(^\text{38}\) Later, a significant similarity (45%) was noted between \( b_5R \) of vertebrate and NADH-nitrate reductase of higher plants.\(^\text{39}\) The concept that these dinucleotide-dependent flavoenzymes belong to the same family has been confirmed by successive reports on the primary structures of the related enzymes\(^\text{40,41}\) and a determination of the three-dimensional structure of FNR at 2.6 Å resolution by Karplus et al.\(^\text{42}\) The functionally important residues as determined by x-ray structure were conserved among the homologous enzymes.\(^\text{43}\) \( b_5R \) may consist of two functional domains: an NH\(_2\)-terminal FAD-binding domain and a COOH-terminal NADH-binding domain, as suggested by the structure of FNR. Sequence alignment of \( b_5R \) with other enzymes of the family suggests that Leu-148 is located at a linker region connecting these two domains.\(^\text{41}\)

We have constructed and purified L148A as well as L148P in order to determine the role of the leucine-148 in the enzyme function. Catalytic properties of the L148A mutant enzyme were almost the same as those of the wild type as observed for L148P mutant. Thus, Leu-148 was shown not to be involved in the catalytic function of the enzyme. These results are consistent with the expectation that this residue resides in the linker region. L148A is much more stable than L148P but is more heat labile than the wild type, suggesting that Leu-148 might play a role(s) in maintaining the native structure of the enzyme. Replacement at position 148 with a proline, which provides a fixed dihedral angle to the main chain, may cause a structural disturbance leading to a more unstable mutant enzyme. Because Leu-148 of \( b_5R \) is replaced only by hydrophobic amino acids such as an isoleucine, a valine, or a phenylalanine in nitrate reductases,\(^\text{36,44}\) hydrophobicity at position 148 may be critical for the maintenance of the structure of \( b_5R \) and nitrate reductases. The x-ray crystallographic analysis, now progressing,\(^\text{45}\) will lead us to further understanding of the role of Leu-148.

ACKNOWLEDGMENT

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