Characterization of a novel nicotinamide adenine dinucleotide-cytochrome b5 reductase mutation associated with canine hereditary methemoglobinemia

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ABSTRACT. Hereditary methemoglobinemia associated with nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) deficiency is a rare autosomal recessive disorder in animals. Recently, nonsynonymous b5R gene (*CYB5R3*) variants have been reported to be associated with canine and feline hereditary methemoglobinemia. However, the underlying molecular mechanisms of canine and feline methemoglobinemia caused by these nonsynonymous variants have not yet been reported. Previously, we reported a Pomeranian dog family with hereditary methemoglobinemia, carrying *CYB5R3* mutation of an A>C transition at codon 194 in exon 7, replacing an isoleucine residue with leucine (p.Ile194Leu). In this study, we investigated the enzymatic and structural properties of the soluble form of wild-type and Ile194Leu canine b5Rs to characterize the effects of this missense mutation. Our results showed that the kinetic properties of the mutant enzyme were not affected by this amino acid substitution. The secondary structure of the wild-type and Ile194Leu b5Rs detected by circular dichroism showed a similar pattern. However, the mutant enzyme exhibited decreased heat stability and increased susceptibility to trypsin hydrolysis. Moreover, the thermostability and unfolding measurements indicated that the mutant enzyme was more sensitive to temperature-dependent denaturation than the wild-type b5R. We concluded from these results that unstable mutant enzyme properties with normal enzymatic activity would be associated with hereditary methemoglobinemia in the Pomeranian dog family.

KEY WORDS: dog, hereditary methemoglobinemia, missense mutation, nicotinamide adenine dinucleotide-cytochrome b5 reductase

Hereditary methemoglobinemia characterized by the deficiency of nicotinamide adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R) (EC.1.6.2.2) is an autosomal recessive disorder [9, 15]. This enzyme transfers electrons from NADH to cytochrome b5 using flavin adenine dinucleotide (FAD) during the reduction of methemoglobin (metHb) [26]. There are two forms of b5R: a membrane-bound form, expressed in the mitochondria and endoplasmic reticulum of the somatic cells, and a membrane anchor domain-lacking soluble form, expressed only in erythrocytes [9, 15]. Both forms are encoded by the cytochrome b5 reductase gene (*CYB5R3*) [15].

In humans, more than 40 *CYB5R3* variants have been reported to cause hereditary methemoglobinemia [15] with two distinct manifestations of the clinical condition: type I and type II [9, 15]. Type I, limited to erythrocyte defects, is related to the soluble b5R form, is relatively benign, and primarily manifests as cyanosis [9, 15]. Type II disease, caused by the defects in the membrane-bound b5R form, is much more severe, resulting in cyanosis, neurological dysfunction, and premature death [9, 15].

In contrast, b5R deficiency-associated hereditary methemoglobinemia has rarely been reported in animals [3, 6, 7, 19]. In the past few years, innovations in massively parallel DNA sequencing have made this approach a cost-effective tool for diagnosing heritable diseases in veterinary medicine, contributing to the identification of nonsynonymous *CYB5R3* variants both in dogs [11] and cats [12, 19]. To date, no information has been available regarding the underlying molecular mechanisms of hereditary methemoglobinemia in veterinary medicine. Recently, we observed an instance of familial hereditary methemoglobinemia in

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Pomeranian dogs in Japan and identified the causative missense variant (c.580A>C) in canine *CYB5R3* [17]. The same mutation allele was found in a Pomeranian dog in Australia [27]. This variant resulted in the replacement of an isoleucine (Ile) residue with leucine (Leu) at amino acid position 194 (p.Ile194Leu) [17]. Dogs homozygous for this missense variant exhibited significantly higher metHb concentrations and lower b5R activities than their healthy counterparts [17]. These findings imply that the c.580A>C variant in canine *CYB5R3* could affect b5R function in erythrocytes, but the precise effector mechanism of this process remains unclear.

As described previously, in the predicted three-dimensional (3-D) model of soluble canine b5R, Ile194 corresponded to a residue located in the first α-helix of the NADH-binding domain at the posterior region of the NADH-binding motif [17]. Although it was suspected that Ile194 binds with a valine residue at the position 190 (Val190) via hydrogen bonding to maintain the spiral conformation of the α-helix, Ile194Leu replacement did not affect the hydrogen bonding with Val190 [17]. Furthermore, some *in silico* algorithms predicted that canine Ile194Leu b5R would likely be tolerated without functionally damaging the enzyme [17].

It is well-known that isoleucine and leucine are structural isomers and differ only in the position of a branching methyl group in the side chain [20]. During the comparison of homologous proteins, such changes between members of a group of amino acids with similar characteristics are often overlooked [24]. Thus, further analysis of Ile194Leu b5R would be required to fully elucidate the underlying molecular mechanisms of the observed clinical manifestations. In this study, we aimed to characterize the mutant enzyme by constructing wild-type and mutant Ile194Leu canine b5R recombinant proteins in their soluble form and analyzing their biochemical and structural properties.

MATERIALS AND METHODS

RNA isolation and reverse transcription polymerase chain reaction

A bone marrow sample from a Jack Russell Terrier without methemoglobinemia, visiting the Veterinary Teaching Hospital at Iwate University for bone marrow examination, was collected with the informed consent of the dog owner. The sample extraction was performed under general anesthesia achieved by the administration of 2% inhaled isoflurane. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA template using the ReverTra-plus-TM cDNA synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

Polymerase chain reaction (PCR) amplification of the canine b5R wild-type and Ile194Leu soluble domains

To generate the wild-type canine b5R soluble domain, the open reading frame corresponding to a methionine residue at the position 24 from a phenylalanine (Phe) at the position 301 was isolated by PCR amplification of the first-strand cDNA obtained as described above. The primers, listed in Table 1, were designed based on the published sequence of the gene encoding *Canis familiaris cyb5r3* (Gene ID: 474479). The primers F1 and R1 included *Bam*HI and *Eco*RI restriction sites, respectively (Table 1). PCR amplification of the wild-type b5R was performed using primers F1 and R1 following the manufacturer's protocol (Platinum *Taq* DNA polymerase, Thermo Fisher Scientific, Waltham, MA, USA). The reaction conditions were as follows: initial denaturation for 15 sec at 94°C, followed by 35 cycles of 15 sec at 94°C, 30 sec at 55°C, and 1 min at 68°C. The PCR product was verified by visualization in 1.0% agarose gel and purified using a commercial kit (Isospin PCR product, Nippon Gene, Toyoma, Japan). The purified wild-type PCR fragment (850 bp) was cloned into a pMD20-T vector (Takara Bio, Kusatsu, Japan).

To generate the mutant enzyme with the codon 194 missense mutation, Ile194Leu b5R, two amplified fragments were generated using the primer pairs F1 and R2 or F2 and R1, using the first-strand cDNA obtained as described above. The primers F2 and R2 included the missense mutation to generate c.580A>C in *CYB5R3* (Table 1). The reaction conditions were as described above. PCR products were verified by visualization on a 1.0% agarose gel and purified. To combine and extend the 5′- and 3′-ends of amplified fragments, these were mixed at an equal volume and used as a template for a second PCR using primers F1 and R1. After electrophoresis, the second PCR product (850 bp) was purified and cloned into the pMD20-T vector as described above.

The *Escherichia coli* DH5α strain was transformed with each recombinant vector, pMD20-T-wild-type, and -Ile194Leu b5R. Selected clones were sequenced using M13 RV or M4 primers and a 3500 Genetic Analyzer device following the Big Dye terminator protocol (Applied Biosystems, Foster City, CA, USA).

Glutathione S-transferase (GST)-tagged b5R expression, purification, and characterization

To construct the GST-tagged b5R expression vector, pMD20-T-wild-type and -Ile194Leu b5R were digested with *Bam*HI and *Eco*RI as described above. The resulting fragments were ligated separately into the GST fusion gene vector, pGEX-6P-1 (GE Healthcare, Chicago, IL, USA) using the Mighty Mix Ligation Kit (Takara). *E. coli* BL21 bacteria harboring the expression plasmid were incubated overnight at 37°C in 50 ml of Luria-Bertani (LB) medium containing 100 µg/ml ampicillin. The culture was inoculated into 500 ml of LB and incubated at 37°C. After an initial incubation for 2.5 hr, isopropyl-β-D-thiogalactopyranoside was added to the medium (at a final concentration of 0.1 mM) and incubated for another 4 hr. The bacterial cells were pelleted by centrifugation (12,000

Table 1. Oligonucleotide primers used to construct DNA fragments of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase soluble forms

Primer	Sequence
F1	5′-*GGATCC*ATGAAGCTGTTCCAG-3′
R1	5′-<u>GAATTC</u>TCAGAAGGCGAAGCAG-3′
F2	5′-GATCCGTGCC**C**TCATCAAAG-3′
R2	5′-CTTTGATGA**G**GGCACGGATC-3′

The F1 and R1 primers included *Bam*HI (italic) and *Eco*RI (underlined) restriction sites, respectively. The F2 and R2 primers included the mutation site in bold.

× g for 5 min), resuspended, and lysed in 50 ml CelLytic B Reagent with lysozyme (0.2 mg/ml), benzonase (50 U/ml), and protease inhibitor (all reagents purchased from Sigma-Aldrich Co., St. Louis, MO, USA), and spun at full speed in a microcentrifuge for 10 min to pellet insoluble materials. The supernatant was diluted with the same volume of phosphate-buffered saline (PBS, pH=7.4), transferred onto a Glutathione Sepharose 4 FF Column (GE Healthcare), and mixed gently at room temperature (20–25°C) for 10 min to ensure the optimal binding of the GST-tagged proteins to the glutathione Sepharose 4FF matrix. After washing the column twice with PBS, the recombinant proteins were cleaved using PreScission protease (GE Healthcare). Elution was performed with the elution buffer according to the manufacturer's protocol. The recombinant proteins were then dialyzed to PBS overnight at 4°C and stored at −80°C until further use. The purity of the recombinant b5Rs was evaluated by electrophoresis on a polyacrylamide gel (10%) in the presence of sodium dodecyl sulfate (SDS). The enzyme concentrations were determined using the Bradford method.

Recombinant b5R protein enzymatic property analysis

To assess steady-state kinetic parameters, the NADH-ferricyanide reductase assay was performed at 340 nm and 25°C using a GENESYS VIS spectrophotometer (Thermo Scientific) as described previously [13] with slight modifications. Briefly, the NADH-ferricyanide activity was measured in 100 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid buffer (pH=8.0) with 0.2 mM NADH and 0.5 mM potassium ferricyanide. The reaction was initiated by the addition of NADH or enzymes. To calculate the kinetic parameters, change in absorbance was assessed as per the variations in NADH and potassium ferricyanide concentrations, and the activity was calculated using 6.25 and 1.02 cm^{-1} mM^{-1} as the molar extinction coefficients, respectively. The assays were performed in triplicate. The Km and $Vmax$ values of each enzyme were calculated using GraphPad Prism software (http://www.graphpad.com/scientific-software/prism/). The $Kcat$ values were calculated by dividing the $Vmax$ by the enzyme concentration, which was determined based on a molecular mass of 32,000 Da. The significance of the differences was evaluated using Student's t-test.

Heat inactivation and protease stability analysis

To evaluate protein stability, the residual activity was measured after incubating the wild-type and Ile194Leu b5Rs at various temperatures (0–60°C) for 10 min, and at 42°C and 50°C for different periods (0–60 min). The residual activity was expressed as a percentage of the activity of the same enzyme before treatment. T_{50} was defined as the temperature at which 50% of the initial activity before heat treatment was retained and was calculated from data at various temperatures (0–60°C) for 10 min. In addition, the activities of wild-type and Ile194Leu b5Rs were measured after incubation at 37°C for 3, 24, and 48 hr. To evaluate the folding status of proteins, trypsin susceptibility was examined by incubating for various periods (0–60 min) at 37°C with 1 µg of trypsin (Sigma-Aldrich Co.) per 50 µg of wild-type or mutant enzyme, following the manufacturer's instructions. The activity of the treated enzyme was evaluated using the NADH-ferricyanide reductase activity as described above. The significance of the differences was evaluated using Student's t-test.

Circular dichroism (CD) spectrophotometric analysis

The secondary structure of the recombinant proteins was spectroscopically evaluated by CD using a spectropolarimeter (Jasco J-820, JASCO Ltd., Tokyo, Japan). Far-UV (200–250 nm) and near-UV (250–300 nm) CD spectra of wild-type and Ile194Leu b5Rs were obtained at 1-nm intervals using protein concentrations of 0.11 and 0.13 mg/ml, respectively, in PBS. The CD spectra were measured at 25°C. The magnitude of the CD spectra was expressed as molar ellipticity [θ], deg·cm^2/dmol, based on the molecular weight of b5R (32,000 Da).

The thermostability and unfolding of the wild-type and Ile194Leu b5Rs were determined according to the changes in the CD signal at 222 nm using temperature scans ranging from 10 to 80°C, increasing at the rate of 1°C/min. The wild-type and Ile194Leu b5Rs were suspended in PBS at protein concentrations of 0.27 and 0.23 mg/ml, respectively. The fraction at which 50% of the total protein was folded and unfolded was determined as the Tm (melting temperature midpoint of the transition) value, and was calculated for both wild-type and mutant b5Rs using the JWTDA-488 software (JASCO Ltd.).

RESULTS

Fusion-expressed b5R protein preparation and identification

To characterize the effect of the Ile194Leu missense mutation on the biochemical properties of the soluble b5R form, the wild-type and mutant b5R coding regions were separately inserted into a GST fusion gene expression vector. Recombinant wild-type and Ile194Leu b5Rs were expressed in *E. coli* BL21 bacteria and purified as described in the "Materials and Methods" section. Electrophoresis on a polyacrylamide gel in the presence of SDS demonstrated that single bands of 32,000 Da were present after purification, in good agreement with the theoretical mass of the soluble wild-type and Ile194Leu b5R forms (Fig. 1).
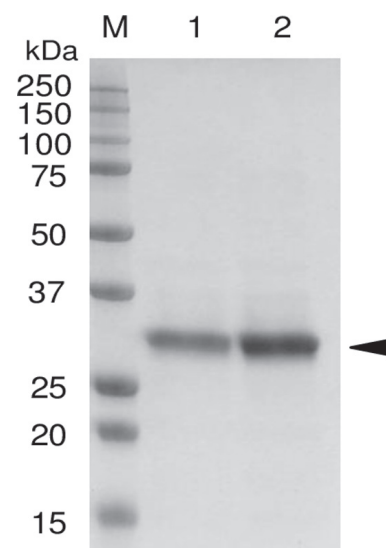

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) soluble-forms. M, size marker; Lane 1, wild-type; Lane 2, mutant b5R. Single bands at 32,000 Da were observed after purification, in good agreement with the theoretical mass of the recombinant soluble-form wild-type and mutant b5Rs.

Table 2. The kinetic properties of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase soluble forms

		Wild-type	Mutant	*P* value
Km [NADH]	μM	13.8 ± 2.4	11.2 ± 6.5	0.497
Kcat	S^{-1}	455.8 ± 143.1	466.8 ± 161.9	0.922
Kcat/Km	$S^{-1}M^{-1}$	$3.4 \pm 1.2 \times 10^7$	$4.7 \pm 1.7 \times 10^7$	0.277
Km [ferricyanide]	μM	14.5 ± 8.8	21.3 ± 2.6	0.271
Kcat	S^{-1}	279.9 ± 52.7	369.8 ± 56.7	0.114
Kcat/Km	$S^{-1}M^{-1}$	$2.3 \pm 0.8 \times 10^7$	$1.8 \pm 0.4 \times 10^7$	0.413

The measurement of the nicotinamide adenine dinucleotide (NADH)-ferricyanide activity were performed in triplicates. Data are represented as mean ± standard deviation. The significance of the differences was evaluated using the Student's *t*-test.

Steady-state kinetic properties of the expressed enzymes

Kinetic analysis was performed using the soluble-form canine b5R wild-type and Ile194Leu recombinant proteins. The enzymatic activity of the mutant b5R was almost identical to that of the wild-type enzyme when measured under standard assay conditions. The kinetic properties of the wild-type and mutant enzymes are summarized in Table 2. The *Km* and the *Kcat/Km* values of the mutant b5R for NADH were 81% and 138% of the wild-type, and the *Kcat* value of mutant b5R was almost the same as that of wild-type b5R. Meanwhile, the *Km*, *Kcat* and *Kcat/Km* values in the mutant b5R for ferricyanide were 147%, 132% and 78% of the wild-type, respectively. In all parameters, however, there was no statistically significant difference between the wild-type and the mutant proteins, suggesting that the b5R catalytic efficiency was not affected by the Ile194Leu replacement.

Structural characterization of the wild-type and mutant b5Rs

The secondary structures of wild-type and Ile194Leu b5Rs were evaluated by CD analysis in the far- and near-UV regions. As shown in Fig. 2, in the far-UV region, the Ile194Leu b5R exhibited CD spectra comparable to those of the wild-type b5R, with positive and negative CD maxima in the ranges of 195–199 nm and 219–222 nm, respectively, consistent with the α-helix-rich structure. The molar ellipticity [θ] of the wild-type and Ile194Leu b5Rs at 222 nm were −5,138.59 and −4,533.98 deg·cm^2/dmol, respectively (Fig. 2). The patterns and intensities of the CD spectra for both b5Rs were comparable, indicating that Ile194Leu replacement did not significantly alter the secondary enzyme structure.

Heat inactivation and protease susceptibility of the b5Rs

We observed distinct differences in the heat stability of the wild-type and Ile194Leu b5Rs. The residual activity of the mutant enzyme after 10 min at 37°C and 50°C was approximately 101% and 23.5% of the initial activity, respectively (Fig. 3A). However, the wild-type b5R retained 105% and 104% of the initial activity after 10 min at 37°C and 50°C, respectively (Fig. 3A). The T_{50} of the I194L b5R (46.2 ± 4.0°C) was significantly lower (*P*=0.001) than that of the wild-type (55.9 ± 0.6°C). Based on these results, we also measured the residual activity for 0, 10, 20, 30, and 60 min incubations at 42°C and 50°C. Enzymatic activity of the wild-type strain was not altered after 60 min of incubation at any temperature (Fig. 3B). However, when the Ile194Leu b5R was incubated under the same conditions, the activity decreased after a 10-min incubation at each temperature, and the residual activities after 60 min of incubation were 56.7% at 42°C and 3.1% at 50°C of the initial activity, respectively (Fig. 3B). In addition, we measured the residual activity of the b5R enzymes after incubation at 3, 24, and 48 hr at 37°C, the normal range of body temperature. As shown in Fig. 3C, the activity of the wild-type b5R retained its high activity up to 24 hr and that at 48 hr was 53.4% of the initial activity. Meanwhile, the activity of Ile194Leu b5R significantly decreased after 24 and 48 hr of incubation, and the residual activities were 4.5% and 2.4% of the initial activity, respectively (Fig. 3C).

We used trypsin to elucidate the protease sensitivity of the mutant Ile194Leu b5R, because trypsin is known to easily access and cleave loosely folded parts of proteins [10, 14]. Consequently, the mutant b5R retained only 22.2% of its initial activity after 60 min of incubation with trypsin at 37°C. However, the wild-type b5R exhibited a considerably higher trypsin resistance, retaining 59.6% of its initial activity under the same conditions (Fig. 4).
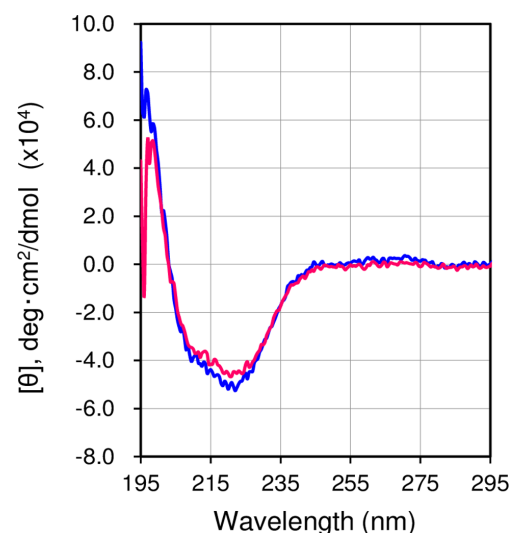

Fig. 2. Steady-state circular dichroism (CD) spectra of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) soluble-forms. The CD spectra were expressed as molar ellipticity [θ], deg·cm^2/dmol of enzyme. The protein concentrations of the canine wild-type (blue) and mutant (pink) b5Rs were 0.11 and 0.13 mg/ml, respectively.
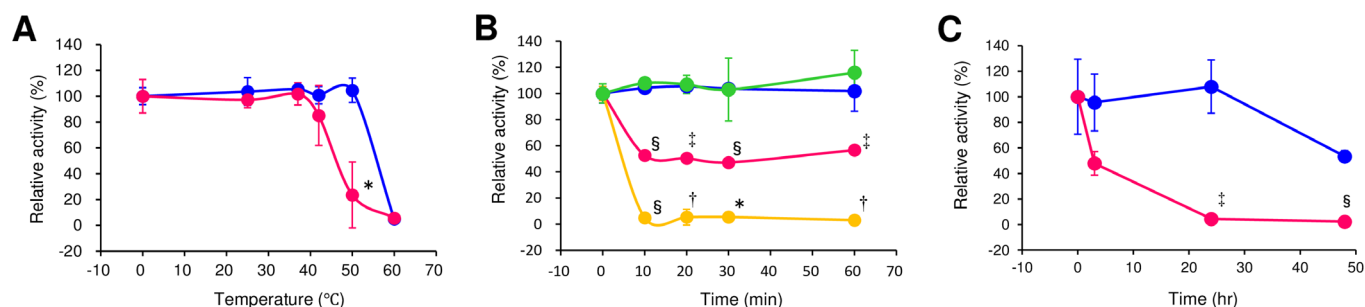
Fig. 3. Heat stability of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) soluble-forms. (A) The wild-type (blue) and mutant (pink) b5Rs were incubated for 10 min at various temperatures. (B) Recombinant b5Rs were incubated at 42°C and 50°C for various periods. The green and blue lines show the values of the wild-type b5R incubated at 42°C and 50°C, respectively. The pink and yellow lines show the values of the mutant b5R incubated at 42°C and 50°C, respectively. (C) Wild-type (blue) and mutant (pink) b5Rs were incubated for 3, 24, and 48 hr at 37°C. These assays were performed in triplicate. The residual activity was expressed as a percentage of the activity of the same enzyme before heat treatment. The points are represented as the mean ± standard deviation. The significance of the differences was evaluated using Student's *t*-test. *$P<0.05$, †$P<0.01$, ‡$P<0.005$, and §$P<0.001$, compared with the values of wild-type b5R under the same treatments.
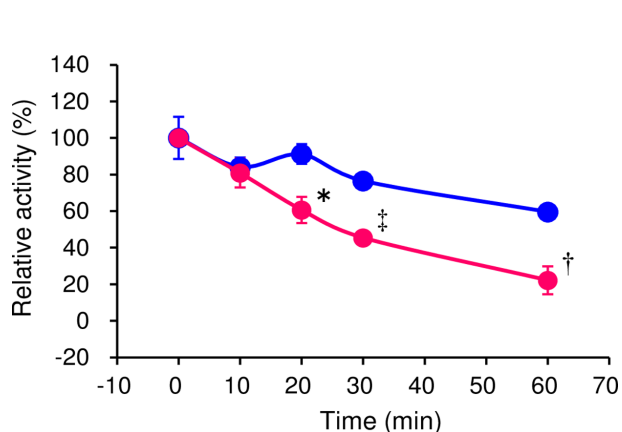

Fig. 4. Trypsin susceptibility of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) soluble-forms. Wild-type (blue) and mutant (pink) b5Rs were treated with trypsin at 37°C for various periods. The assays were performed in triplicate. The residual activity was expressed as a percentage of the activity of the same enzyme before the trypsin treatment. The points are represented as the mean ± standard deviation. The significance of the differences was evaluated using Student's *t*-test. *$P<0.05$, †$P<0.01$, and ‡$P<0.005$, compared with the values of wild-type b5R under the same treatments.
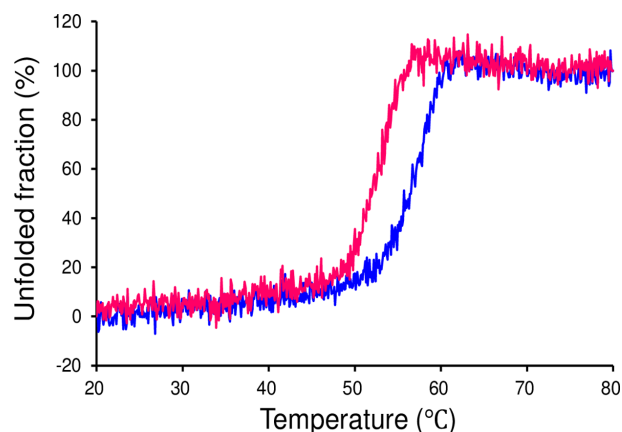

Fig. 5. Thermostability and unfolding analysis of the canine nicotinamide adenine dinucleotide-cytochrome b5 reductase (b5R) soluble-forms. The protein concentrations of canine wild-type (blue) and mutant (pink) b5Rs were 0.27 and 0.23 mg/ml, respectively.

Thermostability and unfolding of the wild-type and Ile194Leu b5Rs

To gain an insight into the effects of the Ile194Leu mutation on the global structural stability, we determined the structural changes of the Ile194Leu b5R and compared them to the structure of the wild-type enzyme using CD analysis at 222 nm under altered temperatures. An increased temperature induced protein denaturation, demonstrating a two-state process. For quantification, the temperature at which half of the recombinant proteins were unfolded was defined as *Tm*. As shown in Fig. 5, the *Tm* values for the wild-type and Ile194Leu b5Rs were 57.0°C and 52.7°C, respectively, suggesting that the mutant enzyme could be more sensitive to temperature-induced denaturation than the wild-type b5R.

DISCUSSION

In human hereditary methemoglobinemia due to *CYB5R3* variants, most mutations are missense, but several premature stop codons and incorrect exon-intron splicing mutations create truncated proteins [15]. Several *in vitro* studies have shown that most missense

mutant proteins exhibit certain dysfunctionalities, including reduced catalytic activity and increased susceptibility to heat and proteases [9, 15, 22]. However, no missense variants affecting the Ile194 residue have been reported in human *CYB5R3*.

The 3-D structures of human [4], rat [5], and porcine [25] b5R were resolved by X-ray crystallography. According to these studies, in the human b5R the FAD cofactor was able to non-covalently adhere to the large, wide boundary cleft between the two major domains [4, 25], whereas the NADH molecule was able to fit into the interdomain cleft on the *re-side* of the FAD isoalloxazine ring [25]. Based on the observation that Ile194 is located in the proximal region of the NADH-binding motif [16, 17], it was suspected that substrate binding would be affected by this amino acid replacement. However, the Ile194Leu mutation did not affect the *Km* for NADH when the b5R activity was measured in the presence of ferricyanide as an electron acceptor, implying that the Ile194 residue does not play a key role in the affinity with NADH (Table 2). In addition, although the differences in the mean values of the other kinetics parameters were observed between the wild-type and the Ile194Leu mutant enzymes, there was no statistically significant difference (Table 2). These results indicated that enzymatic function of the b5R could not affected by Ile194Leu replacement.

Concerning the reductase activities, our study showed remarkable differences in heat stability between the Ile194Leu and wild-type enzymes (Fig. 3A and 3B). The T_{50} value for Ile194Leu b5R was 9.7°C lower than that for the wild-type (Fig. 3A), and the activity of Ile194Leu b5R decreased faster than that of wild-type b5R under the same thermal conditions (Fig. 3B). In the 3 days incubation at 37°C, the activity of mutant b5R was significantly lower than that of wild-type b5R (Fig. 3C). These results indicate that the mutant b5R might be inactivated earlier in the circulating peripheral blood because of Ile194Leu replacement. In addition, the greater susceptibility to trypsin hydrolysis of the Ile194Leu b5R relative to wild-type (Fig. 4) suggested that the folding status of Ile194Leu was looser than that of the wild-type b5R. In human hereditary methemoglobinemia, it has been demonstrated that the characteristics of b5R mutants associated with Type I disorder, p.Ala178Val [9] and p.Cys203Tyr (Ala, alanine; Cys, cysteine; Tyr, tyrosine) [22], are similar to those of Ile194Leu, including heat instability and protease susceptibility but normal enzymatic activity (in Human Genome Variation Society nomenclature, they have been described as p.Ala179Val and p.Cys204Tyr, respectively, [15]). It was concluded that the vulnerabilities of these human b5R mutants might be caused by conformational changes owing to amino acid substitutions [9, 22]. However, the details of this phenomenon remain unclear. Like erythrocyte membrane aspartic proteinase, cathepsin E has been identified previously [21], and it is possible that it is involved in the early degradation of mutant proteins. However, since we did not perform this study using cathepsin E family, further investigation is needed.

In our previous study, the predicted hydrogen bond distances between Ile194 and Val190, and Leu194 and Val190 were 3.07 Å and 3.08 Å, respectively, when measured using the Pymol software (data not shown), suggesting that the replacement with leucine would not alter the structure of the first α-helix in the canine soluble b5R [17]. Consistently, the present study revealed that there was no difference between the secondary structures of the two b5Rs (Fig. 2). Meanwhile, the *Tm* value for Ile194Leu b5R was 4.3°C lower than that for the wild-type (Fig. 5). This result revealed that the mutant protein tertiary structure could be more easily destroyed, owing to Ile194Leu replacement, compared with that of the wild-type enzyme. Despite possessing normal enzymatic functions, the conformational changes of the Ile194Leu b5R mutant might result in metHb accumulation in the erythrocytes of the affected dogs.

MetHb normally forms at low rates of approximately 3.0% of erythrocyte hemoglobin *in vivo* [7, 8], and the NADH-b5R catalytic pathway is responsible for reducing up to 95% of metHb [2]. It is well-known that the nicotine adenine dinucleotide phosphate pathway [1] and antioxidants, including reduced glutathione and ascorbic acid [23], are involved in metHb reduction. *In vivo* comparative studies of b5R function in various mammals reported that b5R enzymatic activities are remarkably lower in dogs and humans than in guinea pigs and rabbits [1]. According to Srivastava *et al.* [18], the erythrocytic concentrations of the b5R enzyme are significantly lower in dogs than in rodents, and decreased concentrations of b5R confer a high degree of susceptibility to metHb accumulation in dog erythrocytes [16]. Although we could not measure the concentrations of erythrocytic b5R in the affected Pomeranian dogs in a previous study [17], dogs with unstable proteins such as the Ile194Leu b5R mutant might collapse the NADH-b5R catalytic pathway in erythrocytes.

Recently, nonsynonymous *CYB5R3* variants have been reported, including p.Gly72Ser and p.Ile190Leu in dogs [11] as well as p.Gly209Ser [12], p.Phe36Leu, and p.Tyr179His [19] in cats (Gly, glycine; Ser, serine; His, histidine). However, the underlying molecular mechanisms of methemoglobinemia caused by nonsynonymous variants in animals have not been completely elucidated yet. Our results provided clear evidence of the instability of an enzyme with otherwise normal catalytic properties resulting in hereditary methemoglobinemia in a Pomeranian dog family that carried the *CYB5R3* missense variant (ATC→CTC at codon 194, Ile194Leu). Since mature erythrocytes cannot synthesize a new protein over a long lifespan, these results indicated that the degradation of the unstable mutant enzyme with normal enzymatic activity might markedly shorten the half-life of the b5R molecules and result in excessive metHb accumulation. Further studies are necessary to clarify the above hypothesis.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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