Identification of Key Residues Determining Isomerohydrolase Activity of Human RPE65*

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RPE65 is the retinoid isomerohydrolase that converts all-trans-retinyl ester to 11-cis-retinol, a key reaction in the retinal visual cycle. We have previously reported that cone-dominant chicken RPE65 (cRPE65) shares 90% sequence identity with human RPE65 (hRPE65) but exhibits substantially higher isomerohydrolase activity than that of bovine RPE65 or hRPE65. In this study, we sought to identify key residues responsible for the higher enzymatic activity of cRPE65. Based on the amino acid sequence comparison of mammalian and other lower vertebrates’ RPE65, including cone-dominant chicken, 8 residues of hRPE65 were separately replaced by their counterparts of chicken RPE65 using site-directed mutagenesis. The enzymatic activities of cRPE65, hRPE65, and its mutants were measured by in vitro isomerohydrolase activity assay, and the retinoid products were analyzed by HPLC. Among the mutants analyzed, two single point mutants, N170K and K297G, and a double mutant, N170K/K297G, of hRPE65 exhibited significantly higher catalytic activity than WT hRPE65. Further, when an amino-terminal fragment (Met1–Arg33) of the N170K/K297G double mutant chicken RPE65 using site-directed mutagenesis. The enzymatic activities of cRPE65, hRPE65, and its mutants were measured by in vitro isomerohydrolase activity assay, and the retinoid products were analyzed by HPLC. Among the mutants analyzed, two single point mutants, N170K and K297G, and a double mutant, N170K/K297G, of hRPE65 exhibited significantly higher catalytic activity than WT hRPE65. Further, when an amino-terminal fragment (Met1–Arg33) of the N170K/K297G double mutant of hRPE65 was replaced with the corresponding hRPE65 fragment, the isomerohydrolase activity was further increased to a level similar to that of cRPE65. This finding contributes to the understanding of the structural basis for isomerohydrolase activity. This highly efficient human isomerohydrolase mutant can be used to improve the efficacy of RPE65 gene therapy for retinal degeneration caused by RPE65 mutations.

Retinal pigment epithelium (RPE)2-specific 65-kDa protein (RPE65) is essential for metabolism of vitamin A in the eye and for maintenance of normal vision. It is the enzyme that catalyzes isomerization of all-trans-retinyl ester to 11-cis-retinol (11cROL), the precursor of the chromophore of visual pigments (see Fig. 1) (1–3). Mutations of RPE65 are associated with inherited retinal dystrophies such as Leber’s congenital amarousis and retinitis pigmentosa (4–17). Previous RPE65 gene replacement therapy in RPE65 null mutants of dog and mouse models displayed promising effects on retinal degeneration (18–23). However, human RPE65 (hRPE65) has lower specific activity than other retinoid-processing enzymes (24–26), and a high abundance of RPE65 in the RPE (11 μg/eye in bovine) is thus required to generate sufficient 11-cis-retinoid for normal vision (1, 27). This demand for high RPE65 levels limits the efficacy of RPE65 gene therapy, and a mutant with increased activity levels would circumvent this limitation.

Several vectors for gene delivery, such as adenovirus (28, 29), recombinant adeno-associated virus (19, 29–33), lentivirus (29, 34–36), plasmid incorporated in nanoparticles (37), and plasmid DNA with electroporation (38, 39) have been used to deliver intact DNA (or reporter genes) to the ocular tissues. Particularly, gene delivery into the subretinal space using adenovirus and lentiviral vectors expressing GFP showed widely distributed GFP expression (29). Recent human clinical trials using recombinant adeno-associated virus expressing WT hRPE65 showed encouraging results; the initial trials showed modest improvements of vision in patients with retinitis pigmentosa and Leber’s congenital amarousis (40–43). However, no present attempts for RPE65 gene therapy have successfully generated full vision recovery despite successful gene delivery. This may be due to low enzymatic activity of hRPE65, meaning that enhancement of hRPE65 enzymatic activity should be considered as an alternative approach.

We have previously reported that RPE65 in cone-dominant chicken has a substantially higher isomerohydrolase activity compared with that in rod-dominant mammals (44). We have cloned chicken RPE65 (cRPE65) and expressed it in the same heterologous system as that for hRPE65. Recombinant cRPE65 showed significantly higher specific isomerohydrolase activity than that of hRPE65 when the activities were normalized by RPE65 protein levels (44), suggesting that RPE65 from cone-dominant chicken is a more efficient isomerohydrolase, in comparison with mammalian RPE65, probably to meet the higher demand for 11-cis-retinal regeneration in the cone-

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2The abbreviations used are: RPE, retinal pigment epithelium; RPE65, RPE-specific 65-kDa protein; 11cROL, 11-cis-retinol; crPE65, chicken RPE65; hRPE65, human RPE65; sIMH, superisomerohydrolase.
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![Diagram of the visual cycle](image)

FIGURE 1. Diagram of the visual cycle. The chromophore, 11-cis-retinal (11cRAL), in visual pigments is isomerized to all-trans-retinal (atRAL) by light, which triggers the phototransduction cascade. atRAL is then released from opsins and reduced to all-trans-retinol (atROL) by an all-trans-retinol dehydrogenase (At-RDH). At-RDH and all-trans-retinol in photoreceptors are transported to the RPE and esterified to all-trans-retinyl ester (atRE) by lecithin retinol acyltransferase (LRAT). All-trans-retinyl ester is converted to 11cROL by the isomerohydrolase RPE65 and further oxidized to 11c-RDH. Finally, regenerated 11-cis-retinal is transported back to photoreceptors and binds to opsins to form visual pigment.

dominant retina. Chicken and human RPE65 proteins share 90% sequence identity (50 different amino acid residues) at the amino acid level (44). We hypothesized that some of the divergent amino acid residues in cRPE65 were responsible for its higher isomerohydrolase activity.

In this study, we substituted the potential key residues in hRPE65 with their counterparts of cRPE65 and evaluated the isomerohydrolase activities of the generated hRPE65 mutants.

EXPERIMENTAL PROCEDURES

Prediction of Candidate Residues Responsible for Higher Enzymatic Activity of cRPE65—In order to predict the key residues responsible for the higher enzymatic activity and/or protein levels of RPE65 in cone-dominant species, RPE65 amino acid sequences from chicken (accession number NP_990215), zebrafish (Taeiophygga guttata, XP_002187720), and American chameleon (Anolis carolinensis, XP_003225885) were aligned with other known RPE65 sequences: human (accession number NP_000320), bovine (NP_776878), dog (NP_001003176), rat (NP_446014), mouse (NP_084263), newt (Q8AXN9), salamander (Q9Y125), and clawed frog (NP_001080269).

Site-directed Mutagenesis—WT hRPE65 and cRPE65 cDNAs were subcloned into cloning vectors as described previously (1, 44). The candidate key residues in hRPE65 were replaced by their counterparts in cRPE65 using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer’s protocol. The introduced mutations were confirmed by sequencing from both strands using an ABI-3730 DNA sequencer (Applied Biosystems, Foster City, CA) and subcloned into an expression vector, pcDNA3.1(−) (Invitrogen). Following the sequence confirmation, the expression constructs were purified by a QIAfilter Maxi Prep kit (Qiagen, Valencia, CA). Further, the hRPE65 and cRPE65 cDNA were individually subcloned into pUC18. To generate six restriction fragments of hRPE65 and cRPE65, unique restriction enzyme sites were introduced without changing the amino acid sequence (see Fig. 5A). Each fragment of hRPE65 was replaced by its counterpart of cRPE65 using the introduced restriction enzyme sites. All primer sets used in this study are summarized in Table 1.

Plasmid Transfection—Constructed plasmids expressing WT hRPE65 and cRPE65 and hRPE65 mutants were purified using a QIAfilter Maxi Prep kit (Qiagen, Valencia, CA) and transfected into 293A-LRAT cells, a cell line stably expressing human LRAT (45), using Fugene 6 transfection reagent (Roche Applied Science) or polyethyleneimine (PEI; Polysciences, Inc. (War- rington, PA)) (1 mg/ml, pH 7.4, 2:1 PEI/DNA ratio), and the cultured media were replaced at 6 h following the transfection. At 48 h post-transfection, cells were harvested by a cell scraper and rinsed twice with ice-cold PBS. Protein levels and enzymatic activities of WT hRPE65 and its mutants were confirmed by Western blot analyses and an in vitro isomerohydrolase activity assay.

Western Blot Analysis—Total cellular protein concentrations were measured using a Bradford assay (46). Equal amounts of total cellular proteins (20 μg) of 293A-LRAT cells expressing WT hRPE65 and cRPE65 and of hRPE65 mutants and bovine RPE microsomal proteins (2.5 μg) as a positive control were resolved by electrophoresis through 8% Tris-glycine SDS-polyacrylamide gel and electrotransferred onto an Immobilon PVDF membrane (Millipore, Billerica, MA) unless specified. The membrane was blocked with 5% (w/v) nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 30 min and subsequently incubated overnight at 4 °C with a 1:1,000 dilution of an anti-RPE65 polyclonal antibody (27) to identify the key residues and a 1:50,000 dilution of an anti-β-actin monoclonal antibody (Sigma-Aldrich). We used another RPE65 antibody (44) to compare the expression levels of cRPE65 and chimeric mutants of RPE65 to avoid the immunoreactivity difference on fragment 3. After four washes with TBST, the mem-
brane was incubated in a light-shielding container for 1.5 h with a 1:25,000 dilution of goat anti-mouse IgG conjugated with DyLight-549 and goat anti-rabbit IgG conjugated with DyLight-649 (Pierce), and the bands were detected using the FluorChem Q imaging system (ProteinSimple, Santa Clara, CA). The signal intensities were semiquantified by densitometry using AlphaView SA software (ProteinSimple) and averaged from at least three independent experiments.

Isomerohydrolase Activity Assay—293A-LRAT cells were separately transfected with plasmids expressing WT human and chicken RPE65 and hRPE65 mutants. 293A-LRAT cells expressing red fluorescence protein (RFP, negative control) were separately transfected into 293A-LRAT cells. Expression levels and enzymatic activities of hRPE65 and cRPE65 were measured by Western blot analysis (Fig. 2A) and an in vitro isomerohydrolase activity assay, respectively. All-trans-[3H]retinol (0.2 μM) was incubated with 125 μg of total cellular protein from the cells expressing RFP (B), hRPE65 (C), and cRPE65 (D) for 2 h, and the generated retinoids were analyzed by HPLC. Peak 1, retinyl esters; peak 2, 11cROL.

RESULTS

Comparison of Expression and Enzymatic Activities of Human and Chicken RPE65—Plasmids expressing RFP (negative control), hRPE65, and cRPE65 were separately transfected into the 293A-LRAT cells and cultured for 48 h. The expression levels and enzymatic activities of hRPE65 and cRPE65 were verified by Western blot analysis (Fig. 2A) and an in vitro isomerohydrolase assay. Both hRPE65 and cRPE65, but not negative control protein, RFP, converted all-trans-retinyl ester into 11cROL, the product of isomerohydrolase (Fig. 2, B–D). cRPE65 produced substantially higher levels of 11cROL than that of hRPE65 after the normalization to its RPE65 protein level, indicating a higher enzymatic activity of cRPE65, consistent with our previous report (44).

Prediction of Key Residues Responsible for Increased Isomerohydrolase Activity of cRPE65—Based on an amino acid sequence alignment of vertebrate RPE65, we selected 8 residues as the candidates that may be responsible for the difference in the isomerohydrolase activity between hRPE65 and cRPE65 (Fig. 3). We divided the candidate residues into two groups in this study: Group A, residues that were found to be identical in the three cone-dominant species but were substituted in other selected vertebrates (Thr39, Cys106, Asn170, Cys330, and Gln497 in hRPE65); Group B, residues that were conserved in lower vertebrates but diverse in mammalians (Lys297, Leu510, and Ser533 in hRPE65). In order to evaluate the contribution of these residues to isomerohydrolase activity, a series of point mutations of the 8 candidate residues (in Groups A and B) in hRPE65 were generated by site-directed mutagenesis. For in vitro enzyme activity assays, we first examined the effect of 4 residues...
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TABLE 2
Expression levels of generated RPE65 mutants in this study

| Mutant Name   | Protein expression (% of WT ± SEM) | N= |
|---------------|-----------------------------------|----|
| wt hRPE65     | 100.0 ±                         | 6  |
| T39R          | 119.2 ± 8.3                     | 6  |
| N170K         | 104.9 ± 3.3                     | 7  |
| C997P         | 106.3 ± 6.5                     | 6  |
| T39R/N170K    | 102.3 ± 10.7                    | 5  |
| N170K/C330T   | 86.2 ± 8.7                      | 5  |
| N170K/C497P   | 92.0 ± 4.4                      | 5  |
| T39R/N170K/C330T | 92.1 ± 3.0                  | 4  |
| T39R/N170K/C497P | 87.9 ± 2.2                  | 4  |
| T39R/N170K/C330T/C497P | 108.8 ± 5.4            | 4  |

TABLE 2
Expression levels of generated RPE65 mutants in this study

| Mutant Name   | Protein expression (% of WT ± SEM) | N= |
|---------------|-----------------------------------|----|
| wt hRPE65     | 100.0 ±                         | 6  |
| C106Y         | 89.0 ± 5.9                      | 5  |
| K297G         | 111.3 ± 7.1                     | 7  |
| L510M         | 171.0 ± 11.7                    | 4  |
| S533A         | 114.9 ± 9.1                     | 5  |
| C106Y/K297G   | 146.3 ± 8.2                     | 3  |
| K297G/L510M   | 180.1 ± 16.8                    | 3  |
| K297G/S533A   | 176.3 ± 12.2                    | 3  |

TABLE 2
Expression levels of generated RPE65 mutants in this study

| Mutant Name   | Protein expression (% of WT ± SEM) | N= |
|---------------|-----------------------------------|----|
| wt hRPE65     | 100.0 ±                          | 6  |
| C106Y         | 99.2 ± 9.8                       | 4  |
| T39R/N170K    | 78.7 ± 2.4                       | 3  |
| T39R/N170K/I220M | 103.3 ± 0.9                | 3  |
| N302I         | 134.0 ± 14.1                     | 3  |
| K297G/N302I   | 145.2 ± 8.0                      | 4  |

Amino acid sequence alignment of RPE65 from different species. RPE65 sequences from the human, bovine, dog, rat, mouse, chicken, zebra finch, clawed frog, salamander, and chameleon are indicated by gray boxes (Group A). Residues conserved in the cone-dominant species are indicated by white boxes (Group B).

(Thr³⁹, Asn¹⁷⁰, Cys³⁰⁰, and Gln⁴⁹⁹ in hRPE65) in Group A as the first experimental set, and the remaining residues (1 residue in Group A (Cys¹⁰⁶) and 3 residues in Group B (Lys²⁹⁷, Leu⁵¹⁰, and Ser⁵³³)) were studied as the second experimental set.

Impacts of Site-directed Point Mutations on Protein Levels and Catalytic Activities of RPE65—Plasmids expressing hRPE65 and cRPE65 and the site-directed mutants of hRPE65 were separately transfected into 293A-LRAT cells, and the transfected cells were cultured for 48 h. Protein expression was confirmed by Western blot analysis (Table 2), and the same batches of total cellular proteins were used for the in vitro isomerohydrolase activity assay. For all of the hRPE65 mutants containing single, double, and multiple mutations, expression levels of RPE65 were comparable (Fig. 4, A–E, Table 2), whereas the two single point mutants N170K and K297G exhibited 1.6- and 1.7-fold higher enzymatic activity, respectively, than WT hRPE65 after normalization by total RPE65 expression levels (Fig. 4, F and G). Furthermore, the tested double, triple, or multiple mutants with N170K (or K297G) in each experimental set did not further enhance catalytic activity of RPE65. These results suggest that mutations of N170K and K297G in hRPE65 may be important for increasing its enzymatic activity.

Construction of Chimeric Human RPE65 and Its Impacts on Its Protein Levels and Catalytic Activity—To further improve the catalytic activity of hRPE65, we constructed chimeric RPE65 by replacing a peptide fragment of hRPE65 with the counterpart of cRPE65 (Fig. 5A, F1–F6). At 48 h post-transfection, the cells were harvested for Western blot analyses and in vitro isomerohydrolase assays. Western blot analysis showed that a F1 chimeric mutant (replaced fragment 1) displayed a higher protein level of RPE65 than did WT hRPE65 and other chimeric mutants (Fig. 5, B and C). Interestingly, F1 and F3 chimeric mutants showed ∼1.5–2-fold higher catalytic activity than did WT hRPE65, whereas the F2, F4, and F5 chimeric mutants substantially decreased the catalytic activities of hRPE65. Following the normalization by RPE65 protein levels, the F1 chimeric mutant showed 1.9C0L production (110.2% of WT hRPE65) comparable with that of WT hRPE65, suggesting that the enhanced catalytic activity of the F1 chimera was prob-
ably due to the increased protein level of the F1 RPE65 chimera (Fig. 5, B and C). It should be noted that the F3 chimera displayed higher normalized enzymatic activity than WT hRPE65, probably due to N170K mutation (159.2% of WT hRPE65). This is well correlated with the result of the N170K single mutation (see Fig. 3).

**Generation of Superisomerohydrolase (sIMH) by Combination of Highly Active Mutants**—Finally, we combined the three highly active mutants identified from the series of site-directed point mutants and the chimeric mutants. As we examined earlier, two single mutants (N170K and K297G) showed significantly higher 11cROL production than did WT hRPE65 (Fig. 6B), although their expression levels were similar to that of WT hRPE65 (Fig. 6A, Table 2). As shown in Fig. 6, a double mutant (N170K/K297G) of hRPE65 demonstrated 3.2-fold higher 11cROL production than WT hRPE65. In addition, a combination of the F1 chimera with the N170K mutations showed even higher protein levels (2.2-fold) and 11cROL production (2.2-fold) than that of N170K single mutant. Finally, after combining all of these three mutations, the 11cROL production of a combined mutant, F1/N170K/K297G (sIMH), was further increased to 4.4-fold of that of WT hRPE65, which is comparable with the activity of WT cRPE65 (5.8-fold in the same assay; Fig. 6B). These results indicate that the three mutations are responsible for the higher activity of cRPE65.

**DISCUSSION**

It has been well established that RPE65 is an essential enzyme for the normal vision because RPE65 deficiency causes blindness and retina dystrophies, such as Leber’s congenital amaurosis or retinitis pigmentosa (4–17). In order to restore the visual function in patients with RPE65 deficiency, RPE65 gene replacement therapy has been performed (40–43). However, none of the gene replacement trials have fully restored the visual function in RPE65-deficient patients, although the gene delivery itself was successful (29). The unsatisfactory efficacy may be ascribed to the weak catalytic activity of RPE65 ($k_{cat} = 1.45 \times 10^{-4} s^{-1}$) (24) in comparison with other retinoid-processing enzymes (25, 26). This hypothesis is supported by the observation that endogenous RPE65 exists at high levels in the RPE (11 μg/eye in bovine), probably to compensate for its relatively low activity (27). It is likely that current RPE65 gene delivery strategies cannot achieve such a high protein level of RPE65. These studies suggest that enhancement of catalytic activity of the delivered hRPE65 may be a means to improve the efficacy of RPE65 gene therapies.

The present study represents the first approach to improve the catalytic activity of hRPE65. Previously, we have shown that RPE65 from cone-dominant chicken has substantially higher isomerohydrolase activity than that of RPE65 from rod-dominant mammals (44). This finding suggests that there is a potential to improve catalytic activity of hRPE65 by replacing...
some key residues with those of cRPE65. In order to identify the key residues responsible for the higher enzymatic activity in cRPE65, three cone-dominant species (two avian and one diurnal-type reptile) were selected as the template for amino acid sequence comparison. Among the divergent residues, 8 amino acid residues were selected as candidates in this study. The residues Glu158, Lys208, and Leu408 in hRPE65 were not selected for the study because their substitutions preserve physicochemical properties in other selected vertebrates (Asp158, Arg208, and Ile408). Although Phe108 is conserved in cone-dominant avian and reptile species, it was also conserved in the clawed frog. Therefore, we did not select the Phe108 residue for

![Image](https://example.com/image1.png)

**FIGURE 5. Schematic diagram of chimeric human RPE65 mutants and their enzymatic activities.** A, the positions of candidate sites in each fragment and specific restriction sites are presented in the diagram (white bar, hRPE65; gray bar, cRPE65; F1–F6, chimeric hRPE65 replaced with the corresponding cRPE65 fragment). B, expression levels of hRPE65 and chimeras were measured by Western blot analysis (Pc, 2.5 μg of bovine RPE microsomal protein; Hum, 20 μg of total cellular protein from hRPE65), and protein levels of RPE65 were semiquantified by densitometry. C, enzymatic activities were measured by the *in vitro* isomerohydrolase assay and quantified using generated 11-cis-[^3H]retinol and normalized by their relative RPE65 protein levels (C).

![Image](https://example.com/image2.png)

**FIGURE 6. Impacts of the site-directed mutations and fragment replacement with those of cRPE65 on protein levels and enzymatic activities of hRPE65.** The identified site-directed mutants in this study (N170K and K297G) and the F1 chimera were combined to produce sIMH (F1/N170K/K297G). The identified point mutants and the F1 chimera of RPE65 were expressed in 293A-LRAT cells. The cells were harvested 48 h after the transfection, and protein levels of RPE65 were confirmed by Western blot analyses (A). Enzymatic activities were measured by the *in vitro* isomerohydrolase assay and quantified by generated 11-cis[^3H]retinol (B). Isomerohydrolase activities were quantified by generated 11-cis[^3H]retinol (pmol/h) and presented as a percentage of WT hRPE65 activity (mean ± S.E., n = 3).

| Mutant name         | Normalized activity (percentage of WT ± S.E.) |
|---------------------|-----------------------------------------------|
| WT hRPE65           | 100.0 ± 10.0                                  |
| I220M               | 62.0 ± 11.0                                   |
| N170K/I220M         | 31.1 ± 7.8                                    |
| T39R/N170K/I220M    | 109.7 ± 4.2                                   |
| T39R/N170K/I220M/Q497P | 108.6 ± 10.4                                 |
| N302I               | 124.8 ± 13.3                                  |
| N302I/K297G         | 107.7 ± 6.9                                   |

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**TABLE 3**

*Isomerohydrolase activities on I220M and N302I mutant series of hRPE65*

*In vitro* activity assays of the indicated mutants were performed as described under “Experimental Procedures,” and isomerohydrolase activities were expressed as relative activity following the normalization by RPE65 levels (percentage of WT hRPE65 activity, mean ± S.E., n = 3).
the mutagenesis study. It is noteworthy that some residue replacements that did not fulfill the above-mentioned criteria of residue selection (I220M and N302I) did not substantially increase the catalytic activity of hRPE65 even in combinations with other mutations (N170K/I220M, T39R/N170K/I220M, and T39R/N170K/I220M/Q497P) (Table 3).

Among the tested mutants, we successfully identified two point mutations, N170K and K297G, which significantly increased 11cROL production compared with that of WT hRPE65. The double mutant that combined these mutations (N170K/K297G) showed a further enhanced 11cROL production (3.2-fold that of WT RPE65) of hRPE65. Furthermore, 11cROL production of this double mutant was even more enhanced by the replacement of the F1 fragment with that of sIMH (Met1–Arg33, containing 3 divergent residues: S2Y, I3S, and L26V). As a result, this superisomerohydrolase mutant (sIMH; F1/N170K/K297G) showed an approximately 4.4-fold higher 11cROL production than did WT hRPE65 under the same experimental conditions. Production of 11cROL by sIMH was near the level of WT cRPE65 (5.8-fold that of WT hRPE65).

As further confirmation of enhanced enzymatic activity of sIMH, we evaluated catalytic efficiencies of WT hRPE65 and sIMH (Fig. 7). sIMH showed a $V_{\text{max}}$ value ~3-fold higher than that of WT hRPE65 (Fig. 7, B and C). It is noteworthy that the estimated concentration of substrate, retinyl esters, in the mouse RPE is ~500 μM (48–50), which is much higher than $K_m$ for both WT RPE65 and sIMH (Fig. 7, B and C). In this case, $K_m$ is not an important factor because the enzyme is saturated by substrate at this concentration.

\[ v = \frac{V_{\text{max}} \times S}{(K_m + S)} \tag{1} \]

$K_m$ can be neglected in the formula when $S \gg K_m$.

The rate of the enzyme reaction is actually equal to $V_{\text{max}}$ in the RPE, meaning that $V_{\text{max}}$ of RPE65 plays a major role in physiological conditions (Fig. 7, B and C).

### FIGURE 7. Analyses of catalytic efficiencies of WT hRPE65 and sIMH.

Adenoviruses expressing hRPE65 and sIMH were prepared as described previously (1, 45). WT hRPE65 and sIMH were separately expressed in 293A (without LRAT) cells for 24 h at a multiplicity of infection of 100, and their expression was evaluated by Western blot analysis (A). Various concentrations of the substrate in liposome were incubated with total cellular protein lysates (125 μg) in the in vitro enzyme assays as described previously (24, 52), and $V_{\text{max}}$ and $K_m$ values of WT hRPE65 (B) and sIMH (C) were calculated through a Michaelis-Menten plot of generated 11cROL.

### FIGURE 8. Locations of Asn$^{170}$ and Lys$^{297}$ and the F1 fragment in an RPE65 three-dimensional structure model.

The locations of two identified key residues and the F1 fragment are shown in the three-dimensional model based on the crystal structure of bovine RPE65 (Protein Data Bank entry 3FSN). The iron binding site, within the catalytic domain, is indicated by an orange dotted circle. The disordered segment (Phe109–Val126), which contains a palmitoylated Cys residue (Cys$^{112}$), is shown by a pink dotted line. The entrance of the hydrophobic tunnel containing an active site is indicated by a red dotted circle. The location of the F1 fragment is indicated by the yellow dotted line. Both Asn$^{170}$ and Lys$^{297}$ residues are located on the surface of the protein (A and B) and more than 20 Å distant from iron(II) in the potential catalytic domain (C).
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To understand the potential contribution of the identified key residues to higher catalytic activity of cRPE65, we analyzed the three-dimensional structure of bovine RPE65 (Protein Data Bank entry 3FSN) by the SwissPDB Viewer version 4.01 (51) and displayed the results by POVRay version 3.61. In the three-dimensional model, both Asn۱۷۰ and Lys۲۹۹ residues are localized on the surface of the RPE65 molecule (Fig. 8, A and B) and distant from the co-factor iron in the RPE65 catalytic site (Fig. 8C), suggesting that these two residues are unlikely to directly participate in the catalysis of the substrate. We speculate that these residue substitutions might improve RPE65 membrane association for more efficient substrate intake and/or product (11cROL) release. Alternatively, these two residues might contribute to proper folding of RPE65 to achieve its active conformation upon association with the membrane. Interestingly, the F1 chimera also revealed a significant improvement of the catalytic activity of hRPE65 due to increased protein levels of RPE65. Unlike the other two residue substitutions, the F1 chimera in which only the F1 fragment is replaced with that of cRPE65 showed a catalytic activity similar to that of WT hRPE65 after normalization by RPE65 protein level. The F1 fragment contains 33 amino acids and only 3 residues that differ between hRPE65 and cRPE65. These residues in the F1 fragment might contribute to proper folding to enhance protein stability of the hRPE65 mutant. Another possibility is the contribution of the nucleotide sequence difference. Among the 99 base pairs encoding the 33 amino acids in the F1 fragment, there are 19 different nucleotides between hRPE65 and cRPE65. These nucleotide substitutions might improve the stability of the hRPE65 mRNA or contribute to more efficient codon usage, leading to higher levels of expression.

In summary, this study generated a highly active hRPE65 mutant, termed sIMH, by replacing only 5 residues with those of cRPE65. This sIMH exhibited higher catalytic activity (4.4-fold higher than WT hRPE65) after normalization by the total protein level of RPE65, the sIMH obtained in this study can be used for the next generation of RPE65 gene replacement therapy and may be more effective than WT hRPE65 in the treatment of retinal dystrophies. These results will also contribute to the understanding of structural basis for the isomerohydrolase activity of RPE65.

Note Added in Proof—Table 2 was inadvertently omitted from the Papers in Press version.

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