Thermostabilization of the Human Endothelin Type B Receptor

Akiko Okuta1,2,†, Kazutoshi Tani2, Shoko Nishimura2, Yoshinori Fujiyoshi2,3 and Tomoko Doi1

1 - Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
2 - Cellular and Structural Physiology Institute, Nagoya University, Chikusa, Nagoya 464-8601, Japan
3 - Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Correspondence to Tomoko Doi: Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa, Oiwake, Sakyo, Kyoto 606-8502, Japan. doi@mb.biophys.kyoto-u.ac.jp

http://dx.doi.org/10.1016/j.jmb.2016.03.024

Edited by J. Bowie

Abstract

The peptide hormone endothelin, produced by the vascular endothelium, is involved in several physiological functions, including maintenance of vascular tone and humoral homeostasis. Endothelin transmits signals through the endothelin receptor, a G-protein-coupled receptor. Structural studies of the endothelin type B receptor (ETBR) have been unsuccessful due to its structural flexibility and instability in detergent-solubilized solution. To overcome these problems, we explored thermostabilization of human ETBR by establishing an ETBR expression system in Escherichia coli, followed by systematic alanine scanning mutagenesis. Among 297 point mutations, 11 thermostabilizing residues were selected and further mutated to other amino acids. The thermostability indices of these residues, represented by the ratios of endothelin-1 (ET-1) binding activities with or without heat treatment at 27 °C for 30 min in a ligand-free form, were compared. The ligand affinity and apparent melting temperature (Tm) of the five most thermostable mutants, R124Y, D154A, K270A, S342A, and I381A, were then examined. The apparent Tm of three single mutants, R124Y, D154A, and K270A, was approximately 7 °C higher than that of the wild type. The apparent Tm value of a combination of the five residues, named the Y5 ETBR mutant, was 17 °C higher than that of the wild type. The Y5 ETBR mutant exhibited an affinity for ET-1 and activated Gq similar to the wild type. Further investigation of the pharmacological properties affected by combinatorial mutations of ET-1, ET-3, TxET-1, and K8794 suggested that Y5 ETBR is highly suitable for representing a ligand-free form of ETBR and is potentially applicable for studying an ET-1-bound form.

© 2016 Elsevier Ltd. All rights reserved.

Introduction

The G-protein-coupled receptors (GPCRs) transmit extracellular signals from hormones, neurotransmitters, and metabolites across the plasma membrane. Endothelin-1 (ET-1), a 21-aa peptide hormone, is involved in a wide range of physiological functions, including vascular regulation, cell proliferation, salt homeostasis, and neural crest development [1–3]. ET-1 transmits signals through two endothelin receptor subtypes, ETAR and ETBR, both of which are class A GPCRs. Both receptors bind ET-1 with subnanomolar affinity [4] and signal via multiple G proteins and β-arrestin [1,5,6]. Several diseases and pathological conditions, such as arterial hypertension, heart failure, renal diseases, diabetes, neuronal dysfunction, and cancer, are caused by a failure of the endothelin system [2,3,7]. Structural information of endothelin receptors is essential toward a better understanding of endothelin signal transduction as well as the rational design of suitable ligands.

The human endothelin type B receptor (ETBR) has been overexpressed in insect cells and purified in milligram scale [8]. Structural and biophysical studies of endothelin receptors have been hampered, however, by their conformational flexibility and instability in...
detergent micelle solution. To overcome these difficulties in purification, crystallization, and biophysical studies, we explored the development of thermostable human ET_B_R by alanine scanning mutagenesis, which was initially used for stabilization of diacylglycerol kinase, an integral membrane protein of Escherichia coli (E. coli) [9,10], and successfully applied for thermostabilization of other GPCRs, such as the β1 adrenergic receptor (β1AR) [11], adenosine A2A receptor (A2A_R) [12,13], neurotensin receptor (NTSR1) [14], corticotropin-releasing factor receptor type 1 (CRF1R) [15], and others [16]. The thermostable β1AR-m23, developed by screening β1AR individual alanine mutants with the antagonist dihydroalprenolol after heat treatment in a ligand-free form, exhibited unchanged affinities for antagonists, but lowered affinities for agonists compared to the wild type. In the thermostabilization of A2A_R, NTSR1, and CRF1R, receptor mutations that allowed for efficient retention of the radioactive ligands after heat treatment in an agonist- or antagonist-bound form were selected to bias the conformation toward either state in addition to the thermostabilization. The ET_B_R binds agonist ET-1 in a virtually irreversible manner [17], but our crystallization trials were unsuccessful. We therefore surveyed thermostable alanine mutants after heat treatment in a ligand-free form by ET-1 binding, which revealed several potential candidates. We further studied the combinatorial and pharmacological properties of individual thermostable mutations that yielded thermostably evolved ET_B_R with an apparent Tm, 12–17 °C higher than that of the wild type in n-dodecyl-β-D-maltopyranoside (DDM)-solubilized solution. Here we describe the development and characterization of these thermostable ET_B_R mutants stabilized in a ligand-free form.

Results and discussion

Expression of ET_B_R in E. coli and identification of thermostabilizing mutations in ligand-free ET_B_R

For systematic screening of thermostable mutants by individual alanine (or leucine if the original residue was an alanine) mutation analyses, we first established the expression of 6hNET_B_R [8], a starting construct (wild-type) containing a hexahistidine tag in the N-terminal tail in E. coli. The pelB leader sequence was introduced to the N-terminus of 6hNET_B_R to facilitate membrane association, and the enhanced green fluorescent protein (EGFP) sequence was fused to its C terminus to confirm full-length expression. In the assays using solubilized proteins, the apparent KD and IC50 values for [125I]ET-1 of 6hNET_B_R expressed in E. coli were 122 ± 38 pM and 0.13 ± 0.01 nM, respectively, comparable to those of 6hNET_B_R expressed in HEK293 (KD: 106 ± 34 pM) or Sf9 cells (IC50: 0.14 ± 0.01 nM; Fig. S1). The maximum number of binding sites calculated from saturation binding of 6hNET_B_R expressed in E. coli was 1.5 ± 0.2 pmol/mg protein, whereas that of 6hNET_B_R expressed in insect cells was 77 ± 9.3 pmol/mg protein [5]. We concluded that 6hNET_B_R expressed in E. coli provides an affinity for ET-1 comparable to that of 6hNET_B_R expressed in HEK293 and insect cells to screen the thermostability of mutants, although its expression level was considerably lower (approximately 1/50) than that in insect cells.

A total of 297 mutations from residues 100 to 399 were prepared, except C171 (involved in a disulfide bond) and D198 and R199 (in the E/DRY motif), which covered seven transmembrane regions and extracellular and intracellular loops. We first evaluated the receptor thermostability in a ligand-free form, because the activity of a ligand-free form would be heat treatment-dependent in contrast to the ET-1 bound ET_B_R, which rarely dissociates under physiological conditions (Fig. S2). The thermostability assay of these mutants was performed on 2% DDM-solubilized receptors by incubating the receptors at 27 °C for 30 min, followed by a ligand binding assay using an agonist, [125I]ET-1 (Fig. 1a). The retained [125I]ET-1 binding activity, reflecting the amount of correctly folded receptor, was compared with that of an unheated sample. The thermostability index, that is, the ratio of residual activities with or without heating at 27 °C to that of the wild-type receptor, was 65.9% ± 2.0%. The Tm of ET-1 binding activity was also used to estimate the expression level in the mutants. Many mutants with mutations located around extracellular loops 1 and 2 had almost no ligand binding activity, which might be due to the lack of an ability to fold correctly. Among the 297 mutants assayed, we selected 11 mutants with a thermostability index greater than 75% and also adequate expression levels, retaining more than 50% [125I]ET-1 binding activity compared with that of the wild type (Fig. 1b and c, and Table S1). The selected mutations were R124A, D154A, V189A, K216A, A219L, I222A, K270A, S279A, L301A, S342A, and I381A, among which four residues were predicted to be at the membrane boundary, as seen for β1AR [11] and A2A_R [12,13] (Figs. 1c and S3).

In the second round of screening, the 11 selected residues were further changed to other amino acids, based on sequences of bovine rhodopsin, human β1AR, turkey β1AR, and human A2A_R. The expression levels and thermostability indices of these mutants were evaluated as described above, and the results are shown in Fig. 2a. The Tm values of the five most thermostable mutants, R124Y, D154A, K270A, S342A, and I381A, are shown in Figs. 2b and S3b. The R124Y, D154A, and K270A mutants...
had a $T_m$ value $\sim 7$ °C higher than that of the wild type, whereas S342A and I381A showed increases of only 1.7 °C and 3.3 °C, respectively. With regard to the ligand binding, R124Y and K270A increased the ET-1 binding activity 1.3- to 2.1-fold that of the wild type, while the D154A, S342A, and I381A mutants retained more than 50% of the activity of the wild type (Fig. 1b).

High ligand affinities are also critically important for maintaining the stable receptor–ligand complex in detergent micelle solution to crystallize them or for screening chemicals to develop drugs. Table 1 shows the apparent IC$_{50}$ values of competitive $[^{125}\text{I}]$ET-1 binding, representing the apparent affinities of the five most thermostable single mutants, R124Y, D154A, K270A, S342A, and I381A for

<table>
<thead>
<tr>
<th>Ballesteros-Weinstein number</th>
<th>ET$_B$R mutant</th>
<th>Thermostability Index (%)</th>
<th>Expression in E. coli (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.55</td>
<td>wt</td>
<td>65.9 ± 2.0</td>
<td>0.8 ± 0.05</td>
</tr>
<tr>
<td>2.57</td>
<td>R124A</td>
<td>86.6 ± 2.7</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>3.40</td>
<td>D154A</td>
<td>98.2 ± 2.6</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>4.40</td>
<td>V188A</td>
<td>77.4 ± 9.7</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>4.43</td>
<td>K216A</td>
<td>77.0 ± 8.7</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>5.35</td>
<td>A219L</td>
<td>84.6 ± 5.1</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>5.44</td>
<td>I222A</td>
<td>84.9 ± 0.8</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>5.66</td>
<td>K270A</td>
<td>117 ± 14.7</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>5.64</td>
<td>S279A</td>
<td>81.8 ± 20</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>7.48</td>
<td>L301A</td>
<td>85.1 ± 2.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>6.54</td>
<td>S342A</td>
<td>103.2 ± 3.3</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>7.48</td>
<td>I381A</td>
<td>97.0 ± 4.9</td>
<td>0.6 ± 0.08</td>
</tr>
</tbody>
</table>

Fig. 1. (a) Schematic outline of the thermostability screening of ligand-free ET$_B$R mutants. (b) The thermostability indices of 11 thermostabilizing mutants displaying more than 75% thermostability in the ligand-free form are summarized. Their expression levels in E. coli are represented as ET-1 binding sites in pmol/mg protein. (c) The thermostabilizing mutations on the homology-modeled ET$_B$R structure based on the rat NTSR1 [26]. The C$_\alpha$s of the five most thermostabilizing mutants are shown as magenta spheres, and those of the remaining 6 mutants are shown as orange spheres. Each residue is labeled with the Ballosteros–Weinstein number in superscript [27]. Blue dotted lines indicate putative membrane surfaces.
agonists ET-1 and ET-3 (an isopeptide of ET-1) and antagonists TxET-1 (Thr18, γ-methylLeu19)ET-1 [18] (Peptide Institute, Osaka, Japan) and K8794 (3-6-[((4-tert-butylphenyl)-sulfonyl]amino)-5-(2-methoxyphenoxo)-2,2′-bipyrimidin-4-yl]oxy)-N-(2,6-dimethylphenyl) propanamide, an ETBR-selective antagonist, a bosentan analogue [19] (Kowa, Co., Ltd.). While most single mutations did not largely reduce the affinity for each ligand, the D154A mutant displayed an approximately 30-fold higher IC50 value for K8794 (Table 1).

**Mutation combinations based on mutant thermostability and ligand-binding affinity**

In the development of thermostable mutants, it is favorable to introduce a minimum number of mutations to study the structure of the native receptor. Based on the evolved thermostable receptor of turkey β1AR [11], human A2AR [12,13], and NTSR [14]; however, the thermostabilizing effects induced by mutations can be additive, but combining mutations of adjacent primary amino acid sequences does not greatly improve thermostability. Among the selected thermostable ETBR mutations, we first constructed a combinatorial mutant, RDK, containing R124Y, D154A, and K270A as core mutations because of their individual high apparent Tm values and better expression of the K270A mutation (Fig. 1b). We then further combined the mutations with one or both of the mutants with a high thermostability index, S342A (RDKS), I381A (RDKI), or both (RDKSI, also named Y5). These mutations are sufficiently independent, with three residues residing in the predicted transmembrane domain and two residues at the membrane boundary (Fig. 1c). To avoid the effect of lower affinity for K8794, we also constructed a set of combinations without D154A to develop a thermostabilized ETBR for ligand screening; RKS containing R124Y, K270A, and S342A; RKL

<table>
<thead>
<tr>
<th>Ballesteros-Weinstein number</th>
<th>ETBR mutant</th>
<th>Apparent Tm (°C)</th>
<th>ΔTm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.55</td>
<td>wt</td>
<td>26.9 ± 0.5</td>
<td>—</td>
</tr>
<tr>
<td>1.55</td>
<td>R124Y</td>
<td>34.3 ± 1.0</td>
<td>7.4</td>
</tr>
<tr>
<td>2.57</td>
<td>D154A</td>
<td>33.8 ± 0.3</td>
<td>6.9</td>
</tr>
<tr>
<td>5.35</td>
<td>K270A</td>
<td>33.7 ± 1.1</td>
<td>6.8</td>
</tr>
<tr>
<td>6.54</td>
<td>S342A</td>
<td>28.6 ± 1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>7.48</td>
<td>D381A</td>
<td>30.2 ± 0.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

**Fig. 2.** (a) Thermostability index representing the ratio of [125I]ET-1 binding activity of heated E. coli lysate at 27 °C for 30 min to the untreated sample normalized as 100%. The bars for the five most thermostabilizing mutants are shown in black, and the bars for the remaining 6 and also other less prominent mutants on the five thermostabilizing residues are shown in gray. (b) Apparent Tm values in 2% DDM-solubilized solution of the five most thermostabilizing mutants expressed in E. coli. Each experiment was repeated twice.
Thermostabilization of the Human ET_{BR}

Table 1. Competitive binding assays of agonists and antagonists for [125I]ET-1 binding of ET_{BR} mutants

<table>
<thead>
<tr>
<th>Ballesteros–Weinstein number</th>
<th>ET-1</th>
<th>ET-3</th>
<th>TxET-1</th>
<th>K8794</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50} (nM)</td>
<td>IC_{50} ratio</td>
<td>IC_{50} (nM)</td>
<td>IC_{50} ratio</td>
</tr>
<tr>
<td>wt*</td>
<td>0.26 ± 0.008</td>
<td>1</td>
<td>0.33 ± 0.02</td>
<td>1</td>
</tr>
<tr>
<td>wt*</td>
<td>0.31 ± 0.02</td>
<td>–</td>
<td>0.33 ± 0.02</td>
<td>–</td>
</tr>
<tr>
<td>1.55</td>
<td>R124Y</td>
<td>0.70 ± 0.08*</td>
<td>2.7</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>2.57</td>
<td>D154A</td>
<td>R7894 0.68 ± 0.07**</td>
<td>2.6</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>5.35</td>
<td>K270A</td>
<td>0.31 ± 0.03</td>
<td>1.2</td>
<td>0.27 ± 0.005</td>
</tr>
<tr>
<td>6.54</td>
<td>S342A</td>
<td>0.42 ± 0.05</td>
<td>1.6</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>7.48</td>
<td>I381A</td>
<td>0.24 ± 0.02</td>
<td>0.9</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>RDK</td>
<td>0.79 ± 0.13*</td>
<td>3</td>
<td>1.2 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>RKS</td>
<td>0.56 ± 0.13</td>
<td>2.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>RKI</td>
<td>0.72 ± 0.05*</td>
<td>2.8</td>
<td>1.1 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>RKS</td>
<td>0.78 ± 0.04*</td>
<td>3</td>
<td>2.9 ± 0.3***</td>
</tr>
<tr>
<td></td>
<td>RDK</td>
<td>0.45 ± 0.08</td>
<td>1.7</td>
<td>1.5 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>RKDS</td>
<td>0.55 ± 0.06</td>
<td>2.1</td>
<td>2.5 ± 0.6**</td>
</tr>
<tr>
<td></td>
<td>Y5</td>
<td>0.38 ± 0.06</td>
<td>1.5</td>
<td>4.8 ± 0.5***</td>
</tr>
</tbody>
</table>

Competitive binding of agonists (ET-1 and ET-3) and antagonists (TxET-1 and K8794) was examined using insect cell membranes expressing point mutations or combinatorial mutations. R124Y, D154A, K270A, and S342A membranes were prepared from SF+ cells, and others were prepared from Sf9 cells. Competitive binding was initiated by adding membranes from Sf9 cells (0.1–0.2 μg) or HEK293 cells (1–5 μg) to an assay mixture comprising 0.1% BSA, 0.03–0.05 mM [125I]ET-1 (2200 Ci mmol⁻¹; PerkinElmer Life Sciences), and 8 concentrations of unlabeled compounds (ET-1, ET-3, and TxET-1 ranging from 1 pM to 1 μM, K8794 ranging from 10 pM to 0.1 mM) in 50 mM Hepes–NaOH (pH 7.5) and 10 mM MgCl₂ (Mg–Hepes). Binding reactions were incubated at 37 °C for 1 h, terminated by dilution with cold Mg–Hepes, and filtered onto glassfiber filters (multiscreen HTS FB, Merck Millipore) to separate unbound [125I]ET-1 [28]. After washing with cold Mg–Hepes, the radioactivity on the filters was measured using a γ-counter. The assays were repeated twice. Data were analyzed by nonlinear regression using GraphPad Prism 6. Statistical analyses were performed by one-way analysis of variance with Dunnett’s multiple comparison post hoc test. *p < 0.05. **p < 0.001. ***p < 0.0001.

* wt: expressed in insect cells (Sf9 and SF+).

⁎ wt: expressed in insect cells (Sf9 and SF+).

containing R124Y, K270A, and I381A; and RKS containing R124Y, K270A, S342A, and I381A.

The ligand-binding properties of these seven combinatorial mutants are shown in Table 1. The RDK mutant of core mutations exhibited 3- to 4-fold higher IC_{50} values for ET-1, ET-3, and TxET-1 compared to that of the wild type, whereas it exhibited a 61-fold higher IC_{50} value for K8794, likely due to the D154A mutation. Surprisingly, further addition of mutations, resulting in RDKI, RKDS, and Y5, additively enhanced the affinity for ET-1, and the IC_{50} value of Y5 was close to that of the wild type. In contrast, the decreases in the affinity for ET-3 by mutations were additive, and Y5 had a 15-fold higher IC_{50} value, although the defects of individual single mutations did not differ much from those for ET-1. The addition of further mutations to RDK did not improve the affinity for TxET-1. Combinatorial mutations without D154A, and both RKS and RKI exhibited an affinity for K8794 similar to that of the wild type.

We then selected candidates with high affinity for the respective ligands, RDK, RKS, and Y5, and examined their thermostability in 1% DDM-solubilized solution prepared from insect Sf9 cell membranes (Fig. 3a and e). The RDK and Y5 mutants containing the core mutations had relatively high T_m values, 47.3 ± 0.5 °C and 47.6 ± 0.4 °C, respectively, while the RKS mutant had a slightly lower T_m of 42.6 ± 0.4 °C, presumably due to the lack of D154A. These mutants had T_m values 12.6–17.6 °C higher than that of the wild type (Fig. 3e).

Characterization of Y5 ET_{BR} in ligand-free and ET-1-bound forms

We studied the properties of the most thermostable Y5 mutant expressed in insect cells. This mutant exhibited a mean apparent K_d value of 25.3 ± 2.9 pM for ET-1, similar to that of the wild type (20.7 ± 1.5 pM), based on a saturation binding assay using insect cell membranes (Fig. 3f). In 1% n-decyl-β-D-maltopyranoside (DM)-solubilized solution, Y5 also exhibited increased thermostability with a T_m value of 47.3 ± 0.5 °C higher than that of the wild type (Fig. 3e).

In the thermal inactivation profile at 45 °C, the wild type had a half-life of 1.0 min and Y5 had a half-life of 92.5 min in 1% DDM-solubilized solution. In contrast, in the thermal denaturation profiles of ET-1-bound states in 1% DDM-solubilized solution, the Y5 ET_{BR} had a T_m value of 65.7 ± 1.5 °C and wild-type ET_{BR} had a T_m value of 75.6 ± 2.6 °C, due to its unusually stable and virtually irreversible ET-1 binding (Figs. 3d and S2).

Although the T_m value of Y5 in the ET-1-bound form...
Thermostabilization of the Human ET_{A}R

Fig. 3 (legend on next page)
Thermostabilization of the Human ET₂R

was approximately 10 °C lower than that of the wild type, it was still stable enough in the range of crystallizable thermostability, because the majority of the previously solved Tₘ values of ligand-bound GPCRs are greater than 55 °C [20]. The Y5 mutant expressed in insect cells was solubilized in detergent and purified as efficiently as the wild type via immunoadfinity and nickel-affinity column chromatography (Fig. S4). Furthermore, they were reconstituted into phospholipid vesicles as described previously and assayed for Gₛ protein coupling [5]. The Y5 mutant exhibited ET-1-dependent Gₛ activation activity similar to the wild type, indicating that Y5 could adopt a fully active conformation via ET-1 binding (Fig. 3g).

We have now successfully created a thermostable ET₂R, suitable as a ligand-free form with a Tₘ value 17.6 °C higher than that of the wild type. It could also be useful for crystallization in an ET-1-bound form with unchanged affinity for ET-1, because agonist-bound forms of GPCRs are generally thought to be more flexible than antagonist-bound or inactive states. Although we evaluated the thermostability of mutants in a ligand-free form, screening the residual activity by ET-1 binding after heat treatment may select for mutations favorable for the ET-1-bound conformation. Investigation of the affinities of thermostable mutants for ET-1 and other ligands suggests that an adequate combination of mutations is important for retaining the wild-type affinity and successive studies, such as drug screening [21].

It is quite surprising that single mutations of R124Y, D154A, or K270A resulted in an apparent Tₘ value ~7 °C higher than that of the wild type, which might reflect the flexibility of these residues in ET₂R. A single Trp₃.₄₁ substitution was successfully introduced for structural analyses of β₂AR and other GPCRs [20]. Replacement of non-conserved residues at the 3.₄₁ position of helix 3 with tryptophan often enhances correct protein folding and thermal stability. We also analyzed the thermostability of the L190W₃.₄₁ ET₂R mutant, in which a single mutation increased the apparent Tₘ by ~7 °C, whereas the addition of the L190W mutation to Y5 decreased the Tₘ by 2 °C (data not shown). Therefore, we did not combine the L190W mutation with Y5. The combination of single thermostabilizing mutations was not always additive and should be optimized for both the thermostability and affinity for each ligand.

When the 11 selected thermostabilized residues in the mutants were mapped onto the homology-modeled structure (Fig. 1c), a group of the five best residues distributed in helix 1, 2, 5, 6, and 7, and the remaining six residues distributed in helix 3, 4, and 5 appeared to localize separately within the structure. Because Y5 showed normal affinity for ET-1 with decreased affinities for ET-3, TxET-1, and K8794 (Table S2), a combination of the remaining six residues might display a distinct preference for these ligands. Although Y5 exhibited a much higher Kᵢ value for K8794 compared to that of the wild type, its value was still within the ordinary Kᵢ range of the previously solved GPCR structures [20]. Only from the point of view of the ligand binding properties, Y5 seems to have the ability to produce all of the above ligand-bound forms in addition to a ligand-free form. Further structural and biophysical analyses are needed, however, to elucidate the detailed reasons for the thermostability conferred by these ET₂R mutations.

Materials and methods

Alanine mutagenesis and expression of ET₂R in E. coli

The human ET₂R gene with a hexahistidine tag between residues Gly57 and Leu66 that we previously established (6hNET₂R) was used as a template for alanine scanning.

Fig. 3. (a) Thermostabilities of the wild-type and ET₂R mutants expressed in Sf9 cells in a ligand-free form. Thermostabilities of the ET₂R mutants were assayed after solubilizing Sf9 membranes in 1% DDM. Closed circles, wild-type; closed diamonds, RKS; open diamonds, RKD; open circles, Y5. (b) Thermostabilities of wild-type and Y5 ET₂R in 1% DM and DDM. Closed circles, wild-type in DDM; closed triangles, wild-type in DM; open circles, Y5 in DDM; open triangles, Y5 in DM. (c) Thermal denaturation rates of ligand-free wild-type (closed circles with a dotted line) and Y5 (open circles with a line) ET₂R in 1% DDM at 45 °C. Half-lives were determined by non-linear regression of a one-phase exponential decay curve using GraphPad Prism 6. Half-lives: wild-type, 1.0 min; Y5, 92.5 min. (d) Thermostabilities of ET-1-bound wild-type and Y5 ET₂R in 1% DDM. Closed circles, wild-type; open circles, Y5. (e) Apparent Tₘ values (°C) determined by nonlinear regression using GraphPad Prism 6 from the results in panels (a), (b), and (d). Differences from the apparent Tₘ value of the wild-type are represented as Δ Tₘ (°C). Expression levels of mutants in insect cells are represented as pmol binding sites per 1 mg membrane proteins, measured using P1 virus-infected cells. (f) Saturation binding curves of specific [₁²⁵I]ET-1 binding to the wild-type and Y5 ET₂R in the binding assays, Sf9 cell membranes containing approximately 2 fmol receptor were incubated with six different concentrations of [₁²⁵I]ET-1 ranging from 1.5 PM to 260 PM in 100 μl of 0.1% BSA, Mg–Hepes buffer at 37 °C for 2 h. Membranes were isolated from unbound [₁²⁵I]ET-1 and washed, and the amount of receptor-bound [₁²⁵I]ET-1 was measured as described in the legend for Table 1. Non-specific binding of the [₁²⁵I]ET-1 in each reaction was assessed by including 100 nM ET-1 in the same reaction and subtracted from the total binding. Apparent dissociation constants (K_d) of [₁²⁵I]ET-1 for wild-type and Y5 ET₂R were determined by Scatchard analysis using GraphPad Prism 6 (inset). Each experiment was performed in duplicate or triplicate. (g) Gₛ protein activation by wild-type and Y5 ET₂R. Closed circles, wild-type with ET-1 (line) or without ET-1 (dotted line); open circles, Y5 with ET-1 (line) or without ET-1 (dotted line).
mutagenesis [5]. The 6hNETbR gene with the N-terminal 30 residues deleted was subcloned into a PET22b vector (Novagen), which confers a pelB leader sequence followed by 9 residues derived from the vector at the N-terminus of 6hNETbR. The EGFP sequence was then fused to the C terminus for stable expression of ETbR in E. coli. Amino acid residues 100 to 399 were each individually changed to alanine using a PrimeSTAR mutagenesis kit (TakaraBio), except that the original alanine residues were changed to leucine. Combinatorial mutants were generated by QuickChange II methodology (Stratagene). Wild-type and 297 mutant genes were expressed in BL21(DE3) E. coli cells (Novagen). Cultures were grown in 10 ml Luria Bertani medium supplemented with 0.2% glucose and 0.1 mg/ml ampicillin in a 100-ml flask at 37 °C to an OD600 of 0.8–1.0. After cooling the cultures on ice, they were induced with 0.3 mM isopropyl β-D-thiogalactopyranoside and shaken for another 18 h at 18 °C. The cells were harvested by centrifugation at 13,000g for 1 min in 1-ml aliquots.

Thermostability and radioligand binding assays

For thermostability screening, E. coli cells harvested from 1 ml culture medium were suspended in 100 µl phosphate-buffered saline containing 20 µg/ml lysozyme and 2.5 units of Benzonase Nuclease (Novagen). The lysates were further solubilized by the addition of 100 µl phosphate-buffered saline containing 4% DDM (Anatrace) and rotated for 1 h at 4 °C, and insoluble materials were removed by centrifugation at 10,000g for 20 min. The supernatants were divided into two aliquots and incubated at 27 °C or on ice for 30 min, respectively, and the residual [125I]ET-1 binding activities were measured by a single-point binding assay using hydroxyapatite resin, as described previously [8,22]. Briefly, 2 µl of the samples (1.5–6 µg solubilized proteins) were incubated with 160 pM [125I]ET-1 in 50 µl binding buffer containing 50 mM sodium phosphate buffer (pH 7.5), 2 mM MgCl₂, 0.1% bovine serum albumin (BSA), and 0.1% digitonin at room temperature for 30 min. Hydroxyapatite resin (30 µl; BioRad) in 15% slurry was added to absorb receptor proteins, and the mixtures were centrifuged at 2000 rpm for 2 min to remove the free [125I]ET-1. The pelleted resins were washed with 0.3 ml of 50 mM sodium phosphate buffer (pH 7.5), 2 mM MgCl₂, and 0.1% digitonin, and measured using a γ-counter. The EGFP-expressing lysate was used to measure non-specific [125I]ET-1 binding in the assay, which was subtracted from the binding activity of each ETbR mutant. The thermostability assays of the 297 mutants were repeated twice.

Thermostability assays were performed using ETbR mutants solubilized in 2% DDM for E. coli cells as described above, and 1% DDM or DM for insect cells. Insect Sf9 cell membranes expressing ETbR mutants were solubilized at a protein concentration of 1.5 ~ 2 mg/ml at 4 °C for 1 h in 1% DDM or DM, 25 mM Hapes–NaOH (pH 7.5), 0.15 M NaCl, and 1 mM EDTA, and the supernatants were obtained by centrifugation at 15,000g for 10 min. For the denaturation of ligand-free ETbR, 20-µl aliquots of the supernatant were placed in 10 polymerase chain reaction (PCR) tubes and incubated at 10 different temperatures (4–60 °C) for 30 min in the absence of ligand in the PCR apparatus and kept on ice. The protein concentrations of solubilized supernatants ranged from 0.5 to 1 mg/ml. The samples were further diluted 100- or 200-fold with binding buffer and their [125I]ET-1 binding activity was measured as described above using hydroxyapatite resin. The residual [125I]ET-1 binding activities were compared to the control [125I]ET-1 binding activity of an unheated sample normalized as 100%. The assays were repeated twice. The thermal denaturation rates of ligand-free ETbR were measured using similarly prepared 1% DDM-solubilized samples. Fifteen-microliter aliquots of the solubilized supernatant were placed into each of 9 PCR tubes, incubated at 45 °C for the indicated times, and kept on ice. The samples were further diluted with binding buffer and their [125I]ET-1 binding activity was measured as described above. The assays were repeated twice. For the denaturation of ligand-bound ETbR, supernatants solubilized in 1% DDM, 25 mM Hapes–NaOH (pH 7.5), 0.15 M NaCl, and 1 mM EDTA were incubated with 0.45 nM [125I]ET-1 at 24 °C for 50 min followed by incubation with 500 nM ET-1 at 24 °C for 30 min, and then 15-µl aliquots were placed into each of 9 PCR tubes. The samples were incubated at nine different temperatures (45–85 °C) for 30 min and kept on ice. Then, the residual [125I]ET-1 binding activities were measured and compared as described above. The assays were repeated three times. The background sample that was first incubated with cold 500 nM ET-1 followed by 0.45 nM [125I]ET-1 at 24 °C for 50 min and then heat treated at 85 °C for 30 min yielded a similar count as the first [125I]ET-1 incubated sample treated at 85 °C for 30 min, described above. The data were analyzed by nonlinear regression using GraphPad Prism 6.

G-protein activation

To purify the wild-type and Y5 ETbR, a Flag epitope tag with the hemagglutinin signal sequence was introduced to the N-terminus of the 6hNETbR construct (F6hN-ETbR) and the resulting F6hN-ETbR and F6hN-Y5 were expressed in Sf9 cells using the Bac-to-Bac expression system (Invitrogen) [23]. Purification and reconstitution of wild-type and Y5 ETbR to phospholipid vesicles were performed as described previously using 0.1% DDM [5]. The activation assay mixtures contained reconstituted receptors at 1 nM, GDP (KeraFAST) at 0.3 µM, purified Gβγ12 (at 0.14 µM), 55 nM [35S]GTPγS, and 1 µM GDP, with or without 1 µM ET-1 in the buffer of 20 mM Hapes–NaOH (pH 8.0), 1 mM EDTA, 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol. The assays were performed at 30 °C for each of the respective times as indicated in Fig. 3g, and 10 µl of the mixtures was filtered into cellulose-mixed ester filters after stopping the reactions (multiscan HTS HA, Merck Millipore) to isolate G proteins from unbound [35S]GTPγS. The assays were repeated twice.

Homology model of ETbR

The amino acid sequences of the human ETbR, rat NTS1, and human orexin 2 receptor were aligned with CLUSTALW [24]. Based on this alignment, MODELLER v9.12 [25] was used to build a model of ETbR starting with the NTSR1 structure (PDB ID: 4BUO) [26]. ETbR belongs to the β-branched class A GPCRs, but there are currently only four experimentally solved structures in this group. Based on the overall sequence similarity of ETbR to
NTSR1 structures, all of which are bound to the agonist NTS1, it seemed to be a good candidate as a homology model template. Because the stability of the thermostabilized ETBr mutants was tested in the absence of any ligands, we preferred to use the NSTR1 structure (4BUO) adopting the inactive-like form at the cytoplasmic side regardless of agonist binding.

Acknowledgments

We greatly thank Ms. K. Hosino for her technical support, Mr. W. Shihoya for preparing insect cell membranes of ETBr point mutants, and Mr. K. Kikuta for figure preparation. We also thank Kowa Co., Ltd. for providing the K8794. This work was supported by JSPS KAKENHI Grant Numbers 15H05775, 26440024, and 26640102; the Japan New Energy and Industrial Technology Development Organization (NEDO); and the Japan Agency for Medical Research and Development (AMED).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2016.03.024.

Received 30 December 2015; Received in revised form 19 March 2016; Accepted 22 March 2016

Keywords:
G-protein-coupled receptor; thermostabilization; alanine scanning mutagenesis; homology model

†deceased after this project.

Abbreviations used:

\( T_m \), melting temperature; GPCR, G-protein-coupled receptor; ET-1, endothelin-1; ETBr, endothelin type B receptor; \( \beta_1 \), \( \beta_2 \) adrenergic receptor; A2AR, adenosine A2A receptor; NTSR1, neurotensin receptor 1; CRF1R, corticotropin-releasing factor type 1 receptor; DDM, n-dodecyl-\( \beta \)-D-maltopyranoside; DM, n-decyl-\( \beta \)-D-maltopyranoside; EGFP, enhanced green fluorescent protein; GTP\( \gamma \)S, guanosine 5'-O-(\( \gamma \)-thio)triphosphate.

References


