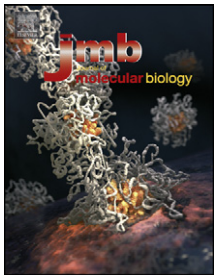


# Thermostabilization of the Human Endothelin Type B Receptor

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## 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 ($ET_BR$) have been unsuccessful due to its structural flexibility and instability in detergent-solubilized solution. To overcome these problems, we explored thermostabilization of human $ET_BR$ by establishing an $ET_BR$ 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 ($T_m$) of the five most thermostable mutants, R124Y, D154A, K270A, S342A, and I381A, were then examined. The apparent $T_m$ of three single mutants, R124Y, D154A, and K270A, was approximately 7 °C higher than that of the wild type. The apparent $T_m$ value of a combination of the five residues, named the Y5 $ET_BR$ mutant, was 17 °C higher than that of the wild type. The Y5 $ET_BR$ mutant exhibited an affinity for ET-1 and activated $G_q$ 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 $ET_BR$ is highly suitable for representing a ligand-free form of $ET_BR$ and is potentially applicable for studying an ET-1-bound form.



## 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, $ET_AR$ and $ET_BR$, 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 ($ET_BR$) 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 $ET_BR$ 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 $\beta_1$ adrenergic receptor ($\beta_1$AR) [11], adenosine $A_{2A}$ receptor ($A_{2A}$R) [12,13], neurotensin receptor (NTSR1) [14], corticotropin-releasing factor receptor type 1 (CRF1R) [15], and others [16]. The thermostable $\beta_1$AR-m23, developed by screening $\beta_1$AR individual alanine mutants with the antagonist dihydroalprenolol after heat treatment in a ligand-free form, exhibits unchanged affinities for antagonists, but lowered affinities for agonists compared to the wild type. In the thermostabilization of $A_{2A}$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 $ET_BR$ 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 $ET_BR$ with an apparent $T_m$ 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 $ET_BR$ mutants stabilized in a ligand-free form.

## Results and discussion

### Expression of $ET_BR$ in *E. coli* and identification of thermostabilizing mutations in ligand-free $ET_BR$

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 $6hNET_BR$ [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 $6hNET_BR$ 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 $K_D$ and $IC_{50}$ values for [$^{125}$I]ET-1 of $6hNET_BR$ expressed in *E. coli* were 122 ± 38 pM and 0.13 ± 0.01 nM, respectively, comparable to those of $6hNET_BR$ expressed in HEK293 ($K_D$: 106 ± 34 pM) or Sf9 cells ($IC_{50}$: 0.14 ± 0.01 nM; Fig. S1). The maximum number of binding sites calculated from saturation binding of $6hNET_BR$ expressed in *E. coli* was 1.5 ± 0.2 pmol/mg protein, whereas that of $6hNET_BR$ expressed in insect cells was 77 ± 9.3 pmol/mg protein [5]. We concluded that $6hNET_BR$ expressed in *E. coli* provides an affinity for ET-1 comparable to that of $6hNET_BR$ 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 $ET_BR$, 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, [$^{125}$I]ET-1 (Fig. 1a). The retained [$^{125}$I]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 [$^{125}$I]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% [$^{125}$I]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 $\beta_1$AR [11] and $A_{2A}$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 $\beta_2$AR, turkey $\beta_1$AR, and human $A_{2A}$R. The expression levels and thermostability indices of these mutants were evaluated as described above, and the results are shown in Fig. 2a. The $T_m$ 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