Many drugs that target G-protein-coupled receptors (GPCRs) induce or inhibit their signal transduction with different strengths, which affect their therapeutic properties. However, the mechanism underlying the differences in the signalling levels is still not clear, although several structures of GPCRs complexed with ligands determined by X-ray crystallography are available. Here we utilized NMR to monitor the signals from the methionine residue at position 82 in neutral antagonist- and partial agonist-bound states of β2-adrenergic receptor (β2AR), which are correlated with the conformational changes of the transmembrane regions upon activation. We show that this residue exists in a conformational equilibrium between the inverse agonist-bound states and the full agonist-bound state, and the population of the latter reflects the signal transduction level in each ligand-bound state. These findings provide insights into the multi-level signalling of β2AR and other GPCRs, including the basal activity, and the mechanism of signal transduction mediated by GPCRs.
**Figure 1** | Analyses of ligand-dependent conformational changes in β2AR with fluorescent probes. (a) Schematic representation of the fluorescent assay. A monobromobimane (mBBr) probe is introduced at C265 of the β2AR without the C265A mutation (orange). After purification, β2AR is in the formoterol-bound state (brown). By the addition of an excess amount of alprenolol (green), formoterol (magenta) is replaced with alprenolol, resulting in β2AR in the alprenolol-bound state (blue). The receptor conformation shift from an active conformation to an inactive conformation was observed as the increase in the fluorescence intensity of mBBr, due to the change in the local environment. (b) Fluorescence spectra of mBBr-labeled β2AR, in the presence of formoterol (cyan), and after the addition of an excess amount of alprenolol (orange). The fluorescence intensities are normalized to the maximal fluorescence intensity in the alprenolol-bound state.

**Results**

**Preparation and characterization of β2AR.** The E122W/N187E/C265A mutant of β2AR (Gly2-Gly365) with an N-terminal FLAG-tag and a C-terminal decahistidine-tag, which exhibited agonist-dependent signalling activities almost identical to the wild type in previous studies\(^{14,22}\), was expressed in a baculovirus–insect cell expression system. β2AR was solubilized by n-dodecyl-β-D-maltopyranoside (DDM), and purified by three chromatography steps, including ligand-affinity chromatography, to more than 95% purity, as judged from SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analyses. The purified β2AR is in the formoterol-bound state, because formoterol was added during the elution from the ligand-affinity chromatography. Radioligand-binding assays with an excess amount of \(^{3}H\)-dihydroalprenolol (DHA), which has a higher affinity for β2AR than formoterol\(^{23,24}\), revealed that more than 80% of the purified β2AR retained the DHA-binding activity. We also confirmed that the purified β2AR exhibited ligand-dependent conformational changes, by experiments using a fluorescent probe introduced at C265 (ref. 8) (Fig. 1a,b).

Methionine residues are frequently observed in TM3, TM5, and TM6 of GPCRs, and these regions exhibit large conformational changes upon activation (Supplementary Fig. S1). β2AR possesses nine methionine residues in extracellular loop 1 (ECL1), TM1, TM2, TM4, TM5 and TM6 (Fig. 2 and Table 1), and M8\(^{22-53}\) (Supplementary Figs S2 and S3), M215\(^{54}\) and M279\(^{41}\) assume distinctly different conformations between the inverse agonist-bound and the full agonist/G-protein-bound crystal structures. Therefore, we utilized the methionine methyl groups to investigate the conformation of the TM region of β2AR in various ligand-bound states. Methionine methyl-selective \(^{13}C\) labeling in the baculovirus–insect cell expression system was accomplished by adding [methyl-\(^{13}C\)] methionine to methionine-deficient medium. We confirmed that about 90% of the methionine methyl groups in thioreredoxin, prepared by the same procedure for β2AR, were labeled with \(^{13}C\) and that the other types of amino-acid residues were not significantly labeled. \(^{1}H\)-\(^{13}C\) heteronuclear multiple quantum coherence (HMOC) spectra of the [methyl-\(^{13}C\)-Met] β2AR in the formoterol-bound state were recorded, and those in the carazolol-bound state were recorded by adding an excess amount of carazolol.
For the assignments of the resonances from the methionine in the TM region, we introduced a further mutation into the methionine residue of interest (Supplementary Fig. S5). For example, M82\textsuperscript{2,5} was assigned by introducing the M82V mutation into the 4Met mutant (Supplementary Fig. S5d). We confirmed that M82V retains the native folding in both the carazolol- and formoterol-bound states, by experiments using a fluorescent probe introduced at C265\textsuperscript{2,27} (Fig. 1b and Supplementary Fig. S6). In addition, the M82V mutation does not affect the affinity of the antagonist \textsuperscript{\textsuperscript{3}H} DHA to β\textsubscript{2}AR (Supplementary Fig. S7). In the carazolol-bound state, two signals were absent in the spectrum of the M82V mutant, revealing that both of these resonances are from M82\textsuperscript{2,5} (Fig. 3c,e). Hereafter, we refer to the downfield and upfield resonances from M82\textsuperscript{2,5} as M82\textsuperscript{D} and M82\textsuperscript{U}, respectively. In the formoterol-bound state, one signal was absent in the spectrum of the M82V mutant, revealing that this resonance is from M82\textsuperscript{2,5} (Fig. 3d,f). The chemical shifts of the resonance from M82\textsuperscript{2,5} in the formoterol-bound state were different from M82\textsuperscript{D} and M82\textsuperscript{U} in the carazolol-bound state (Fig. 4a). Hereafter, this resonance from M82\textsuperscript{2,5} in the full agonist formoterol-bound state is referred to as M82\textsuperscript{A}.

The resonances from M215\textsuperscript{5,54} and M279\textsuperscript{6,41} in the carazolol-bound state were also assigned using the 4Met-based mutants (Supplementary Fig. S5e,f). The spectra of the mutants for M215\textsuperscript{5,54} and M279\textsuperscript{6,41} in the formoterol-bound state were not significantly different from those without these mutations, suggesting that the resonances from M215\textsuperscript{5,54} and M279\textsuperscript{6,41} were not observed in the formoterol-bound state (Fig. 4a).

M82 in the states bound with antagonist and partial agonists. To investigate the structures of the TM region in the neutral antagonist-bound and partial agonist-bound states, the HMBC spectra of the 4Met mutant labeled with methyl-\textsuperscript{13}C-Met were recorded in the state bound with the neutral antagonist, alprenolol, and in those bound with the partial agonists, tulobuterol and clenbuterol (Fig. 4b,c). Alprenolol does not alter the basal activity of β\textsubscript{2}AR\textsuperscript{16,19}. Clenbuterol reportedly has higher efficacy than tulobuterol\textsuperscript{24} (Supplementary Table S1). The tulobuterol- and clenbuterol-bound states were achieved by extensive washing with these ligands on cobalt-affinity resin after the ligand-affinity purification steps. The alprenolol-bound state was attained by adding an excess amount of alprenolol over clenbuterol. As a result, in the alprenolol-bound state, a major and a minor resonance that slightly shifted from M82 in the states bound with antagonist and partial agonists, the HMQC spectra of the 4Met mutant labeled with methyl-\textsuperscript{13}C-Met were recorded (Supplementary Table S1). For the assignments of the resonances from the methionine in the TM region, we introduced further mutations into the methionine residue of interest (Supplementary Fig. S5). For example, M82\textsuperscript{2,5} was assigned by introducing the M82V mutation into the 4Met mutant (Supplementary Fig. S5d). We confirmed that M82V retains the native folding in both the carazolol- and formoterol-bound states, by experiments using a fluorescent probe introduced at C265\textsuperscript{2,27} (Fig. 1b and Supplementary Fig. S6). In addition, the M82V mutation does not affect the affinity of the antagonist \textsuperscript{\textsuperscript{3}H} DHA to β\textsubscript{2}AR (Supplementary Fig. S7). In the carazolol-bound state, two signals were absent in the spectrum of the M82V mutant, revealing that both of these resonances are from M82\textsuperscript{2,5} (Fig. 3c,e). Hereafter, we refer to the downfield and upfield resonances from M82\textsuperscript{2,5} as M82\textsuperscript{D} and M82\textsuperscript{U}, respectively. In the formoterol-bound state, one signal was absent in the spectrum of the M82V mutant, revealing that this resonance is from M82\textsuperscript{2,5} (Fig. 3d,f). The chemical shifts of the resonance from M82\textsuperscript{2,5} in the formoterol-bound state were different from M82\textsuperscript{D} and M82\textsuperscript{U} in the carazolol-bound state (Fig. 4a). Hereafter, this resonance from M82\textsuperscript{2,5} in the full agonist formoterol-bound state is referred to as M82\textsuperscript{A}.

The resonances from M215\textsuperscript{5,54} and M279\textsuperscript{6,41} in the carazolol-bound state were also assigned using the 4Met-based mutants (Supplementary Fig. S5e,f). The spectra of the mutants for M215\textsuperscript{5,54} and M279\textsuperscript{6,41} in the formoterol-bound state were not significantly different from those without these mutations, suggesting that the resonances from M215\textsuperscript{5,54} and M279\textsuperscript{6,41} were not observed in the formoterol-bound state (Fig. 4a).

To examine whether the resonances from M82\textsuperscript{2,5} in the ligand-bound states undergo conformational exchange, we also recorded the spectra at a lower temperature, 283 K (Fig. 4d and Supplementary Fig. S8). As a result, the resonances from M82\textsuperscript{2,5} in the tulobuterol- and clenbuterol-bound states significantly shifted away from M82\textsuperscript{U}, and the M82\textsuperscript{A} resonance in the formoterol-bound state slightly shifted away from M82\textsuperscript{U} at 283 K (Fig. 4d and Supplementary Fig. S8a–c), whereas the resonances from the methyl groups of tulobuterol and clenbuterol did not shift (Supplementary Fig. S8d,e). In the spectra of β\textsubscript{2}AR with lower concentrations of clenbuterol and tulobuterol, the signals were observed at the same chemical shifts as those at higher ligand concentrations (Supplementary Fig. S9), suggesting that the NMR signals are not significantly affected by the exchange between the free and bound states or non-specific effect of the ligands, because β\textsubscript{2}AR is only in the ligand-bound state under the present solution conditions. This is consistent with the estimation of the bound population of β\textsubscript{2}AR by the ligand concentration of the NMR samples and the reported affinities of the ligands (Supplementary Table S1).
Table 1 | Summary of the differences in β2AR methionine resonances in the states with various efficacies.

<table>
<thead>
<tr>
<th>Position in the β2AR structure</th>
<th>M36</th>
<th>M40</th>
<th>M82</th>
<th>M96</th>
<th>M98</th>
<th>M156</th>
<th>M171</th>
<th>M215</th>
<th>M279</th>
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<tr>
<td>Structural element*</td>
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<td>TM1</td>
<td>TM2</td>
<td>TM2</td>
<td>ECL1</td>
<td>TM4</td>
<td>TM4</td>
<td>TM5</td>
<td>TM6</td>
</tr>
<tr>
<td>Extracellular half (E) or intracellular half (I)</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>E</td>
<td>I</td>
<td>E</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Large conformational change upon activation</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
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</table>

Number of observed resonances in each ligand-bound state

<table>
<thead>
<tr>
<th>Ligand-bound state</th>
<th>Carazolol</th>
<th>Alprenolol</th>
<th>Tulobuterol</th>
<th>Clenbuterol</th>
<th>Formoterol</th>
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<tr>
<td>Number of observed resonances</td>
<td>1</td>
<td>1</td>
<td>1</td>
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Efficacy-dependent changes

<table>
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<tr>
<th>In chemical shifts</th>
<th>No‡</th>
<th>—</th>
<th>Yes</th>
<th>No‡</th>
<th>—</th>
<th>No‡</th>
<th>No‡</th>
<th>No‡</th>
<th>Yes</th>
<th>Yes</th>
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<tr>
<td>In signal intensities†</td>
<td>No</td>
<td>—</td>
<td>Yes§</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

†| TM and ECL stand for transmembrane helix and extracellular loop, respectively.
‡| The signal intensities were normalized by dividing the observed intensities with the concentrations of β2AR, estimated from the SDS-PAGE, and the numbers of scans.
§| Normalized chemical shift differences were < 0.1 p.p.m.
◦| In a complex manner, depending on both the populations and the exchange rates between the m82H chemical shift (p.p.m.)

Figure 3 | 1H-13C SOFAST-HMQC spectra of [methyl-13C-Met] β2AR and their assignments. (a, b) 1H-13C SOFAST-HMQC spectra of [methyl-13C-Met] β2AR in the carazolol-bound state (a) and in the formoterol-bound state (b). (c, d) 1H-13C SOFAST-HMQC spectra of the [methyl-13C-Met] β2AR 4Met mutant in the carazolol-bound state (c) and in the formoterol-bound state (d). (e, f) 1H-13C SOFAST-HMQC spectra of the [methyl-13C-Met] β2AR 4Met/M82V mutant in the carazolol-bound state (e) and in the formoterol-bound state (f). The regions with methionine chemical shifts are shown, and the assigned resonances are indicated. The resonances from M40 in the carazolol-bound state, and M40, M215 and M279 in the formoterol-bound state were not observed. Resonances indicated with single asterisk are derived from minor impurity proteins from insect cell membranes, but not from β2AR. Double asterisks are residues from m40 in the carazolol-bound state, and m40, m215 and m279 in the formoterol-bound state.

Signals from the residues except for M82. The resonances from M215, which is in the intracellular side of TM5, in the states with carazolol, alprenolol, tulobuterol, clenbuterol and formoterol are shown in Fig. 5a,b. M215 exhibited single resonance in the carazolol-, alprenolol-, tulobuterol- and clenbuterol-bound states (Fig. 5a,b). The chemical shifts in the alprenolol- and tulobuterol-bound states were between those in the carazolol- and clenbuterol-bound states, and the chemical shifts in the alprenolol-bound state were closer to those in the carazolol-bound state. The intensities were in the following order: carazolol-bound state > alprenolol-bound state > tulobuterol-bound state > clenbuterol-bound state and the resonances from M215 were not observed in the formoterol-bound state (Table 1). As the temperature was lowered from 298 K to 283 K, the M215 resonances in the carazolol- and alprenolol-bound states shifted towards that in the carazolol-bound state (Supplementary Fig. S10). The chemical shifts and intensities of the resonances from M279, which is in the intracellular side of TM6, also exhibited similar bound ligand-dependent changes (Fig. 5c).

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Figure 4 | The difference in $\beta_2$AR M82$^{2,53}$ resonances in the states with various efficacies. (a) Overlay of the $^1$H-$^1$C SOFAST-HMQC spectra of [methyl-$^1$C-Met]$\beta_2$AR/4Met at 298 K in the carazolol-bound state (black) and the formoterol-bound state (red). (b) $^1$H-$^1$C SOFAST-HMQC spectra of [C(235)H$_2$, methyl-$^1$C-Met]$\beta_2$AR/4Met at 298 K in the carazolol-bound (black), alprenolol-bound (green), clenbuterol-bound (red) and formoterol-bound (state) states. Only the regions with M82 resonances are shown. (c) Overlay of the spectra shown in panel b, with the same colors. The centers of the resonances from M82 are indicated with dots. (d) $^1$H-$^1$C SOFAST-HMQC spectra of [C(235)H$_2$, methyl-$^1$C-Met]$\beta_2$AR/4Met at 283 K, with the same colors as in panel b. In panels a–d, the assigned resonances are indicated with the names of the bound ligands in parentheses (a,c). M82 resonances are indicated with the superscripts used in the main text. Resonances indicated with single asterisk are derived from impurities. Double asterisks are $t_1$ noises derived from the intense DDM signal with an $^1$H chemical shift of 1.6-1.7 p.p.m.

The HMQC spectra of the $\beta_2$AR without the 4Met mutation were also recorded in the state bound with alprenolol, tulobuterol and clenbuterol, as well as carazolol and formoterol (Supplementary Fig. S1). As a result, the chemical shift changes observed for the $\beta_2$AR without the 4Met mutation were almost identical to those for the 4Met mutant, although the M82 resonances partially overlapped with the resonances from M96 or M98. Therefore, we can rule out the effect of the 4Met mutation on the efficacy in each ligand-bound state.

The differences in the other methionine resonances in each ligand-bound state are summarized in Fig. 6 and Table 1. The resonances from M36, M96/M98, M156 and M171 exhibited only small chemical shift differences in each ligand-bound state (Fig. 6 and Table 1).

**Discussion**

Different spectrum patterns from the M82$^{2,53}$ methyl group are observed, depending on the efficacy of the bound ligand: the resonances of M82$^U$ and M82$^D$ in the inverse agonist-bound state, two resonances with slightly different chemical shifts from M82$^U$ and M82$^D$ in the neutral antagonist-bound state, one resonance with a chemical shift between M82$^U$ and M82$^A$ in the partial agonist-bound state and the resonance of M82$^A$ in the full agonist-bound state (Fig. 4b–d).

Based on the comparison between the $\beta_2$AR crystal structures in the forms bound with an inverse agonist and with both a full agonist and a G-protein, the following structural mechanism for GPCR activation was proposed: full agonists induce a conformational change of S207$^{-5,46}$ and a subsequent rearrangement of the interactions between the TM helices in the middle of the TM region; the inward shifts of TM5 and TM7 at P211$^{5,45}$ and N318, respectively, and the axial shifts of TM3 and TM6 at I121$^{3,40}$ and F282$^{26,44}$, respectively, resulting in a large outward movement of the cytoplasmic half of TM6 (Supplementary Fig. S2). The M82$^{2,53}$ side chain also exhibits a dramatic conformational change upon activation, and this conformational change strongly correlates with the rearrangement
Figure 5 | The difference in $\beta_2$AR M215$^{5,54}$ or M279$^{6,41}$ resonances in the states with various efficacies. (a) $^1$H,$^13$C SOFAST-HMQC spectra of $[\alpha,\beta,\beta,^2$H$_3$, methyl,$^13$C-Met]$\beta_2$AR/4Met at 298 K in the carazolol-bound (black), alprenolol-bound (cyan), tubulobuterol-bound (green), clenbuterol-bound (blue) and formoterol-bound (red) states. Only regions with M36 and M215 resonances are shown. (b) Overlay of the spectra shown in panel a, except for the formoterol-bound state, with the same colors. In panel b, only the regions with M215 resonances are shown. The centers of the resonances from M215 are indicated with dots. In panel c, only the regions with M215 resonances are shown. The centers of the resonances from M279 are indicated with dots. The line shapes of the M279 resonances are distorted due to the overlaps with the $t_1$ noises derived from the intense DDM signal with an $^1$H chemical shift of 1.6–1.7 p.p.m.

Figure 6 | Normalized chemical shift differences of the methionine methyl resonances between the carazolol- and formoterol-bound states. Normalized chemical shift differences, $\Delta \delta$, were calculated by the equation $\Delta \delta = (\Delta \delta_{1H}^2 + \Delta \delta_{13C}^2)/\sqrt{3}$. The normalization factor (3.5) is the ratio of the s.d. of the methionine methyl $^1$H and $^13$C chemical shifts, deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/). The error values were calculated by the formula $\Delta \delta = (\Delta \delta_{1H} + \Delta \delta_{13C})/\sqrt{3}$, where $\Delta \delta_{1H}$ and $\Delta \delta_{13C}$ are the digital resolutions in p.p.m. in the $^1$H and $^13$C dimensions, respectively. The number of replicates is greater than two. For MB2, both of the $\Delta \delta$s between M82U and M82A (M82U/A), and M82D and M82A (M82D/A) were calculated. For M215 and M279, we could not calculate the $\Delta \delta$s because the resonances were not observed in the formoterol-bound state, although $\Delta \delta$s between the carazolol- and clenbuterol-bound states, which should be smaller than those between the carazolol- and formoterol-bound states, were $>0.1$ p.p.m.

of the interactions between 1121$^{3,40}$ and P282$^{6,44}$ (Supplementary Fig. S3), via the inward shift of TM7 at Y316$^{43}$ and S319$^{7,46}$ and the axial shift of TM3 at C116$^{3,35}$ (Supplementary Fig. S2). Therefore, the side-chain conformation of M82$^{2,53}$ would be sensitive to the activation state of $\beta_2$AR. On the other hand, M82$^{2,53}$ is 5–8 Å away from the conserved $\beta$-OH groups of the $\beta_2$AR ligands, and is not directly involved in the ligand binding. Therefore, the resonances from M82$^{2,53}$ would be less affected by the direct interactions with ligands. This is supported by further observation that the affinity of the antagonist [3H] DHA for the 4Met/M82V mutant was almost identical to that for the 4Met mutant (Supplementary Fig. S7).

The $^13$C and $^1$H chemical shifts of the methionine methyl signals are reportedly affected by its side-chain conformation and the local environments, including the ring current effects from the neighbouring aromatic rings, respectively$^{25–27}$. Based upon the crystal structures of $\beta_2$AR, we propose that the M82U and M82D signals correspond to the inactive states that cannot directly activate G-proteins, and the M82A signal corresponds to the active state that can interact with G-proteins (Supplementary Discussion). Therefore, the differences between the conformations corresponding to M82U and M82D are found in a limited region close to the ligand-binding site. In contrast, the differences between the conformations corresponding to M82U/D and M82A are found in the TM region. Hereafter, we refer to these conformations corresponding to M82U, M82D and M82A as the M82U conformation, the M82D conformation and the M82A conformation, respectively.

Previous comprehensive analyses of efficacies in various ligands revealed that the efficacies increase in the following order$^{24}$: an inverse agonist, carazolol; a neutral antagonist, oxprenolol, which is an analogue of alprenolol; a weak partial agonist tubulobuterol; a partial agonist, clenbuterol; and a full agonist, formoterol (Supplementary Table S1). In the neutral agonist-bound and partial agonist-bound states, the M82$^{2,53}$ resonances exhibited chemical shifts between M82U and M82A in an efficacy-dependent manner (Fig. 4c). The resonances from M82$^{2,53}$ in the partial agonist-bound states shifted towards M82A as the temperature was lowered from 298 K to 283 K (Supplementary Fig. S8a,b). The temperature-dependent shifts, together with the chemical shifts in
an efficacy-dependent manner, suggest that \(\beta_2\text{AR} \) exists in equilibrium between the M82\(^{1}\) and M82\(^{3}\) conformations in the partial agonist-bound states, with faster exchange rates than the chemical shift difference. The observation of two resonances from M82\(^{2,3}\) in the carazolol- and alprenolol-bound states suggests that \(\beta_2\text{AR} \) exists in equilibrium between the M82\(^{2}\) and M82\(^{3}\) conformations in the inverse agonist- and neutral antagonist-bound states, with slower exchange rates than the chemical shift difference. The two resonances from M82\(^{2,3}\) in the alprenolol-bound state were closer to M82\(^{3}\) than those in the carazolol-bound state, suggesting that the minor population of the M82\(^{3}\) conformation, in equilibrium with the M82\(^{3}\) conformation, would also exist in this state, as supported by the simulation of M82\(^{2,3}\) resonances (Supplementary Discussion). The incomplete linearity of the efficacy-dependent chemical shift change might be derived from another equilibrium within the M82\(^{3}\) conformation.

Similar phenomena were also observed for the M215 and M279 resonances. The M215 and M279 resonances exhibited the chemical shifts and intensities in an efficacy-dependent manner (Fig. 5 and Table 1), and these resonances shifted towards those in the carazolol-bound state at lower temperature (Supplementary Fig. S10a and b). The simulations of these resonances, with the same parameter as that of the M82 resonances, were in good agreement with the observed spectra (Supplementary Discussion). Therefore, the efficacy-dependent conformational equilibrium between the M82\(^{2}\) and M82\(^{3}\) conformations observed for M82\(^{2,3}\) accompanies large conformational changes on TM5 and TM6.

Based on the above structural interpretation of the resonances from M82, we propose the following signal regulation mechanism. In the full agonist formoterol-bound state, most of the \(\beta_2\text{AR} \) molecules assume the active conformation (Fig. 7a). In the partial agonist tulobuterol- and clenbuterol-bound states, \(\beta_2\text{AR} \) exists in an equilibrium between the inactive and active conformations (Fig. 7b), and the populations of the two conformations determine the efficacies. This is in good agreement with the significant, but reduced, efficacy for tulobuterol and clenbuterol\(^{24}\). In the neutral antagonist alprenolol-bound state, \(\beta_2\text{AR} \) exists in equilibrium between two major inactive conformations, which are different only in the region close to the ligand-binding site, and one minor active conformation (Fig. 7c). The weak basal activity is due to the existence of the minor M82\(^{3}\) conformation. In the inverse agonist-bound state, \(\beta_2\text{AR} \) exists in equilibrium between the two locally different inactive conformations (Fig. 7d). Considering that the basal activity partly remained even in the carazolol-bound state\(^{16,24}\), there may be a minor population of the M82\(^3\) conformation in this state, in an equilibrium with one of the inactive conformations.

Two mechanisms have been proposed for the partial efficacies in the partial agonist-bound states: the equilibrium between the active and inactive states, and the distinct conformation of the partial agonist-bound state from those of both the active and inactive states\(^3\), although there have been no experimental evidence for them. The chemical shifts of the M82\(^{2,3}\) resonances in the efficacy-dependent and the temperature-dependent manner in the partial agonist-bound states suggest the equilibrium between the active and inactive states.

The M82\(^D\) resonance was observed only in the inverse agonist- and neutral antagonist-bound states, and the intensity of the M82\(^D\) resonance is higher in the inverse agonist-bound state than in the neutral antagonist state! In addition, the \(^{13}\)C chemical shifts of M82\(^U\) and M82\(^D\) suggest that the M82\(^U\) conformation flexibly adopts both the \textit{trans} and \textit{gauche} conformations, whereas the M82\(^D\) conformation mainly adopts the \textit{trans} conformation (Supplementary Discussion); therefore, the M82\(^D\) conformation may be more rigid than the M82\(^U\) conformation. It is thus tempting to speculate that the M82\(^D\) conformation is more refractory to shifting to the activated state than the M82\(^U\) conformation, and the higher population of the M82\(^D\) conformation in the inverse agonist-bound state causes the inhibition of the basal activity.

The resonances from M215\(^{5,54}\) and M279\(^{6,41}\), which are located on the cytoplasmic side of the TM region, were not observed in the formoterol-bound state, suggesting that these signals were broadened due to the conformational exchange between the multiple
conformations in the formoterol-bound state. The multiple conformations on the cytoplasmic side of the TM region in the full agonist-bound state may be effective for interacting with various effectors in the conformational selection manners\(^\text{28,32}\). A number of \(\beta_2\)AR ligands have been shown to impart differing degrees of signalling in G-protein and arrestin pathways, a phenomenon called ‘functional selectivity’ or ‘biased signalling’\(^\text{20,28–32}\). The biased signalling is important for both understanding the functions of GPCRs and drug development, and it is quite interesting to investigate the conformations of \(\beta_2\)AR in the biased ligand-bound states. Although biased signalling was not reported for the ligands chosen in this study\(^\text{30}\), the biased signalling may be due to the diversity of the conformational dynamics in the cytoplasmic side of the TM region in each ligand-bound state.

The conformational differences between the crystal structures of \(\beta_2\)AR were also basically observed in those of adenosine \(A_2\)A receptor and rhodopsin\(^\text{33–35}\). In addition, methionine residues are highly abundant in TM3, TM5 and TM6, as described above (Supplementary Fig. S1), and can be observed without any chemical modification. Therefore, the methionine \(^1\)H–\(^13\)C resonances, including Met\(^\text{2}\), are broadly applicable for the analyses of the bound ligand efficacy-dependent conformational differences in various GPCRs.

**Methods**

**Reagents and buffers.** All reagents were from Nacalai Tesque, unless otherwise noted. The (\(\alpha\)\(\beta\)\(\beta\)\(\beta\))\(^{-}\)His\(^\text{1}\)–\(^13\)C) methionine was prepared by the dehydrated mixture of [\(^\text{13}\)C\(^\text{1}\)C\(^\text{1}\)] methionine (ISOTEC or Cambridge Isotope Laboratories) with Esherichia coli cystathionine-\(\gamma\)-synthase, as previously described\(^\text{36}\). Alprenolol-cysteine (AlpC) was synthesized as previously described\(^\text{37}\). Affi-gel/AlpC was prepared by coupling 5 mg ml\(^{-1}\) AlpC solution with Affi-gel10 (Bio-Rad) in a dimethyl sulfoxide (DMSO, Dojindo) solution, according to the manufacturer’s instructions. Carazolol (Wako Chemicals) and formoterol (Sigma) were solubilized at 100 mM in DMSO-d\(_4\), (ISOTEC), and diluted with buffers. Tulobuterol (Wako Chemicals) was solubilized at 400 mM in methanol-d\(_4\), (ISOTEC), and diluted with buffers. Alprenolol (Sigma) and clenbuterol (Wako Chemicals) were solubilized at 100 mM in D\(_2\)O (Cambridge Isotope Laboratories), and diluted with buffers.

**Generation of \(\beta_2\)AR recombinant baculovirus.** The complementary DNA fragment encoding human \(\beta_2\)AR (Gly2-Gly365), with an N-terminal FLAG-tag (DYKDDDA) and a C-terminal decahistidine-tag was amplified by PCR and cloned into the pFastBac1 vector (Invitrogen) via the restriction fragment encoding human \(\beta_2\)AR in the pFastBac1 vector (Novagen),Mutations were introduced by either a QuikChange site-directed mutagenesis kit or a QuikChange multi site-directed mutagenesis kit (Strategene), E122W, N167E and C265A mutations were introduced in all constructs, unless otherwise stated.

**SD cells (Invitrogen) were routinely maintained at 27 °C in Grace’s supplemented medium (GIBCO) containing 10% fetal bovine serum (Biowest), 0.1% Phorous F-68 (GIBCO), 50 international units per ml penicillin, 50 \(\mu\)g ml\(^{-1}\) streptomycin and 0.125 \(\mu\)g ml\(^{-1}\) amphotericin B. Recombinant baculoviruses were generated and amplified with the Bac-to-Bac system (Invitrogen), according to the manufacturer’s instructions.

**Expression and purification of \(\beta_2\)AR.** The expressSF + cells (SF + cells, Protein Sciences) were routinely maintained at 27 °C in 100 ml SF-900 II serum-free medium (GIBCO), with 50 international units per ml penicillin, 50 \(\mu\)g ml\(^{-1}\) streptomycin and 0.125 \(\mu\)g ml\(^{-1}\) amphotericin B, in a 250 ml Erlenmeyer flask (Corning) on an orbital shaker (130 rpm). For the expression of [\(^\text{13}\)C\(^\text{1}\)C\(^\text{1}\)] Met\(^\text{397–406}\) (2007). The ligand exchange from formoterol to clenbuterol was accomplished by further washing of the resin for about 3 h, with 50 ml of buffer B, supplemented with 0.1% DDM and 1 mM ligands (clenbuterol or tulobuterol). The protein was eluted with 10 ml of buffer B, supplemented with 0.08% DDM, 200 mM imidazole and the ligands (100 mM formoterol, 1 mM clenbuterol or 1 mM tulobuterol).

**The eluate from the second TALON affinity step was concentrated using a centrifugal filter device (AmiconUltra 4, 30 KDa molecular weight cutoff, Millipore), which was successively exchanged to buffer C (20 mM sodium phosphate, pH 7.1, H\(_2\)O/D\(_2\)O = 1/99), supplemented with the ligands (100 mM formoterol, 1 mM clenbuterol or 1 mM tulobuterol). In total, 50–400 \(\mu\)g of \(\beta_2\)AR and its mutants were obtained per 0.81 insect cell culture.

**NMR experiments.** All of the spectra were recorded with a Bruker Avance 800 spectrometer equipped with a cryogen probe, and were processed by Topspin 2.1 (Bruker).

The assignment of the methionine \(^1\)H–\(^13\)C resonances of \(\beta_2\)AR was accomplished by the following procedure. \(^1\)H–\(^13\)C SOFAST-HMQC spectra were recorded for 298 K. For all the NMR experiments, carazolol was added to a final concentration of 500 \(\mu\)M, and \(^1\)H–\(^13\)C SOFAST-HMQC spectra and \(^1\)H–\(^13\)C HMQC spectra with echo/anti-echo gradient coherence selections were recorded for 5–40 \(\mu\)M [\(^\text{13}\)C\(^\text{1}\)C\(^\text{1}\)] Met\(^\text{397–406}\) (2007). The ligand exchange from formoterol to clenbuterol or tulobuterol was accomplished by further washing of the resin for about 3 h, with 50 ml of buffer B, supplemented with 0.1% DDM and 1 mM ligands (clenbuterol or tulobuterol). The protein was eluted with 10 ml of buffer B, supplemented with 0.08% DDM, 200 mM imidazole and the ligands (100 mM formoterol, 1 mM clenbuterol or 1 mM tulobuterol).

**The assignment of the methionine \(^1\)H–\(^13\)C resonances of \(\beta_2\)AR was accomplished by the following procedure. \(^1\)H–\(^13\)C SOFAST-HMQC spectra were recorded for 298 K. Our assignment of the methionine \(^1\)H–\(^13\)C resonances in the carazolol-bound states. \(^1\)H–\(^13\)C HMQC spectra with echo/anti-echo gradient coherence selections were recorded for 298 K, for the assignment of the methionine \(^1\)H–\(^13\)C resonances from the \(\gamma\)7 noise derived from the intense DDM signals in the \(^1\)H–\(^13\)C SOFAST-HMQC spectra.

**The structures of \(\beta_2\)AR in the various ligand-bound states were investigated by the following procedure. \(^1\)H–\(^13\)C SOFAST-HMQC spectra were recorded for 298 K [\(^\text{13}\)C\(^\text{1}\)C\(^\text{1}\)] Met\(^\text{397–406}\) (2007). The ligand exchange from formoterol to clenbuterol and clenbuterol-bound states, carazolol and alprenolol were added to final concentrations of 500 \(\mu\)M and 1 mM, respectively, and \(^1\)H–\(^13\)C SOFAST-HMQC spectra were recorded at 283 and 298 K.

**\(^1\)H–\(^13\)C SOFAST-HMQC spectra were recorded by excitation with a 4 ms PC9 120 degree pulse\(^\text{38}\) and the inversion of a 2 ms Q3 180 degree pulse\(^\text{39}\). For all of the spectra, the spectral widths were set to 12,800 Hz and 4,800 Hz for the \(^1\)H and \(^13\)C dimensions, respectively, and the inter-scan delays were set to 1 s. For the spectra recorded at 298 K in Supplementary Figs 5S–h, 1,024×128 complex points were acquired, with 256 scans/peak and a gradient rise time of 10 h for each spectrum. For the other spectra recorded at 298 K, 1,024×256 complex points were recorded, and 128 scans/FID gave rise to an acquisition time of 10 h for each spectrum. For the spectra recorded at 283 K, 1,024×192 complex points were recorded and 256 scans/FID gave rise to an acquisition time of 15 h for each spectrum. All of the spectra were referenced with 3-(trimethylsilyl)-1-propanesulfonylic acid sodium salt in both \(^1\)H and \(^13\)C dimensions.

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Author contributions
Y.K. designed the experiments, constructed the mutants for the resonance assignments, expressed and purified the labeled β2AR, collected and processed the NMR data, performed the fluorescent and radioligand binding assays, and wrote the manuscript. T.U. designed the experiments, processed the NMR data, and wrote the manuscript. K.K. investigated the 13C incorporation rates in thioredoxin. M.M. and H.T. constructed the plasmid, produced deuterated amino acids. M.R. and Y.S. expressed and purified the non-labeled β2AR. A.K.K. investigated the 13C incorporation rates in thioredoxin. M.M. and H.T. constructed the plasmid, produced recombinant baculoviruses, and expressed the non-labeled β2AR. I.S. supervised the overall project, designed the experiments, processed the NMR data, and wrote the manuscript.

Additional information
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