# SUPPLEMENTARY INFORMATION

**Multiple Ligand-Specific Conformations of the $\beta_2$-Adrenergic Receptor**

Alem W. Kahsai[1,3,4], Kunhong Xiao[1,4,5], Sudarshan Rajagopal[1], Seungkirl Ahn[1], Arun K. Shukla[1,3], Jinpeng Sun[1], Terrence G. Oas[2], and Robert J. Lefkowitz[1,2,3,5]

Departments of [1]Medicine and [2]Biochemistry, and the [3]Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina, 27710, USA

[4] Contributed equally to this work

[5] To whom correspondence should be addressed to R.J.L. (lefko001@receptor-biol.duke.edu) or K.X. (khxiao@receptor-biol.duke.edu)

Table of contents





Nature Chemical Biology: doi:10.1038/nchembio.634

## Supplementary Methods

### Reagents

Isoproterenol, salmeterol, salbutamol, propranolol, pindolol, carazolol, ICI 118,551, carvedilol, *N*-ethylmaleimide-$H_5$ (NEM-$H_5$), succinic anhydride-$H_4$ (SA-$H_4$) and α-chymotrypsin were purchased from Sigma-Aldrich (St Louis, MO). THRX-144877 was a generous gift from Theravance, Inc. (South San Francisco, CA). Sequencing grade, modified trypsin was supplied from Promega (Madison, WI). *N*-ethylmaleimide-$D_5$ (NEM-$D_5$, 99% atomic D) and succinic anhydride-$D_4$ (SA-$D_4$, 99% atomic D) were obtained from Cambridge Isotopes Laboratories (Andover, MA). All other reagents were of analytical grade obtained from various suppliers and used without further purification unless indicated otherwise.

### Expression and purification of the human $\beta_2$-adrenergic receptor

Expression and purification of the human $\beta_2$AR has been described elsewhere[1]. Briefly, recombinant baculovirus was generated by co-transfection of Sf9 cells with linearized BaculoGold DNA (PharMingen, San Diego, CA) and the pVL1392 (Invitrogen, Carlsbad, CA) transfer vector containing N-terminus cleavable influenza-hemagglutinin signal sequence followed by FLAG epitope tag and C-terminus hexa-histidine (6xHis) tag of human $\beta_2$AR cDNA[2,3]. Four to five days after infection, culture supernatants were collected and amplified once before plaque purification. The plaque-purified viruses were amplified several times to generate a high-titer virus stocks (about $1 \times 10^9$ plaque-forming units per ml of culture). Purification of the $\beta_2$AR was carried out using Sf9 cells, in 1L SF 900 II medium (Life Technologies, Inc.) supplemented with 5% (v/v) fetal calf serum (Gemini Bio-products, Calabasas, CA) and 0.1mg/ml gentamicin (Roche Molecular Biochemicals, Mannheim, Germany). Cells were infected with high viral titer (1:40 dilution, cell density of 3.5–5.5 × $10^6$cells/ml) virus for 72 hr at 27 °C, then harvested (10 min at 5,000 × g) and pellets were lysed in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml benzamidine, 10 µg/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride. Following centrifugation (30 min at 30,000 ×g), the lysed cells were resuspended in solubilization buffer (20 mM Tris, pH 7.4, with 0.35% n-dodecyl-β-D-maltoside (DDM; Calbiochem, San Diego, CA), 1 mM EDTA, 100



Nature Chemical Biology: doi:10.1038/nchembio.634

mM NaCl, 10 µg/ml benzamidine, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and 10 µg/ml soybean trypsin inhibitor, dounce homogenized (20 strokes) and then stirred for 1 h at 4 °C. The solubilized receptor was purified by a three-step affinity chromatography, using alprenolol (Sigma, St Louis, MO), Ni-NTA (Invitrogen, Carlsbad, CA), and M1 anti-FLAG columns as described[1,3]. Purified receptor was dialyzed with Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) twice against 2 L volume of 50 mM potassium phosphate buffer (pH 7.5), containing 50 mM NaCl and 0.02% (m/v) DDM at 4°C. The amount and purity of the protein were analyzed by using Bio-Rad protein assay reagent and Coomassie Blue-staining after SDS-PAGE. Small aliquots of purified receptor were then deglycosylated with PNGase F (NEB, Ipswich, MA), flash-frozen in liquid nitrogen, and stored at –80 °C. Approximately 15 nmol of pure protein generally could be obtained from a 1 L culture. The specific activity for the purified receptor was ~10 nmol/ml protein as determined by radio-ligand binding assay.

**Differential labeling reactions for identification of cysteine and lysine-containing peptides**

Differential labeling reactions of cysteines and lysines of the $\beta_2$AR were done under identical conditions. Purified $\beta_2$AR (2.5 µM) in 50 mM potassium phosphate buffer (pH 7.5), containing 50 mM NaCl and 0.02% (m/v) DDM, was incubated with carrier solvent or indicated ligand (50 µM) for 30 min at 25 °C in a 110 µl total reaction volume.

The kinetics of reaction of *N*-ethylmaleimide (NEM) with $\beta_2$AR cysteine residues were measured by mixing a reference reaction mixture made with *N*-ethylmaleimide-$H_5$ (NEM-$H_5$) with time point samples taken from a reaction made with *N*-ethylmaleimide-$D_5$ (NEM-$D_5$). The reference reaction mixture (110 µl) was made by adding NEM-$H_5$ to a final concentration of 2 mM in the $\beta_2$AR solution described in the previous paragraph (**Supplementary Fig. 4a**). This reference reaction was incubated for 60 min at 25 °C. 10 µl aliquots were prepared from this mixture and added to a series of pre-chilled 0.6-ml tubes containing 1 µl of dithiothreitol (DTT, 20 mM final concentration from 50× stock



Nature Chemical Biology: doi:10.1038/nchembio.634

in $NH_4HCO_3$) to quench the reaction and were then flash frozen in liquid nitrogen. Similarly, to the second sample tube containing $\beta_2$AR–ligand complex, NEM-$D_5$ was added at a concentration of 2 mM, but at specified time points 10-µl aliquots were withdrawn from the reaction mixture and quenched as described for the reference reaction (**Supplementary Fig. 4b**). Equal aliquots from both the NEM-$H_5$ reference reaction and the NEM-$D_5$ time point samples were mixed to produce a sample with various ratios of protiated and deuterated NEM-derivatized cysteines (**Supplementary Fig. 4a,b**). For experiments involving differential labeling of lysines of $\beta_2$AR on ligand binding with light and heavy succinic anhydride (SA-$H_4$ and SA-$D_4$), reactions were set up very similarly with the exception that a final concentration of 100 mM free L-lysine in 200 mM Tris-Cl (pH 8.0) was used instead of DTT for quenching. Inverse stable-isotope labeling experiments of the $\beta_2$AR sites in the receptor alone sample were also performed by using the deuterated-reagent (NEM or SA) labeled receptor as a reference instead of the protiated-reagent (NEM or SA) labeled receptor.

**Fraction of sites labeled in the different ligand-bound $\beta_2$AR**

To quantitate labeling of sites in the different ligand-bound $\beta_2$AR complexes, percent intensity ratios (%R) for each site/ligand pair were computed by dividing the signal intensity of the monoisotopic peak of the deuterated reagent (NEM-$D_5$ or SA-$D_4$)-labeled peptide from different time points by the intensity of the monoisotopic peak of the protiated reagent (NEM-$H_5$ or SA-$H_4$)-labeled sample, which was allowed to react for 60 minutes. This would result in the time course for all residue/ligand pair reaching 100% intensity ratio at 60 minutes (**Supplementary Fig. 4a,b,c,d**). Kinetic analysis of such data (*i.e.,* %R vs. t) indicated that however, the labeling reaction itself is not complete for all residue/ligand pairs in the arbitrarily chosen longest time point of the experiment, 60 minutes. To account for this difference in our analysis, we determined a correction factor (reactivity ratio, $R_r$) for every residue/ligand pair by comparing the extent of labeling between the native $\beta_2$AR with different ligands, at 60 minutes, to that of proteolyzed-$\beta_2$AR sample, labeled overnight. The fraction of each site labeled after 60 min for each ligand were therefore performed by the following experiment: Two separate labeling reaction pools were prepared, a native ligand-bound $\beta_2$AR (2.5 µM)



Nature Chemical Biology: doi:10.1038/nchembio.634

preparation reacted with a deuterated (NEM-$D_5$ or SA-$D_4$) version of the labeling derivatives and proteolyzed $\beta_2$AR (2.5 μM) reacted with protiated (NEM-$H_5$ or SA-$H_4$) version of the reagents. In the native preparation, $\beta_2$AR in the different ligand-bound complexes were reacted in with 2 mM NEM-$D_5$ or SA-$D_4$ for one hour at 25°C (reactions were terminated either by adding DTT or free L-lysine as described above). For the proteolyzed $\beta_2$AR preparation, exactly equal amount of aliquot of $\beta_2$AR was treated with either chymotrypsin or trypsin (37°C, 10 h) and then modified with NEM-$H_5$ or SA-$H_4$ (5 mM, 25°C, 14 h). Equal sized aliquots of the native (heavy) and proteolyzed (light) samples were mixed, subjected to proteolytic digestion and analyzed by MS. The ratios of the heavy (deuterated) and light (protiated) reagent-modified peptides for each site/ligand pair were calculated by measuring the relative signal intensities of the monoisotopic peaks to yield a reactivity ratio ($R_r$). These results were used to compute the percent of sites labeled (%F) as a function of time via the following equation:

$$\%F(t) = \%R(t) * R_r \quad (1)$$

where %F(t) is the percent of site labeled, %R(t) is the ratio of the heavy:light signal intensity for time point t sample, prepared as described above and $R_r$ is the heavy:light reactivity ratio from the reactivity experiment described above.

**Linearity of intensity ratio measurements**

Accuracy of peptide ratio measurements were tested by comparing between measured peptide intensity ratios and expected peptide intensity ratios for labeled $\beta_2$AR by MALDI-TOF-MS. Briefly, two separate stable isotope-labeling reaction pools of $\beta_2$AR (2.5 μM) were prepared, one with NEM-$H_5$ (2 mM) and the other with NEM-$D_5$ (2 mM) each incubated for 1 hr at room temperature, and quenched as described above. Eight serial two-fold concentration dilutions or mole factions (1:1 to 1:128) of the deuterated NEM-labeled $\beta_2$AR were prepared in volumes of 10 μl and titrated against a fixed quantity of the protiated NEM-labeled $\beta_2$AR (2.5 μM; 10 μl). Protiated and deuterated NEM labeled-$\beta_2$AR samples, mixed at different ratios, were then subjected to proteolytic digestion and MS analysis. The same stable-isotope labeling experiment of the receptor was also performed in an inverse experimental paradigm.



Nature Chemical Biology: doi:10.1038/nchembio.634

**In-solution digestion of $\beta_2$AR complexes**

Proteolytic digestion of the differentially labeled $\beta_2$AR samples were achieved using precipitation with methanol/cholorform followed by re-solubilization of the protein pellet using urea and in-solution digestion with appropriate enzyme. Briefly, each of the labeled protein solution (20 µl total volume from light and heavy reaction mixtures) from the different time points were diluted immediately with four volumes of methanol (1:4; v/v) and mixed briefly. To each tube, 25 µl $CHCl_3$ was added and mixed. Phase separation was accomplished by adding water (3 × volumes) followed by vigorous mixing, and centrifugation (12000 × g, 2 min). The upper aqueous phase was carefully aspirated and then to the organic phase, methanol (4 × volumes) was added to recover the sample as precipitate after centrifugation (12000 × g, 4 min), removal of the supernatant and drying in a speed vacuum (Savant Instruments Inc., Farmingdale, NY). The resulting precipitates were re-solubilized in a buffer (50 mM Tris-HCl (pH 8.0), 4 M urea, 5 mM EDTA, and 0.001% DDM), reduced with 2 mM DTT (final concentration) at 37 °C for 30 min, and alkylated with 20 mM iodoacetamide (IAA) at room temperature for 40 min in dark.

In-solution digestion was performed using either α-chymotrypsin (Sigma, St. Louis, MO) or trypsin (modified, sequencing grade, Promega, Madison, WI) at a 1:15 and 1:25 enzyme-to-substrate ratios, respectively, in a 40 µl total volume in 50 mM $NH_4HCO_3$ (pH 8.0) for 18 h at 37 °C. To each digested sample, equal volume of 100% acetonitrile ($CH_3CN$) was added, and dried under speed vacuum. Peptide mixtures (35 µl) were then acidified by adjusting the pH below 2 in aqueous trifluoroacetic acid (TFA; Pierce, Rockford, IL) solution prior to desalting. The peptide samples were desalted by using reversed-phase $C_{18}$ resin ZipTip micropipette tips (Millipore, Bedford, MA) following the manufacturer's instructions for peptide loading, clean up and elution. Elution was achieved with 2 µl of $CH_3CN$/0.25% TFA [50:50(*v*:*v*)] into clean 0.6-ml Eppendorf microcentrifuge tubes (Eppendorf, Westbury, NY). Eluted peptide samples were then subjected to mass spectra analyses using the procedure described below.



Nature Chemical Biology: doi:10.1038/nchembio.634

**Mass-spectrometry analysis of peptides**

Mass-spectrometry (MS) analysis of the peptide digests were performed on an ABI 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser operating at 337 nm, a video system, and a 4000 Series Explorer software for spectra acquisition and instrument control. Saturated solution of α-cyano-4-hydroxycinnamic acid in 20 mM ammonium phosphate, 50% $CH_3CN$ and 0.2% TFA was used as ionization matrix for analysis. Peptide samples (0.3 µl) were spotted onto MALDI target plate followed by addition of equal volume of the matrix solution; and the mixture was allowed to co-crystallize at room temperature. All mass spectra were obtained in the reflection positive-ion mode, at an acceleration voltage of 20 kV and a detector voltage of 2 kV. MS data was automatically acquired over the mass-to-charge (*m/z*) range of 700 - 4000 Da and laser intensity was adjusted to obtain optimized resolution.

Mass spectra data analyses were performed with Data Explorer software (version 4.3.0.0, Applied Biosystems, Framingham, MA). Peptide peaks from known trypsin or chymotryptic auto-proteolytic products were used as internal standards for calibration. After calibration, data lists of mono-isotopic peptide peak mass and intensity were extracted from baseline corrected spectra. Mass list of the peptides were used for peptide mass fingerprinting analysis using program of the Protein Prospector database search algorithm (http://prospector.ucsf.edu, UCSF, San Francisco, CA) in mass range of 600 to 4,000 Da. Search parameters allowed for maximum of two missed cleavage and a mass tolerance of ±50 ppm. For identification of NEM modified peptides, oxidation of methionines, *N*-ethylmaleimide alkylation or carbamidomethylation of cysteines were used as variable modifications. For identification of SA modified peptides, oxidation of methionines as well as succinylation, and carbamylation of lysines were used as variable modifications; while carbamidomethylation of cysteines as constant modification. The appearance of corresponding deuterated peak with a mass shift of 5 Daltons (for NEM labeling) or 4 Daltons (for SA labeling) was used to confirm the identification of labeled peptides. In addition, to further confirm sites of modification by the specific modifying reagents, MS/MS amino acid sequence analyses of all the



Nature Chemical Biology: doi:10.1038/nchembio.634

differentially labeled peptides were performed and MS/MS spectra were obtained in a MALDI-TOF/TOF mode. The resulting spectra were interpreted manually with the help of MS-PRODUCT program in Protein Prospector.

**Quantitation of site-specific labeling kinetics**

One major advantage of using residue specific stable-isotope labeling followed by mass spectrometry analysis is in its ability to providing accurately quantitative extent of labeling of residue side chains in proteins[4,5]. Here, the time course of NEM modification of cysteines or SA modification of lysines on ligand binding to the $\beta_2$AR was measured by determining the fraction of each site labeled versus time as described in the previous section. To determine the appropriate fitting model, the progression curves (fraction of sites labeled, %F *vs.* time in min) for each site/ligand pair were fit to each of three different models. The first model allows for a burst phase occurring within the dead time of the experiment (~10 seconds) and a phase with a defined relaxation time and has the form:

$$F(t) = 100 - A \exp(- t/\tau_1) \quad (2)$$

where t is the labeling time; $\tau_1$ is the relaxation time; A is the amplitude of the observable phases; and 100 - A is the burst phase amplitude. The second model has two distinct observable phases but does not allow for a burst phase. This model is:

$$F(t) = 100 - A \exp(- t/\tau_1) - (100-A) \exp(- t/\tau_2) \quad (3)$$

where $\tau_1$ and $\tau_2$ are the time constants of each phase; and A is the fractional amplitude of the $\tau_1$ phase. The third model allows for a burst phase and two additional phases and has the form:

$$F(t) = 100 - A \exp(- t/\tau_1)) - B \exp(- t/\tau_2) \quad (4)$$

where A and B are the amplitudes of the phases with relaxation times $\tau_1$ and $\tau_2$, respectively and the burst phase amplitude is (100 - A - B). All three models force $F(\infty)=100$, which is expected from the experimental design.

The models used to extract kinetic parameters from the data were chosen on the basis of the simplest model that produced a goodness of fit equal to or better than the more



Nature Chemical Biology: doi:10.1038/nchembio.634

complicated model. On this basis, the single exponential model was chosen for only K227/propranolol. All other data sets required double exponential fits. When these data were fitted with the third model (Eq. 4), the quality of the fits were not significantly better than the fits with no burst phase. Therefore, the second model (Eq. 3) was used to determine best-fit parameter values for all data sets except K227/propranolol. The two time constants ($\tau_1$ and $\tau_2$) obtained from the fits give the time constants for the fast and slow phases ($\tau_{fast}$ and $\tau_{slow}$) of the labeling reactions. Numerical values for the relaxation kinetic parameters and amplitudes are shown in **Supplementary Material Table 3**.

**Competitive radio-ligand binding experiments**

Competition binding assays were performed with purified $\beta_2$AR using [$^{125}$I]-cyanopindolol (CYP) (2200 Ci/mmol; PerkinElmer, Boston, MA) as the radioligand. Purified $\beta_2$AR treated with DMSO, 2 mM NEM or 2mM SA was incubated with [$^{125}$I]-CYP in the binding buffer (50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 2mM EDTA, 0.1% DDM) and various concentrations of competing ligands (THRX-144877 or carvedilol; $10^{-12}$ M to $10^{-5}$). After incubation for 2 h at room temperature, binding reactions were quickly precipitated using 20% PEG 400 and 0.1% BSA followed by rapid filtration of receptor bound radioactivity on a Whatman GF/B filter using a 96-well Brandel cell harvester (Brandel, Gaithersburg, MD). The filter was washed 5 times with 1 ml of binding buffer each and subsequently, radioactivity was measured in a gamma counter (Packard, Downers Grove, IL). All the values were converted into percent bound after normalization to the value measured at $10^{-11}$ M dose of the competing ligand. $IC_{50}$ values for the ligands were determined by fitting the data from the competition studies to nonlinear regression analysis (one-site competition model) using Graphpad Prism.

**Cyclic AMP generation**

Adenylyl cyclase activity was assayed indirectly by determining the accumulation of second messenger cAMP measured by using ICUE2[6], an improved version of a fluorescence resonance energy transfer (FRET)-based cAMP biosensor, ICUE[7]. HEK



Nature Chemical Biology: doi:10.1038/nchembio.634

293 cells stably expressing $\beta_2$AR and ICUE2 were stimulated with various concentrations of $\beta_2$AR for 3 minutes. Cyclic AMP concentrations were quantified as a FRET ratio between CFP and YFP intensity.

**β-arrestin recruitment**

b-arrestin recruitment to ligand activated $\beta_2$AR was assessed by using a luciferase based reporter gene assay known as "Tango" in a 96-well format, as previously described by Barnea et al.[8]. In this assay, the C-terminus of the human b2AR is replaced with the C-terminal tail of the V2 vasopressin receptor tail (to increase a signal-to-noise ratio) followed by a TEV protease cleavage site and a tTA transcription factor. This construct was stably transfected in HEK293 cells along with a construct encoding b-arrestin 2 fused to TEV protease. Upon ligand stimulation, the recruitment of b-arrestin to the receptor results in making tTA free from the receptor by TEV cleavage. The tTA translocates to the nucleus where it transcribes a stably expressing luciferase reporter gene. HEK293 cells stably transfected with these constructs were seeded at 25,000 cells per well in a 96 well plate. The next day, compounds diluted in HBS were added to the cells to their final concentration followed by incubation at 37 $^0$C for 14-16 hours. The next day, the plate was cooled to room temperature, and an equal amount of Bright-Glo luciferase reagent (Promega, Madison, WI) was added to each well. After 5 min, luminescence was read using a NOVOstar microplate reader (BMG Labtech, Cary, NC).

**ERK activation**

ERK1/2 phosphorylation was assessed using a cellular activation of signaling enzyme-linked immunosorbent assay (ELISA) kit (SABiosciences, Frederick, MD) according to the manufacturer's protocol. Briefly, HEK-293 cells stably expressing $\beta_2$AR were plated in 96-well plates at 50,000 cells/wells, serum starved overnight, and treated with vehicle or a range of concentrations of different $\beta_2$AR ligands for 5 minutes. Stimulated cells were then fixed in 4% formaldehyde and incubated with p-ERK antibody. After washing, incubation with secondary antibody conjugated to horseradish peroxidase (HRP) for 60



Nature Chemical Biology: doi:10.1038/nchembio.634

minutes at room temperature and treatment with color developer, absorbance was read at 450 nm using a NOVOstar microplate reader.

**Estimation of ligand efficacy**

The operational model developed by Black and Leff [9] was used to estimate efficacy by calculation of the coupling coefficient $\tau$. In the operational model, the response of the system to ligand stimulation is based on receptor occupancy alone, as the ligand:receptor complex is coupled to downstream signaling pathways without any allosteric component. The response of the system is then related to ligand concentration for Hill coefficient of 1 by:

$$\frac{E}{E_m} = \frac{\tau\,[A]}{\tau\,[A] + ([A] + K_D)}$$

where $E_m$ is the maximal response of the system to a full agonist, $K_D$ is the agonist dissociation constant and $\tau$ is "coupling efficiency" between the agonist:receptor complex and its downstream signaling partners.

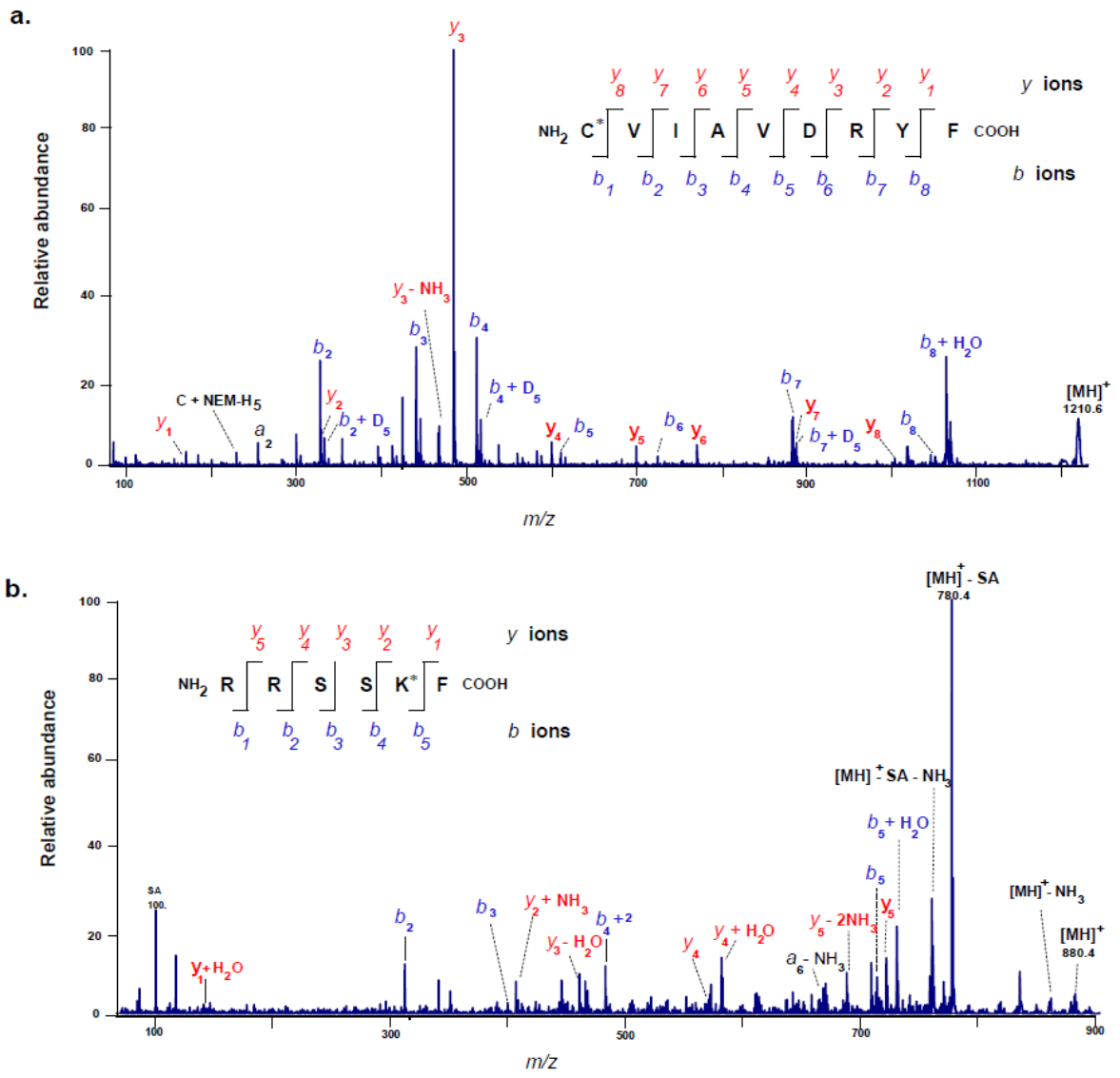
**Statistical analysis.**

Statistical analysis and curve fitting were done using Prism 5.01 (GraphPad Software, San Diego, CA). For statistical comparison, one-way analysis of variance (ANOVA) or a student's two-tailed unpaired *t*-test were used, with p-values of $< 0.05$ considered significant.



Nature Chemical Biology: doi:10.1038/nchembio.634

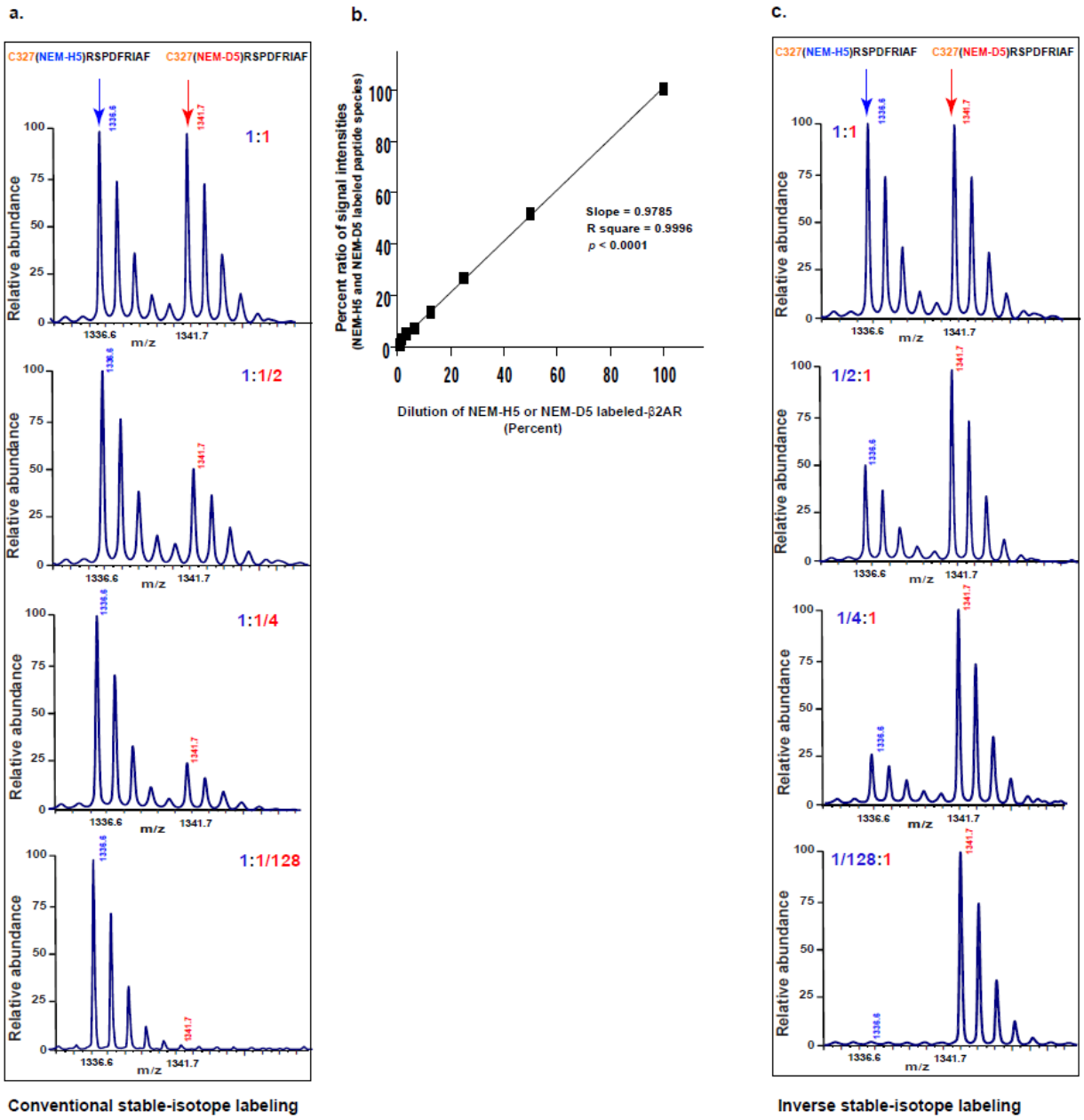
## Supplementary Results




**Supplementary Figure 1: Representative MS/MS spectra of NEM and SA-modified peptide fragments of the $\beta_2$AR.** Fragment ions (MS/MS spectra) of (**a**) $^{125}$-C(NEM-$H_5$)VIAVDRYF-$^{133}$ with *m/z* 1210.6, and (**b**) $^{259}$-RRSSK(SA-$H_4$)F-$^{264}$ with *m/z* 880.5, after chymotryptic digestion. Insets in each show the accepted nomenclature of the *y* and *b* ions produced by differential fragmentation of peptide bonds. Sequence specific ions produced by consecutive fragmentation reaction of the precursor ions such *m/z* 328.1 (or with a +5 m/z shift deuterated NEM, 333.1), 512.3 (or with a +5 *m/z* shift deuterated NEM, 517.3) in (**a**) and *m/z* 780.4 in (**b**) clearly confirm the presence NEM-labeled Cys 125 and SA-labeled Lys 263, respectively.



Nature Chemical Biology: doi:10.1038/nchembio.634




**Supplementary Figure 2: Quantitative comparison of measured versus theoretical peptide intensity ratios for labeled $\beta_2$AR by MALDI-TOF MS.** Shown are comparison between measured intensity ratios (NEM-$H_5$ /NEM-$D_5$) and expected ratios (eight serial two-fold concentration dilutions, 1:1 to 1:128) of the deuterated NEM-labeled $\beta_2$AR, titrated against a fixed quantity of the protiated NEM-labeled $\beta_2$AR performed in both conventional (**a**) and inverse (**c**) stable-isotope labeling experiment of the receptor. Percent ratios of signal intensities between deuterated and protiated NEM-labeled

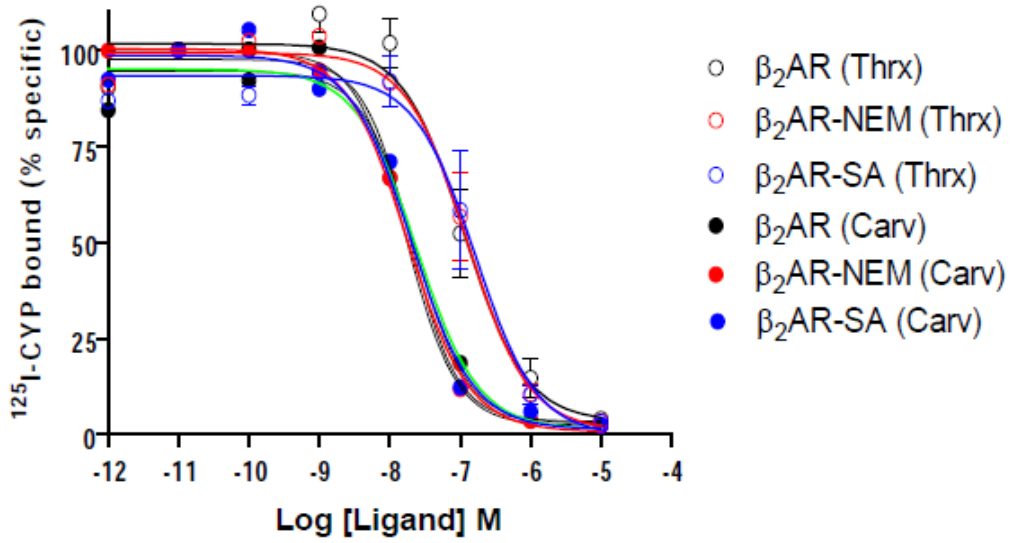


Nature Chemical Biology: doi:10.1038/nchembio.634

peptide peaks were plotted as shown in (**b**) for a singly charged ion ($[M+H]^+$) peak pair at *m/z* 1336.6 and 1341.7 corresponding to peptide $^{327}$CRSPDFRIAF$^{336}$ modified at Cys 327 by NEM-$H_5$ and NEM-$D_5$, for the conventional and inverse labeling strategies, respectively (**a** and **c**). The linear relation between signal intensities and dilution of labeled-$\beta_2$AR protein concentrations (slope = 0.9785 $\pm$ 0.13; $R^2$ = 0.9996; $p < 0.0001$) demonstrates the quantitative nature of the MS platform with a linear dynamic range of more than 2 orders of magnitude. Data are means ± s.e.m. of four independent experiments.



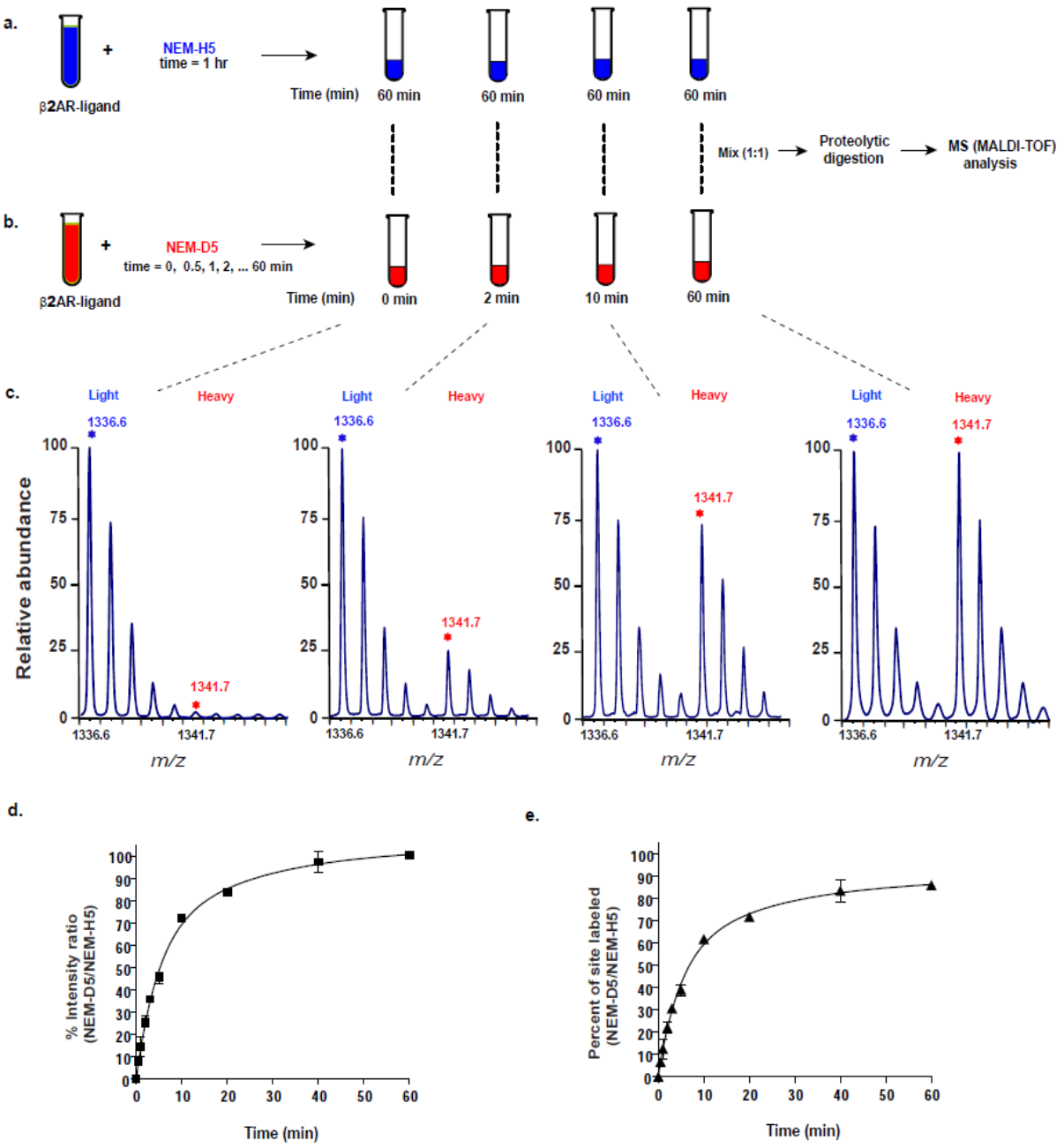
Nature Chemical Biology: doi:10.1038/nchembio.634




**Supplementary Figure 3: Binding properties of $\beta_2$AR in the different labeling conditions**. Competition binding assays were performed as described under Supplementary Methods. Data shown are the means ± s.e.m of specific binding of eight determinations assayed in duplicate. $IC_{50}$ values of test ligands (THRX-144877 or carvedilol) for the displacement of specific [$^{125}$I]-CYP binding in the different $\beta_2$AR treatments were determined from non-linear regression fits of a single binding site model. The $pIC_{50}$ values ± s.e.m. in the $\beta_2$AR treated with DMSO, NEM and SA are 6.95 ± 0.15, 6.90 ± 0.10, and 6.78 ± 0.10 (THRX-144877) or 7.62 ± 0.16, 7.75 ± 0.04, and 7.69 ± 0.12 (carvedilol), respectively.



Nature Chemical Biology: doi:10.1038/nchembio.634




**Supplementary Figure 4: Schematic illustration of the labeling experiment designed to monitor conformational changes in the $\beta_2$AR.** (**a-b**) Labeling of cysteines is initiated in two purified $\beta_2$AR pools (2.5 μM; bound to the same ligand; 30 min at 25 °C) by adding 2 mM of either NEM-$H_5$ as in (**a**) or NEM-$D_5$ as in (**b**) (see Supplementary Methods for details). In (**a**) reactions are performed for an hour at 25 °C (terminated by adding DTT) and equal sized aliquots are prepared. In (**b**) exactly equal

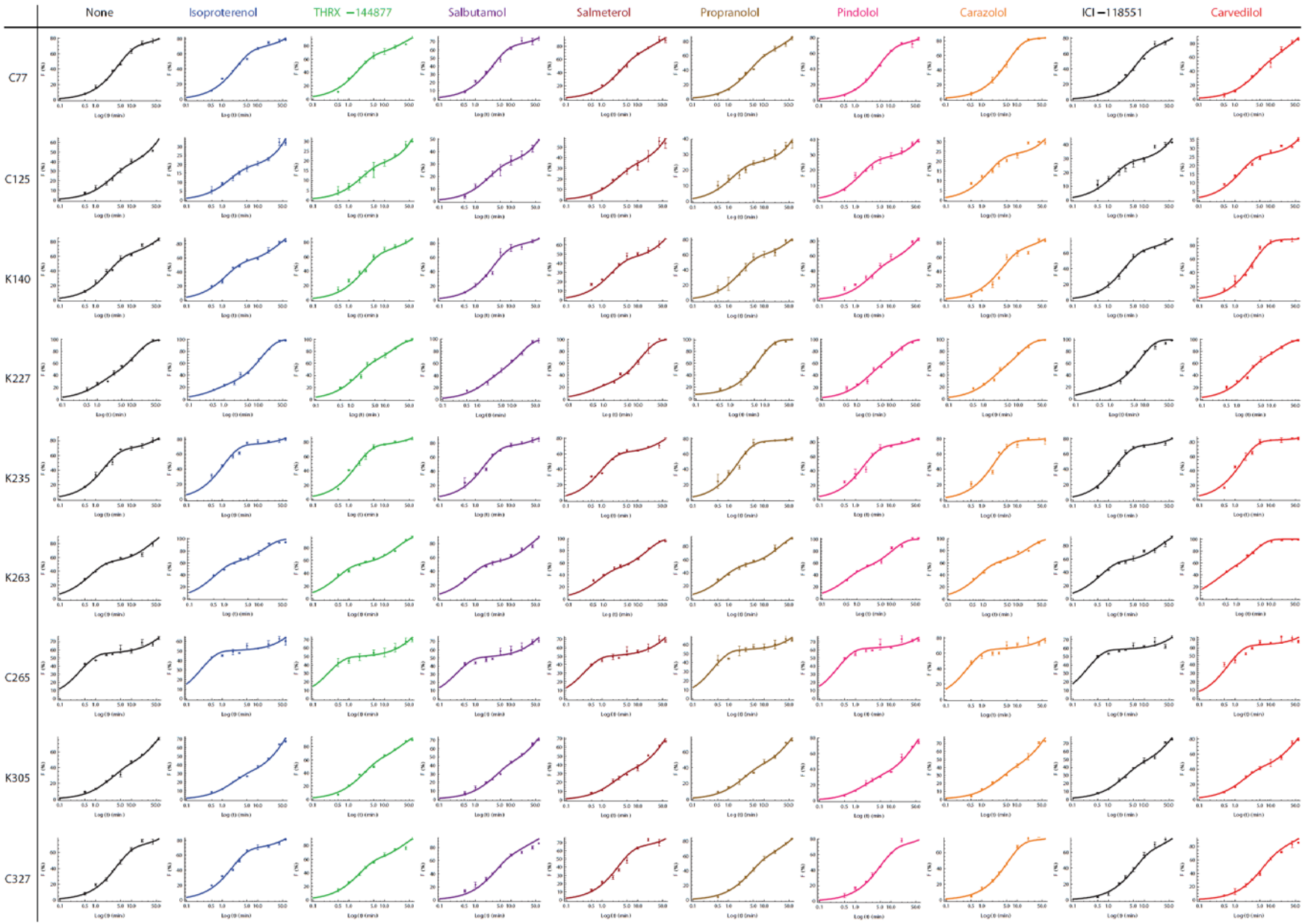


Nature Chemical Biology: doi:10.1038/nchembio.634

sized aliquots are withdrawn at different time points and quenched. Equal amounts of the two pools are then mixed, subjected to proteolysis with appropriate enzyme (chymotrypsin or trypsin), and analyzed with MALDI-TOF-MS to determine peptide fragments that have been modified. (**c**) Shows representative spectra with singly charged ion ($[M+H]^+$) peaks at *m/z* 1336.6 and 1341.7 corresponding to peptide $^{327}$CRSPDFRIAF$^{336}$ modified at Cys 327 by NEM and exhibiting a mass difference of 5 Da because of modification with either NEM-$H_5$ or NEM-$D_5$, respectively. (**d**) Shows representative plot of the change in the intensity ratios (%R) between peptide signal intensities containing NEM-$D_5$ and NEM-$H_5$ over time. Four selected time points (0, 2, 10, and 60 min) in this time series curve correspond to the four representative spectra in (**c**). Each percent intensity ratio (%R) data point was corrected by a specific site/ligand pair reactivity ratio ($R_r$) to obtain percent of site labeled (%F) as represented by the curve in (**e**) for the percent intensity ratios (%R) plot in (**d**). Details for performing fraction residues reacted in the different ligand bound-$\beta_2$AR complexes at 1 hr and computing percent of sites labeled are described in **Supplementary Methods**.



Nature Chemical Biology: doi:10.1038/nchembio.634



**Supplementary Figure 5: Time-progress curves for the extent of labeling of different cysteines and lysines in the $\beta_2$AR**. Each section shows percent of each site (Cys or Lys) labeled (%F) by specific reagent in the different ligand bound-$\beta_2$AR plotted versus labeling time in minutes on a logarithmic scale. The solid lines in each plot are the best fit obtained after fitting to double exponential function except for K227/Propanalol (single phase kinetics) as described in **Supplementary Methods**. The plotted %F values correspond to the means ± standard errors of values obtained from 3-6 independent experiments.



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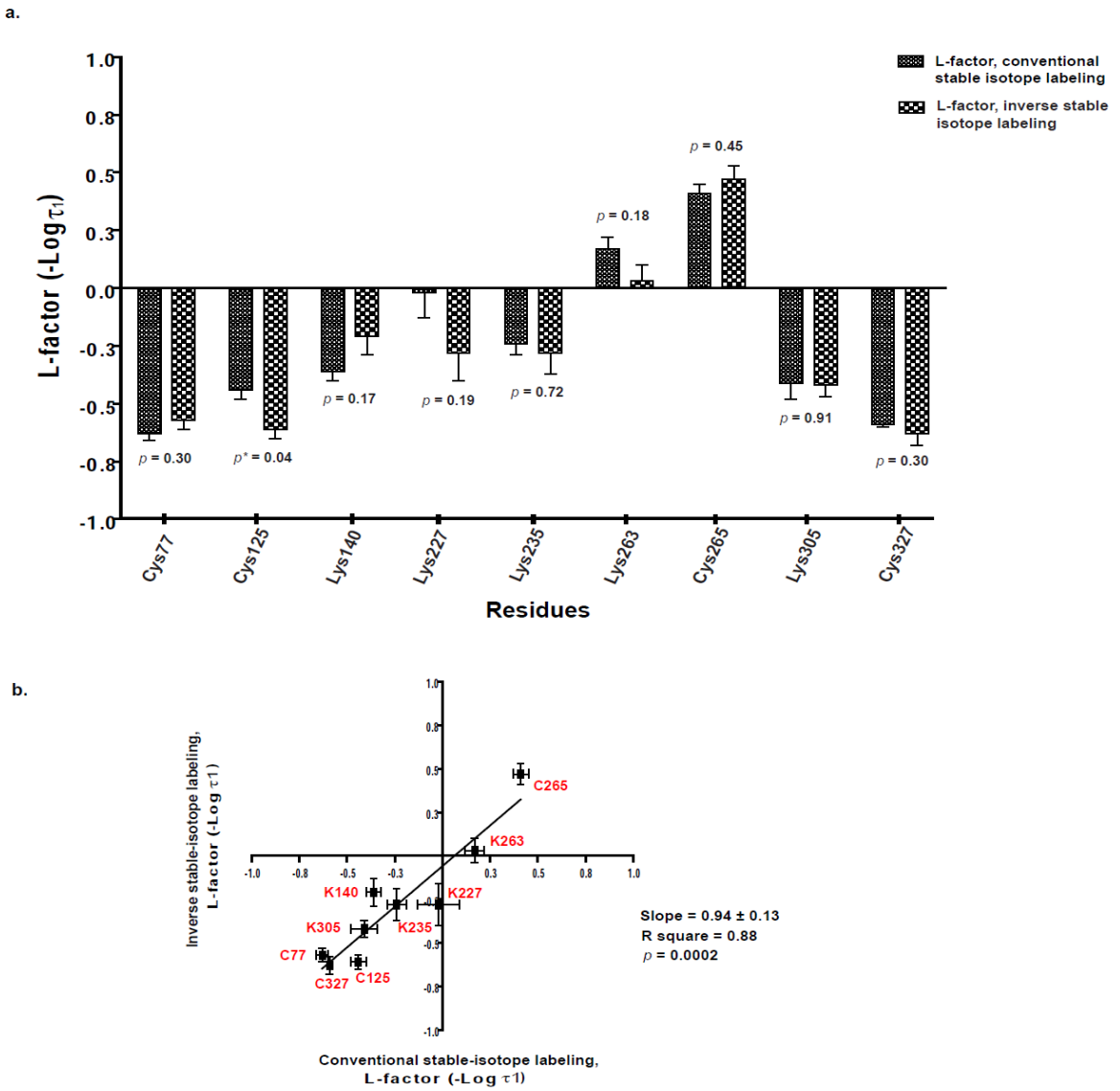


**Supplementary Figure 6: Comparison of L-factors of $\beta_2$AR residues between two stable-isotope labeling strategies.** (**a**) L-factors (-log $\tau_1$) of the nine sites of the $\beta_2$AR control sample as determined using conventional and inverse stable-isotope labeling strategies where the deuterated-reagent labeled receptor was used as a reference. An unpaired Student's t test was used for comparison of L-factors between the two groups for each site and *p* values are shown. (**b**) Linear correlation of the L-factors between the two stable isotope labeling strategies of the $\beta_2$ adrenergic receptor sites in the control sample with a slope of 0.94 $\pm$ 0.13 (mean ± s.e); $R^2$ = 0.88; intercept = -0.06 ± 0.06; and *p* value of 0.0002. Data are means from at least three independent experiments and error bars indicate uncertainties from the standard error (s.e.) of the fitted parameter.



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**Supplementary Table 1: $\beta_2$AR peptides modified with *N*-ethylmaleimide or succinic anhydride.**

| Modified peptide sequence | Coordinates on the $\beta_2$AR (Start-End) [c] | Monoisotopic peptide mass of $[MH]^+$ [d] With NEM-$H_5$ or SA-$H_4$ *m/z* (calc) | *m/z* (obs.) | With NEM-$D_5$ or SA-$D_4$ *m/z* (calc.) | *m/z* (obs.) | Residue | % max. overlap of isotope peaks [e] |
|---|---|---|---|---|---|---|---|
| **C**AD*LVM*GLAV[a] | 75-86 | 1189.56 | 1189.58 | 1194.59 | 1194.62 | Cys 77 | 1.0 |
| **C**VIAVDRYF[a] | 125-133 | 1210.59 | 1210.59 | 1215.61 | 1215.62 | Cys 125 | 0.7 |
| AITSP*F**K**Y[a] | 134-141 | 1050.51 | 1050.48 | 1054.54 | 1054.55 | Lys 140 | 1.1 |
| QEA**K**RQL[a] | 224-230 | 972.51 | 972.51 | 976.54 | 976.56 | Lys 227 | 0.7 |
| KID**K**SEGRF[a] | 232-240 | 1179.48 | 1179.48 | 1183.51 | 1183.53 | Lys 235 | 1.5 |
| RRSS**K**F[a] | 259-264 | 880.46 | 880.46 | 884.49 | 884.50 | Lys 263 | 0.5 |
| F**C**LKEHKALK[b] | 264-273 | 1341.77 | 1341.74 | 1346.80 | 1346.77 | Cys 265 | 1.1 |
| IR**K**EVY[a] | 303-308 | 907.49 | 907.50 | 911.52 | 911.53 | Lys 305 | 0.6 |
| **C**RSPDFRIAF[a] | 327-336 | 1336.65 | 1336.62 | 1341.68 | 1341.65 | Cys 327 | 0.9 |

Modified peptide sequences containing either NEM-labeled at cysteine or SA-labeled at lysine are shown in bold fonts. [a] Denotes chymotryptic while [b] tryptic peptides. *Asterisks* (*) indicate carbamidomethylation with iodoacetamide, oxidation, or methylation of sites. [c] Indicates starting and ending amino acid residues. [d] Indicates monoisotopic peptide masses for the ion $[MH]^+$, *i.e.*, *m/z* values of protiated (NEM-$H_5$ or SA-$H_4$) or deuterated (NEM-$D_5$ or SA-$D_4$)-labeled peptides at either cysteines or lysines. [e] Indicates maximum signal interference at the monoisotopic peak $[MH]^+$ of the deuterated (NEM-$D_5$ or SA-$D_4$)-labeled peptides as computed using Protein Prospector tool, MS-isotope, from the protiated (NEM-$H_5$ or SA-$H_4$)-labeled peptide sequence.

**Supplementary Table 2A: Functional properties of different $\beta_2$AR ligands.**

| | G-protein activation (cAMP accumulation) | | β-arrestin recruitment | | ERK1/2 activation | |
|---|---|---|---|---|---|---|
| Ligand | % $E_{max}$ (mean ± s.e.m) | $pEC_{50}$ ± s.e.m | % $E_{max}$ (mean ± s.e.m) | $pEC_{50}$ ± s.e.m | % $E_{max}$ (mean ± s.e.m) | $pEC_{50}$ ± s.e.m |
| **Isoproterenol** | 100.0 ± 3.2 | 9.8 ± 0.1 | 100.0 ± 5.6 | 8.1 ± 0.2 | 100.0 ± 2.6 | 10.8 ± 0.1 |
| **THRX-144877** | 102.7 ± 2.4 | 9.8 ± 0.1 | 142.1 ± 8.5 | 9.2 ± 0.2 | 104.8 ± 3.1 | 10.60 ± 0.1 |
| **Salbutamol** | 106.4 ± 2.9[a] | 8.7 ± 0.1 | 41.3 ± 4.4 | 6.4 ± 0.1 | 96.8 ± 4.0 | 10.02 ± 0.2 |
| **Salmeterol** | 113.8 ± 2.7[a] | 9.1 ± 0.1 | 59.2 ± 4.7 | 8.1 ± 0.3 | 104.2 ± 3.4 | 9.6 ± 0.2 |
| **Propranolol** | N.D. | — | N.D. | — | 10.0 ± 5.0 | — |
| **Pindolol** | 27.4 ± 3.2 | 9.0 ± 0.4 | N.D. | — | 68.6 ± 7.8 | 9.2 ± 0.5 |
| **Carazolol** | N.D. | — | N.D. | — | 25.8 ± 3.0 | 7.9 ± 0.3 |
| **ICI-118551** | N.D. | — | N.D. | — | 7.9 ± 3.0 | — |
| **Carvedilol** | N.D. | — | 21.3 ± 0.6* | — | 26.2 ± 4.5 | 7.6 ± 0.6 |

**Supplementary Table 2B: Operational efficacy (Tau, $\tau$) values for different $\beta_2$AR ligands.**

| Ligand | G-protein activation (cAMP accumulation) | β-arrestin recruitment | ERK1/2 activation |
|---|---|---|---|
| | Log $\tau$ ± s.e.m Log $\tau$ | Log $\tau$ ± s.e.m Log $\tau$ | Log $\tau$ ± s.e.m Log $\tau$ |
| **Isoproterenol** | 4.29 ± 0.08 | 1.48 ± 0.13 | 4.79 ± 0.15 |
| **THRX-144877** | 2.64 ± 0.09 | 1.28 ± 0.13 | 2.01 ± 0.21 |
| **Salbutamol** | 2.45 ± 0.08 | 0.21 ± 0.1 | 3.48 ± 0.29 |
| **Salmeterol** | 1.16 ± 0.07 | 0.04 ± 0.07 | 1.80 ± 0.19 |
| **Propranolol** | N.D. | N.D. | N.D. |
| **Pindolol** | -0.31 ± 0.07 | -2.134 ± 0.91 | 0.23 ± 0.08 |
| **Carazolol** | N.D. | N.D. | -0.26 ± 0.02 |
| **ICI-118551** | N.D. | N.D. | N.D. |
| **Carvedilol** | N.D. | N.D. | -0.26 ± 0.03 |

Potency ($pEC_{50}$) and maximal ligand effect ($E_{max}$) in activating adenylyl cyclase activity, recruiting β-arrestins and activating ERK1/2 were measured in $\beta_2$AR-overexpressing HEK-293 cells as described under Supplementary Methods. Potency described as negative logarithm of the ligand concentration required to elicit half the maximal response. Maximal response elicited by ligand ($E_{max}$) was calculated as percentage of the isoproterenol stimulated response. Data for $pEC_{50}$ and $E_{max}$ represent the means ± s.e.m. obtained from at least three independent experiments. N.D. denotes no response

detected. *$EC_{50}$ could not be determined because of low response. [a]Salbutamol and salmeterol are partial agonists as described[10,11], however, in this experimental paradigm each has full agonist activity, as a result of receptor over-expression, *i.e.*, increased receptor reserve. In Supplementary Table 2B, ligand dose-response curves were fitted to the operational model of drug agonism (black and leff, 1983)[9] as described in the Supplementary Methods and the $\tau$ values shown are the fitted values from the analysis of mean ± s.e.m.

**Supplementary Table 3A: Fast phase relaxation time constants (min).**

| Residue | None $\tau_1 \pm$ s.e. | Iso $\tau_1 \pm$ s.e. | Thrx $\tau_1 \pm$ s.e. | Salb $\tau_1 \pm$ s.e. | Salm $\tau_1 \pm$ s.e. | Prop $\tau_1 \pm$ s.e. | Pind $\tau_1 \pm$ s.e. | Caraz $\tau_1 \pm$ s.e. | ICI $\tau_1 \pm$ s.e. | Carv $\tau_1 \pm$ s.e |
|---|---|---|---|---|---|---|---|---|---|---|
| **C77** | 4.26 ± 0.3 | 2.43 ± 0.3 | 1.76 ± 0.3 | 3.08 ± 0.3 | 3.04 ± 0.4 | 3.68 ± 0.5 | 4.62 ± 0.4 | 5.40 ± 0.2 | 5.26 ± 0.7 | 3.67 ± 0.7 |
| **C125** | 2.75 ± 0.3 | 1.61 ± 0.3 | 2.22 ± 0.3 | 2.36 ± 0.3 | 2.67 ± 0.3 | 1.23 ± 0.2 | 1.44 ± 0.2 | 1.58 ± 0.4 | 1.37 ± 0.3 | 1.42 ± 0.2 |
| **K140** | 2.28 ± 0.2 | 1.24 ± 0.2 | 2.71 ± 0.5 | 3.17 ± 0.7 | 1.72 ± 0.3 | 2.10 ± 0.3 | 2.61 ± 0.5 | 3.61 ± 0.7 | 2.74 ± 0.2 | 3.26 ± 0.5 |
| **K227** | 1.05 ± 0.3 | 0.48 ± 0.2 | 1.45 ± 0.3 | 1.56 ± 0.3 | 0.44 ± 0.2 | 6.70 ± 0.3 | 1.71 ± 0.4 | 1.41 ± 0.4 | 0.17 ± 0.1 | 1.79 ± 0.5 |
| **K235** | 1.73 ± 0.2 | 1.10 ± 0.1 | 1.70 ± 0.2 | 1.76 ± 0.2 | 0.96 ± 0.1 | 1.83 ± 0.2 | 1.76 ± 0.3 | 2.32 ± 0.4 | 1.60 ± 0.1 | 1.57 ± 0.2 |
| **K263** | 0.68 ± 0.1 | 0.50 ± 0.1 | 0.47 ± 0.1 | 0.66 ± 0.1 | 0.65 ± 0.1 | 0.66 ± 0.1 | 0.46 ± 0.1 | 0.68 ± 0.1 | 0.59 ± 0.1 | 0.22 ± 0.1 |
| **C265** | 0.39 ± 0.0 | 0.27 ± 0.1 | 0.28 ± 0.0 | 0.31 ± 0.1 | 0.33 ± 0.1 | 0.38 ± 0.1 | 0.34 ± 0.1 | 0.41 ± 0.1 | 0.27 ± 0.1 | 0.66 ± 0.1 |
| **K305** | 2.59 ± 0.5 | 1.85 ± 0.4 | 2.36 ± 0.4 | 3.24 ± 0.6 | 2.10 ± 0.5 | 2.36 ± 0.5 | 1.72 ± 0.5 | 2.78 ± 0.7 | 2.62 ± 0.4 | 1.66 ± 0.3 |
| **C327** | 3.87 ± 0.1 | 1.89 ± 0.2 | 1.84 ± 0.3 | 3.41 ± 0.6 | 2.82 ± 0.3 | 3.95 ± 0.3 | 4.93 ± 0.7 | 5.18 ± 0.4 | 5.32 ± 0.6 | 5.32 ± 0.5 |

**Supplementary Table 3B: Slow phase relaxation time constants (min).**

| Residue | None $\tau_2$ ± s.e | Iso $\tau_2$ ± s.e | Thrx $\tau_2$ ± s.e | Salb $\tau_2$ ± s.e | Salm $\tau_2$ ± s.e | Prop $\tau_2$ ± s.e | Pind $\tau_2$ ± s.e | Caraz $\tau_2$ ± s.e | ICI $\tau_2$ ± s.e | Carv $\tau_2$ ± s.e |
|---|---|---|---|---|---|---|---|---|---|---|
| **C77** | 142.2 ± 47.3 | 103.0 ± 36.6 | 40.7 ± 10.0 | 163.3 ± 72.0 | 35.38 ± 7.36 | 57.8 ± 11.5 | 155.5 ± 67.7 | 459.9 ± 565.8 | 103.6 ± 45.0 | 41.3 ± 7.1 |
| **C125** | 98.6 ± 13.8 | 264.2 ± 37.3 | 323.7 ± 38.5 | 176.5 ± 20.8 | 120.4 ± 24.2 | 258.8 ± 37.7 | 295.4 ± 54.1 | 486.7 ± 175.8 | 226.1 ± 51.4 | 422.6 ± 99.0 |
| **K140** | 64.0 ± 17.14 | 44.8 ± 6.8 | 69.4 ± 24.3 | 93.54 ± 69.2 | 113.04 ± 20.2 | 70.24 ± 14.2 | 50.24 ± 10.9 | 72.74 ± 50.0 | 107.94 ± 20.8 | 405.74 ± 2068.0 |
| **K227** | 12.5 ± 1.6 | 12.0 ± 1.0 | 16.9 ± 4.5 | 17.0 ± 2.1 | 12.0 ± 1.3 | — | 13.5 ± 4.5 | 10.6 ± 2.2 | 7.4 ± 0.8 | 16.1 ± 5.1 |
| **K235** | 84.8 ± 23.8 | 153.1 ± 88.2 | 107.1 ± 55.3 | 86.1 ± 42.7 | 93.7 ± 24.1 | 331.1 ± 520.5 | 95.0 ± 54.0 | 488.2 ± 1478.0 | 146.1 ± 53.2 | 259.7 ± 403.3 |
| **K263** | 41.4 ± 6.4 | 14.8 ± 2.7 | 24.4 ± 2.9 | 39.4 ± 4.9 | 17.9 ± 4.9 | 28.6 ± 2.8 | 9.9 ± 1.8 | 19.71 ± 3.6 | 33.0 ± 5.0 | 2.9 ± 0.3 |
| **C265** | 102.8 ± 22.4 | 181.1 ± 46.7 | 98.5 ± 11.9 | 87.6 ± 18.3 | 102.3 ± 17.0 | 165.1 ± 42.2 | 135.8 ± 44.2 | 113.6 ± 52.7 | 140.3 ± 42.0 | 210.0 ± 150.4 |
| **K305** | 58.0 ± 4.1 | 64.9 ± 6.2 | 31.4 ± 4.8 | 72.4 ± 9.4 | 70.6 ± 7.6 | 56.6 ± 6.7 | 49.2 ± 4.8 | 56.1 ± 7.4 | 62.2 ± 7.3 | 49.7 ± 4.5 |
| **C327** | 142.2 ± 47.3 | 103.0 ± 36.6 | 40.7 ± 10.0 | 35.4 ± 7.4 | 163.3 ± 72.0 | 57.8 ± 11.5 | 155.5 ± 67.7 | 459.9 ± 565.8 | 103.6 ± 45.0 | 41.3 ± 7.1 |

**Supplementary Table 3C: Fractional amplitudes for the fast phase (%).**

| Residue | None | Iso | Thrx | Salb | Salm | Prop | Pind | Caraz | ICI | Carv |
|---|---|---|---|---|---|---|---|---|---|---|
| | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e | $A_1$ ± s.e |
| **C77** | 68.6 ± 2.8 | 64.7 ± 3.9 | 64.2 ± 4.5 | 63.4 ± 3.6 | 59.9 ± 5.8 | 56.6 ± 4.8 | 68.7 ± 3.5 | 81.9 ± 2.1 | 64.0 ± 6.0 | 48.5 ± 6.1 |
| **C125** | 32.9 ± 3.1 | 17.4 ± 1.4 | 17.0 ± 1.1 | 28.6 ± 1.6 | 30.2 ± 3.9 | 23.2 ± 1.3 | 26.7 ± 1.5 | 22.6 ± 1.9 | 27.2 ± 2.3 | 25.5 ± 1.4 |
| **K140** | 58.9 ± 5.0 | 51.6 ± 3.9 | 63.6 ± 5.3 | 73.5 ± 7.4 | 43.5 ± 3.1 | 55.3 ± 3.9 | 44.9 ± 6.0 | 67.1 ± 11.2 | 61.8 ± 2.5 | 88.0 ± 5.7 |
| **K227** | 28.5 ± 5.5 | 18.4 ± 3.2 | 54.1 ± 8.1 | 32.1 ± 5.6 | 18.6 ± 4.0 | 93.5 ± 1.4 | 42.1 ± 15.7 | 35.9 ± 11.1 | 12.3 ± 4.4 | 57.0 ± 10.4 |
| **K235** | 66.5 ± 3.1 | 72.0 ± 3.0 | 75.0 ± 3.5 | 73.4 ± 4.3 | 60.6 ± 2.6 | 76.0 ± 3.9 | 73.4 ± 4.6 | 77.2 ± 5.1 | 69.2 ± 2.3 | 82. 0 ± 3.5 |
| **K263** | 51.3 ± 2.7 | 56.0 ± 3.5 | 51.4 ± 2.2 | 49.3 ± 2.3 | 44.8 ± 2.3 | 47.6 ± 2.1 | 44.3 ± 5.0 | 56.9 ± 3.5 | 54.3 ± 2.6 | 38.7 ± 5.1 |
| **C265** | 55.0 ± 2.0 | 50.0 ± 1.7 | 49.2 ± 1.3 | 50.2 ± 2.3 | 49.8 ± 1.7 | 53.5 ± 1.7 | 61.4 ± 2.1 | 64.8 ± 3.1 | 57.9 ± 2.0 | 62.4 ± 3.4 |
| **K305** | 36.4 ± 3.6 | 26.5 ± 2.8 | 53.3 ± 5.9 | 36.6 ± 3.6 | 29.1 ± 3.0 | 35.9 ± 3.6 | 25.9 ± 3.4 | 32.4 ± 4.5 | 38.9 ± 3.3 | 37.3 ± 2.6 |
| **C327** | 65.20 ± 5.0 | 67.5 ± 3.0 | 55.6 ± 4.5 | 61.6 ± 5.8 | 63.2 ± 4.3 | 52.8 ± 2.3 | 68.5 ± 6.6 | 74.3 ± 3.6 | 58.8 ± 5.3 | 64.2 ± 5.1 |

**Supplementary Table 3D: Burst phase amplitudes (%).**

| Residue | None | Iso | Thrx | Salb | Salm | Prop | Pind | Caraz | ICI | Carv |
|---|---|---|---|---|---|---|---|---|---|---|
| | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e | $A_b \pm$ s.e |
| **C77** | 1.0 ± 1.3 | 2.0 ± 2.7 | 0.0 ± 0.0 | 1.5 ± 2.2 | 0.8 ± 2.2 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.6 ± 0.9 | 1.1 ± 2.0 | 0.0 ± 0.0 |
| **C125** | 2.2 ± 1.8 | 0.8 ± 1.5 | 0.9 ± 0.9 | 0.3 ± 1.3 | 0.8 ± 2.7 | 1.8 ± 1.5 | 0.8 ± 1.7 | 2.2 ± 2.0 | 2.4 ± 2.5 | 1.6 ± 1.5 |
| **K140** | 1.4± 2.9 | 1.0 ± 2.8 | 3.3 ± 2.7 | 0.0 ± 0.0 | 3.3 ± 2.0 | 1.0 ± 2.4 | 4.3 ± 2.8 | 0.0 ± 0.0 | 0.0 ± 0.0 | 2.2 ± 3.3 |
| **K227** | 6.8 ± 2.5 | 5.6± 2.8 | 2.3 ± 3.6 | 4.5 ± 1.8 | 2.0 ± 3.6 | 5.0 ± 2.0 | 6.5 ± 3.5 | 6.0 ± 2.4 | 8.0 ± 2.0 | 3.4 ± 3.1 |
| **K235** | 1.7 ± 2.7 | 3.9 ± 3.6 | 1.4 ± 3.4 | 4.5 ± 3.4 | 3.9 ± 3.3 | 3.3 ± 3.6 | 4.3 ± 3.7 | 3.0 ± 4.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **K263** | 0.7 ± 3.8 | 2.4 ± 3.4 | 1.4 ± 2.9 | 0.9 ± 3.1 | 1.0 ± 1.8 | 1.1 ± 2.5 | 1.8 ± 3.9 | 2.2 ± 3.4 | 1.7 ± 3.5 | 2.0 ± 2.0 |
| **C265** | 2.9 ± 3.7 | 2.5 ± 3.4 | 2.5 ± 2.5 | 2.0 ± 4.4 | 1.4 ± 3.2 | 2.4 ± 3.3 | 4.4 ± 4.0 | 2.6 ± 5.8 | 2.5 ± 4.0 | 8.7 ± 5.2 |
| **K305** | 2.1 ± 1.9 | 1.4 ± 2.2 | 0.0 ± 0.0 | 2.4 ± 1.6 | 1.9 ± 2.1 | 2.0 ± 2.1 | 0.5 ± 2.6 | 0.4 ± 2.4 | 0.3 ± 2.0 | 0.6 ± 2.1 |
| **C327** | 1.8 ± 2.2 | 3.5 ± 2.3 | 1.1 ± 3.0 | 4.3 ± 1.6 | 3.7 ± 2.7 | 0.0 ± 0.0 | 2.5 ± 1.7 | 0.1 ± 1.2 | 0.0 ± 0.0 | 0.9 ± 1.3 |

All relaxation times and amplitudes were obtained by fitting to Eq. 3 except for K227/prop using Eq. 2. The burst phase amplitudes listed in 3D were determined by fitting to Eq. 4. s.e. denote uncertainties from the standard error of the fitted parameter. Single-letter code used to represent the amino acids.

**Supplementary Table 4: Kinetic parameters for control $\beta_2$AR from inverse labeling experiment.**

| Residue | Fast phase relaxation time constants (min) $\tau_1 \pm$ s.e. | Slow phase relaxation time constants (min) $\tau_2 \pm$ s.e. | Fractional amplitudes for the fast phase (%) $A_1 \pm$ s.e. |
|---|---|---|---|
| **C77** | 3.69 ± 0.5 | 69.84 ± 9.5 | 47.88 ± 3.2 |
| **C125** | 4.07 ± 0.5 | 190.40 ± 31.7 | 55.96 ± 1.5 |
| **K140** | 1.63 ± 0.4 | 63.18 ± 15.1 | 52.40 ± 5.1 |
| **K227** | 1.92 ± 0.7 | 50.13 ± 33.6 | 66.42 ± 10.7 |
| **K235** | 1.89 ± 0.5 | 70.73 ± 28.0 | 58.35 ± 6.6 |
| **K263** | 0.94 ± 0.2 | 34.09 ± 6.4 | 55.00 ± 4.7 |
| **C265** | 0.34 ± 0.1 | 135.60 ± 26.6 | 53.05 ± 3.1 |
| **K305** | 2.64 ± 0.1 | 76.28 ± 8.5 | 39.97 ± 2.7 |
| **C327** | 4.22 ± 0.6 | 84.95 ± 34.7 | 68.58 ± 5.1 |

All relaxation times and amplitudes were obtained by fitting to Eq. 3. s.e. denote uncertainties from the standard error of the fitted parameter. Single-letter code used to represent the amino acids.

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