

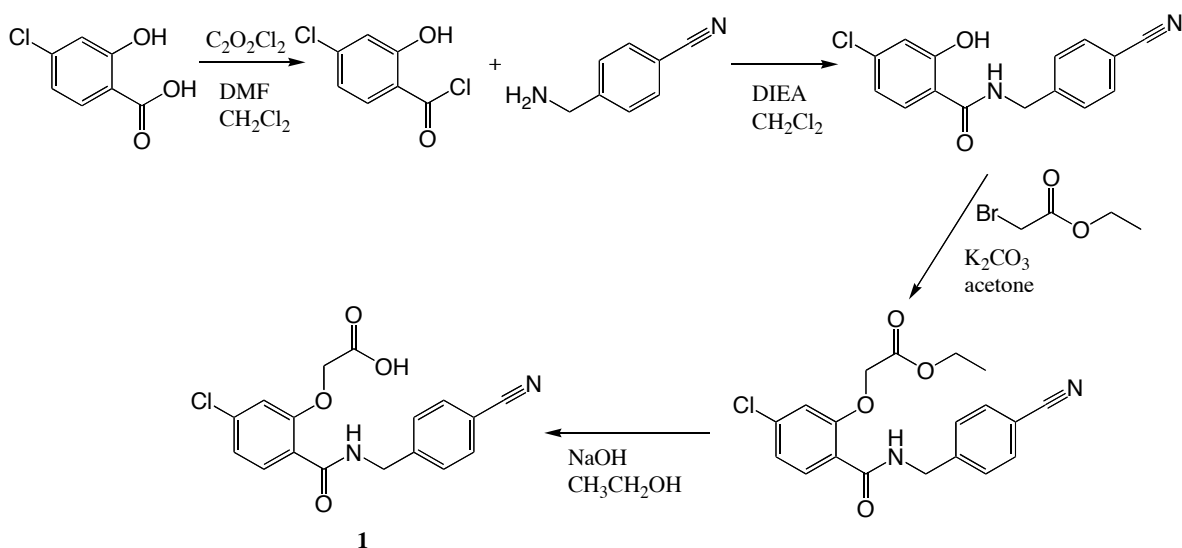
## Supplemental Information

### Site Specific Conversion of Cysteine Thiols into Thiocyanate Creates an IR Probe for Electric Fields in Proteins

#### Contents:

#### I. Synthesis of Nitrile-containing Inhibitor of *h*ALR2

The nitrile-containing inhibitor of human aldose reductase, (5-chloro-2-[(4-cyanobenzyl)amino]carbonylphenoxy) acetic acid, **1**, was synthesized according to the procedure described in Scheme 1.<sup>1</sup> Unless noted, all starting materials were used without further purification, and all reactions were carried out under an atmosphere of N<sub>2</sub>(g). Flash column chromatography (FCC) was carried out with silica gel (Aldrich, 230 - 400 mesh, 60 Å) equilibrated in heptane. The proton nuclear magnetic resonance spectrum of product **1** was obtained on a 200 MHz Varian Gemini spectrometer.



*4-chloro-2-hydroxybenzoyl chloride*: A slurry of 5.96 g of 4-chloro-2-hydroxybenzoic acid (Aldrich, 93%) in 20 mL of  $CH_2Cl_2$  was prepared and 50 mL of oxalyl chloride was added dropwise over several minutes. A drop of dimethylformamide (DMF) was added and the solution was stirred at room temperature for 24 hr. Excess oxalyl chloride was removed under vacuum, the solution was filtered, and excess  $CH_2Cl_2$  was removed under vacuum. The acid chloride product was used in the next reaction without further purification.

*4-chloro-N-(4-cyanobenzyl)-2-hydroxybenzamide*: The acid chloride starting material was dissolved in 30 mL  $CH_2Cl_2$  and chilled to 0 °C. Diisopropylethylamine (DIEA, 11 mL) was added dropwise. 6.28 g of 4-(aminomethyl)benzonitrile hydrochloride (Aldrich, 97%) was added and the solution was stirred at room temperature for 24 hr. The  $CH_2Cl_2$  was then removed under vacuum and the amide product was extracted with ethyl acetate, washed sequentially with 1-2 M HCl(aq) and saturated NaCl(aq), and dried over  $MgSO_4$ . The  $MgSO_4$  was removed by filtration, excess ethyl

acetate was removed under vacuum, and product was separated and isolated by FCC (product eluted with 20% (v/v) ethyl acetate in heptane). Yellow fractions were combined and excess solvent was removed under vacuum.

*ethyl (5-chloro-2{[(4-cyanobenzyl)amino]carbonyl}phenoxy) acetate*: The amide starting material was dissolved in 15 mL of acetone and 9.5 mL of 2 M K<sub>2</sub>CO<sub>3</sub>(aq) was added. Ethyl bromoacetate (500  $\mu$ L) was added and the solution was heated at 50 °C for 2 hr. After returning to room temperature, the solution was cooled to 0 °C and 1 M HCl(aq) was added until the solution was pH = 1. The product was extracted with ethyl acetate, washed with saturated NaCl(aq) and dried over MgSO<sub>4</sub>. The MgSO<sub>4</sub> was removed by filtration, and the resulting product was purified by FCC (product was eluted at 20% (v/v), 60% (v/v) and 100% (v/v) ethyl acetate in heptane). Yellow fractions were combined, and excess solvent was removed under vacuum.

*(5-chloro-2{[(4-cyanobenzyl)amino]carbonyl}phenoxy) acetic acid (1)*: The acetate product from the previous reaction were dissolved in 12 mL ethanol and 2 mL of 2 M NaOH(aq) were added. The solution was stirred at room temperature for 4 hr, after which the ethanol was removed under vacuum. 1 M HCl(aq) was added to the solution until pH = 0, resulting in a white precipitate. The precipitate was extracted into ethyl acetate, washed with saturated NaCl(aq) and dried with MgSO<sub>4</sub>. After removing the MgSO<sub>4</sub> by filtration the ethyl acetate was removed under vacuum. The resulting white powder was washed with CH<sub>3</sub>CN and H<sub>2</sub>O (neutral pH), and dried. (9.2 ppm, t, 1H; 7.9 ppm, d, 1 H; 7.8 ppm, d, 2 H; 7.5 ppm, d, 2 H; 7.3 ppm, s, 1 H; 7.2 ppm, d, 1 H; 4.9 ppm, s, 2 H; 4.6 ppm, d, 2H. 345 m/z (ES+), 344.6 calculated.  $\bar{\nu}_{C\equiv N} = 2236.2 \text{ cm}^{-1}$  (H<sub>2</sub>O).)

## II. *h*ALR2 Expression and Purification

The expression and purification of human Aldose reductase (*h*ALR2) has been described previously.<sup>2,3</sup> Briefly, the gene for wild-type (WT) *h*ALR2 was obtained from Alberto Podjarny in the pET-15b expression vector (Novagen). The gene was sequenced to confirm the location of cysteine residues. The plasmid was transformed into the *E. coli* strain BL21(DE3) (Novagen) for protein expression. In a 6 L shake flask, 2 L of sterilized LB media with 100  $\mu$ g/mL ampicillin added were inoculated with the plasmid-containing *E. coli* and grown for approximately 4 hr at 37 °C. Expression of *h*ALR2 was then induced with the addition of IPTG to a concentration of 1 mM. After induction, the growth was maintained at 37 °C for approximately 4 hr. The cells were then pelleted, resuspended in a lysis buffer of 50 mM phosphate pH 8.0, 300 mM NaCl, and 10 mM imidazole, and lysed with a cell homogenizer operating between 15-20 kpsi. The cell debris was removed by 2 cycles of centrifugation at 10 kg for 20 min.

The lysate was then loaded directly onto a Ni-NTA agarose column (Qiagen), washed with buffer containing 20 mM imidazole, and eluted with buffer containing 250 mM imidazole. The recovered protein was exchanged into thrombin cleavage buffer (20 mM Tris pH 8.0, 150 mM NaCl, 2.5 mM CaCl<sub>2</sub>) and thrombin was added to a concentration of approximately 4 U thrombin per mg of His-tagged protein. The cleavage reaction was left in the dark at room temperature for 12-16 hr, then exchanged into 20 mM Tris pH 8.0. The resulting solution was loaded onto a 1 mL HiTrap Q HP anion exchange column (GE Biosciences) and eluted with a salt gradient stepped to 80

mM NaCl 20 mM Tris pH 8.0. Typical yields of *hALR2* were approximately 10 mg of purified WT *hALR2* per liter of growth media.

The purity of the final cleaved product was confirmed by SDS-polyacrylamide gel electrophoresis and mass spectrometry. Protein intact masses were obtained by nanoESI-MS performed in the Vincent Coates Foundation Mass Spectrometry Lab, Stanford (<http://mass-spec.stanford.edu>). Aliquots (5  $\mu$ L) of approximately 10  $\mu$ M protein in 1:1 acetonitrile:water, 0.1% formic acid were analyzed by direct nanospray infusion using an Advion NanoMate chip-based nanoelectrospray source (Advion Biosciences, Ithaca, NY); 0.3 psi, 1.55 kV MS data were collected on a Q-ToF API US quadrupole-time of flight mass spectrometer (Micromass-Waters Corp., Milford, MA); source temperature 30° C, cone voltage 30, standard source pressure. The MaxEnt1 algorithm was used for MS data deconvolution.

### III. *hALR2* Kinetics and Inhibition

All kinetic measurements were performed with DL-glyceraldehyde as substrate and NADPH as cofactor. All reactions were performed in 20 mM phosphate buffer pH 7.0 at room temperature. Enzyme reaction kinetics were measured by monitoring the decrease in NADPH adsorption at 340 nm over 5 min of at least 2 replicates of each sample. Typical reactions contained 100-200 nM *hALR2*, 100  $\mu$ M NADPH, and 5-100  $\mu$ M DL-Glyceraldehyde. Data were fit directly to the Michaelis-Menten equation to determine  $k_{cat} = 0.31 \text{ s}^{-1}$  for WT *hALR2*, similar to what has been reported previously.<sup>4</sup> The inhibition constant of **1** in WT *hALR2*,  $K'_i$ , was determined under conditions of saturating concentrations of DL-glyceraldehyde and NADPH, and concentration of **1** typically in the range of 0.01-10  $\mu$ M. The decrease in adsorption of NADPH at 340 nm was observed for 5 min, and the slope ( $V_i$ ) was divided by that obtained with no added inhibitor ( $V_0$ ). This ratio was fit to the equation:

$$\frac{V_i}{V_0} = \frac{E - I - K'_i + [(E - I - K'_i)^2 + 4EK'_i]^{\frac{1}{2}}}{2E}$$

where  $E$  is the enzyme concentration,  $I$  is the inhibitor concentration, and  $K'_i$  is the apparent inhibition constant, as has been described previously.<sup>3,5</sup>  $K'_i$  was determined to be 2.1  $\mu$ M.

### IV. SCN-Labeling of Ribonuclease S

*S-protein*: Bovine ribonuclease A and subtilisin Carlsberg were obtained from Sigma-Aldrich and used without further purification. Ribonuclease A was dissolved to 2% in 0.1M TRIS buffer pH 8 and subtilisin was added to a final concentration of  $8 \cdot 10^{-3}$ %. The reaction proceeded for 18 hrs on ice and was stopped with the addition of HCl to pH 2<sup>6</sup>. The reaction mixture was then purified by HPLC on a C-18 column, using a gradient of 5-50% acetonitrile in water with 0.1% trifluoroacetic acid over 60 minutes. The fraction containing S-protein was lyophilized.

*SCN-S-peptide*: The N-terminal peptide of ribonuclease A residues 1-15 with the

substitution of homocysteine (FMOC-homocys from Peptech Corp) for the native methionine was synthesized on an Applied Biosystems 431A peptide synthesizer using standard HBTU/HOBt activation protocols. The product was purified by HPLC on a C-18 column, using a gradient of 5-35% acetonitrile in water with 0.1% trifluoroacetic acid over 60 minutes, and lyophilized. The resultant powder was dissolved to 20 mM in 1M ammonium carbonate pH 9.5 and 1.2 equivalents of DTNB were added.<sup>7</sup> The reaction was followed by absorbance at 412 nm until completion (less than one minute), and immediately lyophilized. The lyophilized intermediate peptide was dissolved in water to 2 mM, reacted with 10 equivalents of KCN, and followed by absorbance at 412 nm to completion (less than one minute). This product was then purified by HPLC on a C-18 column, using a gradient of 5-35% acetonitrile in water with 0.1% trifluoroacetic acid over 60 minutes, and lyophilized (53% yield based on peptide). LC-MS: 1748.0 Da [Theoretical = 1748.0 Da].

*SCN-RNase S*: 1.5 equivalents of SCN-S-peptide in pH 7 phosphate buffer were added to 1 equivalent S-protein in pH 7 phosphate buffer, and the complex was then subjected to 3 cycles of dilution and concentration using a Vivaspin500, 5-kDa molecular weight cutoff (MWCO) centrifugal concentrator (Vivascience) to ensure that unbound SCN-S-peptide was removed. The resulting SCN-RNase S was lyophilized and subsequently dissolved to 11 mM in 50:50 (v/v) glycerol:water for infrared experiments.

## V. SCN-Labeling of *hALR2*

*Singly-labeled hALR2*: To 280  $\mu\text{M}$  *hALR2* in 20 mM phosphate pH 7 was added 1.1 molar equivalents of DTNB (17 mM, in 100 mM phosphate pH 7.5) When the reaction was initiated, a rapid rise ( $t_{1/2} < 1$  min) in TNB concentration was observed by monitoring the absorbance at 412 nm. The solution was incubated at room temperature for 10 min, by which point 1 molar equivalent of TNB was observed. 20 equivalents of KCN were added and the reaction incubated at room temperature for 30 min (by which point an additional molar equivalent of TNB is observed) before applying it to a size exclusion column (PD-10, GE Biosciences) equilibrated with 20 mM phosphate pH 7. The isolated protein was then concentrated using a Vivaspin500, 10-kDa MWCO concentrator (Vivascience).

The intact masses of labeled and wild type reference samples were obtained by nanoESI-MS as described. Masses of 36158 and 36132 Da, respectively, were obtained. The theoretical value for wild type is 36135 Da.

To the concentrated, labeled enzyme was added 1.5 equivalents of NADP<sup>+</sup> (Sigma-Aldrich) and 1.5 equivalents of the nitrile-containing inhibitor (**1**). This was then subjected to 3 cycles of dilution and concentration to wash away unbound inhibitor and cofactor. The final concentration of 1mM was used for infrared experiments.

*Multiply-labeled hALR2 using DTNB*: The following is a representative reaction. To 280  $\mu\text{M}$  *hALR2* in 20 mM phosphate pH 7 was added DTNB (17 mM in 100mM phosphate pH 7.5) to a final concentration of 10mM DTNB. The solution was incubated at room temperature for 4 hrs, by which point 2.2 molar equivalents of TNB were observed by absorbance at 412 nm. When the reaction was initiated, a rapid rise ( $t_{1/2} < 1$

min) in TNB concentration was observed which corresponded to the reaction of DTNB with approximately 1 cysteine residue; this was followed by a much slower rise ( $t_{1/2} > 30$  min) corresponding to the reaction of additional cysteine residues in the protein. Unreacted DTNB was removed by size exclusion as above. 70 equivalents of KCN were added and the reaction incubated at 4° C for 12 hours before purifying again by size exclusion. The isolated protein was then concentrated by centrifugation. Samples for infrared experiments with inhibitor bound were prepared as above for singly-labeled *hALR2*.

*Multiply-labeled hALR2 using NTCB:* To 280  $\mu$ M *hALR2* in 20 mM phosphate pH 7 was added NTCB (20 mM, in 100mM pH 7.5 phosphate) to a final concentration of 10 mM in NTCB followed immediately by 70 molar equivalents of KCN. The solution was incubated at room temperature for 4 hrs at which point 4 molar equivalents of TNB were observed by absorbance at 412 nm. The mixture was then purified by size exclusion and concentrated by centrifugation. Samples for infrared experiments with inhibitor bound were prepared as above for singly-labeled *hALR2*.

## VI. SCN-Labeling of Photosynthetic Reaction Centers

*Generation of single-cysteine mutant RCs:* Wild-type reaction centers from *Rhodobacter capsulatus* contain five cysteines located at residues L92, L98, L108, L246, and L247. These cysteines were mutated to alanine in three successive rounds of site-directed mutagenesis on a cloning plasmid containing the L gene (Quikchange mutagenesis kit, Stratagene). The room-temperature absorption spectrum and  $P^+Q_A^-$  recombination kinetics of the resulting mutant were indistinguishable from those of wild type.

A unique cysteine was introduced at Ile(L150) in a fourth mutagenesis step. Based on the *Rb. capsulatus* RC homology structure<sup>13</sup>, Ile(L150) is located between the C and D transmembrane helices, at the N-terminus of a short helix along the periplasmic surface of the protein. It is moderately buried beneath the sidechains of both neighboring residues and residues in the loop between the A and B helices. All subcloning, expression and purification of RCs were performed as previously described.<sup>8,9</sup> Typical yields were 20-25 ODV per liter. Wild-type vectors and host strains were kindly provided by D. K. Hanson (Argonne National Laboratory).

*RC labeling:* 50-100 nmol of RCs in labeling buffer (0.05% Deriphath 160-C, 10 mM Tris pH 8, 300 mM NaCl), were incubated with 10 molar equivalents of DTNB for 8-16 hours at room temperature. TNB and excess DTNB were removed by size exclusion (PD-10, GE Biosciences). The RCs were then incubated in labeling buffer with 500 molar equivalents of KCN (<sup>12</sup>C or <sup>13</sup>C as noted) for 8-16 hours at room temperature followed by size exclusion purification. Reaction efficiency was calculated by measuring the absorbance at 412 of the TNB released from the second reaction. For infrared spectra, RCs were concentrated to 1-1.5 mM using Ultra-4 concentrators with 50 kDa MWCO (Millipore).

## VII. Stark Spectroscopy of SCN-RNase S

Vibrational Stark experiments were performed using 11 mM protein in 50% v/v glycerol water. This sample was pipetted into an infrared spectroscopy liquid cell consisting of a pair of 1-mm thick, 13-mm diameter sapphire windows (Meller Optics) with 40 Å of nickel vacuum deposited on the surfaces facing the sample<sup>8,9</sup>. The nickel electrodes were connected to a high voltage DC power supply (Trek Instruments Inc.), whose output voltage was synchronized to the FTIR scan timing with a home-built control unit. The windows were separated from each other by a pair of 26 micron thick Teflon spacers and held in place with a metal clamp. The sample was rapidly frozen by immersing the cell in a custom liquid nitrogen cryostat<sup>12</sup> to form a glass. Spectroscopy was carried out on a Bruker Vertex FTIR, with a nitrogen cooled indium antimonide detector and 1 cm<sup>-1</sup> resolution. 512 scans with an applied field of 1 MV/cm were alternated with 512 scans with no applied field. Field-off scans were averaged and subtracted from the averaged field-on scans. The Stark tuning rate reported is the average of three separate experiments.

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