Published in final edited form as:

Biochemistry. 2011 October 4; 50(39): 8311–8322. doi:10.1021/bi200930f.

Electrostatic Fields Near the Active Site of Human Aldose Reductase: 2. New Inhibitors and Complications due to Hydrogen Bonds†

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Abstract

Vibrational Stark effect spectroscopy was used to measure electrostatic fields in the hydrophobic region of the active site of human aldose reductase (*h*ALR2). A new nitrile-containing inhibitor was designed and synthesized, and the x-ray structure of its complex, along with cofactor NADP⁺, with wild-type *h*ALR2 was determined at 1.3 Å resolution. The nitrile is found to be in close proximity to T113, consistent with a hydrogen bond interaction. Two vibrational absorption peaks were observed at room temperature in the nitrile region when the inhibitor binds to wild-type *h*ALR2, indicating that the nitrile probe experiences two different microenvironments, and these could be empirically separated into a hydrogen bonded and non-hydrogen bonded population by comparison with the mutant T113A, where a hydrogen bond to the nitrile is not present. Classical molecular dynamics simulations based on the structure predict a double-peaked distribution in protein electric fields projected along the nitrile probe. The interpretation of these two peaks as a hydrogen bond formation-dissociation process between the probe nitrile group and a nearby amino acid side chain is used to explain the observation of two IR bands, and the simulations were used to investigate the molecular details of this conformational change. Hydrogen bonding complicates the simplest analysis of vibrational frequency shifts as being due solely to electrostatic interactions through the vibrational Stark effect, and the consequences of this complication are discussed.

The organization of charged, polar and polarizable residues in the tertiary structure of a protein creates large internal electrostatic fields, which are expected to affect many aspects of protein function (1–3). Vibrational Stark effect (VSE) spectroscopy has been developed in recent years as a strategy to measure the magnitude and direction of protein electrostatic fields (4–9). This method has been applied in several systems, including myoglobin (4), human aldose reductase (*h*ALR2) (5, 6), *Pseudomonas putida* ketosteroid isomerase (KSI) (7, 8) and ribonuclease S (RNase S) (9). *h*ALR2 is a particularly attractive target for such studies as a large number of very high resolution structures are available (10–12). *h*ALR2 is a 36 kDa aldo-keto reductase that catalyzes the conversion of aldehydes derived from

†This work was supported in part by a grant from the NIH (GM27738) to SGB. Portions of this research were carried out at the Stanford Synchrotron Radiation Light source, a national user facility operated by Stanford University on behalf of the U.S. Dept. of Energy, Office of Basic Energy Sciences. The SSRL Structural Molecular Biology Program is supported by the Dept. of Energy, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program, and the National Institute of General Medical Sciences.

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Supplementary Information Available at <http://pubs.acs.org/>

1. Analysis of Stark Spectroscopy Data
2. Crystallographic Data
3. Temperature Dependent IR Data
4. Parameterization of Inhibitor 2

carbohydrate metabolism to corresponding alcohols using NADPH as the reducing reagent (13, 14). Since its involvement in the polyol pathway is believed to be responsible for many long-term diabetic complications, researchers have been working for decades on developing tight-binding inhibitors of *h*ALR2 for potential pharmacological treatment of diabetes (10–20). Among a variety of *h*ALR2 inhibitors, there is a class of carboxylate-type inhibitors developed at the Institute for Diabetes Discovery (IDD), whose molecular scaffold allows for a variety of chemical modifications while still maintaining tight binding affinity (19). This provides us the starting point to design novel IDD-type inhibitors with Stark probes such as nitriles (5, 6) in order to study the electrostatic environment in *h*ALR2.

The quantitative basis of this approach derives from the relationship between changes in protein electric fields, $\Delta F_{protein}^t$ (in MV/cm) and observed molecular vibrational frequency shifts, $\Delta \bar{\nu}^{obs}$ (in cm^{-1}):

$$hc\Delta \bar{\nu}^{obs} = -\Delta \mu^r \cdot \Delta F_{protein}^t \quad (1)$$

where $|\Delta \mu'|$ (in units of $\text{cm}^{-1}/(\text{MV}/\text{cm})$) is the Stark tuning rate, a measure of the sensitivity of the transition frequency to an electric field, h is Planck's constant and c is the speed of light. We have shown that nitriles have a desirable combination of extinction coefficient in a relatively uncluttered region of the IR spectrum and a relatively large Stark tuning rate (21). Nitriles can be incorporated on unnatural or modified amino acids or inhibitors (5, 6, 22). The dot product in Eqn. 1 can be reduced to a measure of the projection of the projection of the protein electric field long the $-\text{CN}$ bond vector, $\Delta F_{||}^{t, protein}$ since $\Delta \mu'_{\text{CN}}$ has been shown to be parallel to the transition dipole moment which in turn is parallel to the $-\text{CN}$ bond (23). The magnitude of the Stark tuning rate, $|\Delta \mu'_{\text{CN}}|$, is obtained by measuring the vibrational Stark spectrum in an external electric field, either for closely related compounds in organic glasses or in the protein (9), and the direction of the $-\text{CN}$ bond is obtained by x-ray crystallography. Thus observed frequency shifts $\Delta \bar{\nu}_{\text{CN}}^{obs}$ can be translated into values for the projection of changes in the protein electric field along the $-\text{CN}$ bond axis, $\Delta F_{||}^{t, protein}$, and this can be compared with simulations that are widely used to estimate such fields.

In part I of this series, a nitrile probe was delivered to the hydrophobic specificity pocket of *h*ALR2 through the binding of an IDD-type inhibitor, **1**, (5-chloro-2-[(4-cyanobenzyl)amino]carbonyl]phenoxy) acetic acid that we prepared (Figure 1) (6). Changes in electrostatic fields due to mutations in the vicinity of the nitrile based on a modeled structure were quantified using nitrile stretching frequency shifts and compared with calculation results. According to the modeled structure of the *h*ALR2/1/NADP⁺ complex, a hydrogen bond donor from an amino acid side chain in the vicinity of the nitrile probe might complicate interpreting observed frequency shifts as perturbations in electric fields in the sense described by Eqn. 1, where $\Delta F_{protein}^t$ is meant to describe the electric field change due to a change in the organized environment, not specific, local chemical interactions such as hydrogen bonds, which may have an electrostatic component, but may also depend on overlap of the wavefunctions on the donor and acceptor, a contribution that is not included in Eqn. 1 (8, 9).

In the current work we have modified the molecular structure of **1** by introducing the nitro functionality to the meta position of the amine moiety (inhibitor **2**, (5-chloro-2-[(4-cyano-3-nitrobenzyl)amino]carbonyl]phenoxy) acetic acid (Figure 1)). This molecule was initially designed for the purpose of increasing inhibitor-binding affinity because it has been shown that adding a nitro group in the meta position increases the binding affinity of *h*ALR2 by 200-fold (19, 24). This modification also makes the orientation of the probe more unambiguous in the active site by preventing phenyl ring rotation. As described in the

following, the structure of the tertiary complex of wild-type (WT) *h*ALR2/2/NADP⁺ was determined by x-ray crystallography at 1.3 Å resolution. This structure shows that the nitrile probe is buried deep in the hydrophobic region of the binding pocket and well protected from water. It also shows that the probe forms a short contact with the side chain of residue T113. Interestingly, we found two IR peaks in the nitrile region when **2** bound to WT *h*ALR2, and IR spectra of two *h*ALR2 mutants helped to assign their origin. Classical molecular dynamics (MD) simulations were performed to provide a molecular-level understanding of observed IR features. The complications due to hydrogen bonds for using VSE spectroscopy to study protein electrostatic fields are discussed.

MATERIALS AND METHODS

A. Inhibitor synthesis

The nitrile-containing inhibitor **2**, (5-chloro-2-[[[(4-cyano-3-nitrobenzyl)amino]carbonyl]phenoxy] acetic acid was synthesized according to the procedure described in Scheme 1. All starting materials were purchased from Aldrich and used without further purification. All reactions were carried out under nitrogen protection. Products from each step were purified by either flash column chromatography (FCC) using silica gel (Aldrich, 230–400 mesh, 60 Å) or high performance liquid chromatography (HPLC, Shimadzu). Proton nuclear magnetic resonance spectra (¹H NMR) were determined on a 300 MHz Varian Gemini instrument, and mass spectra on ZQ single quadrupole electron spray mass spectrometry (MS).

4-cyano-3-nitrobenzyl bromide—2.4 g N-bromosuccinimide (NBS) and 180 mg azobisisobutyronitrile (AIBN) were added to a 16 mL solution of 2 g 4-methyl-2-nitrobenzonitrile in anhydrous carbon tetrachloride (CCl₄). The reaction was heated at 80 °C overnight. The solution was filtered to remove solids and put under vacuum to remove excess CCl₄. The residue was dissolved in ethyl acetate (EtOAc) and applied to FCC equilibrated in hexane. The product was eluted with 60% (v/v) EtOAc in hexane. The product was identified by the appearance of new peaks on NMR spectra: 4-methyl-2-nitrobenzonitrile (8.3 ppm, s, 1 H; 8.1 ppm, d, 1 H; 7.8 ppm, d, 1 H), *vs.* 4-cyano-3-nitrobenzyl bromide (8.5 ppm, s, 1 H; 8.2 ppm, d, 1 H; 8.1 ppm, d, 1 H; 4.9 ppm, s, 2 H).

4-cyano-3-nitrobenzyl azide—The bromide product was dissolved in 60 mL anhydrous dimethylformamide (DMF). 500 mg sodium azide was added in small portions over 10 min. The reaction was stirred at room temperature for 24 hr. Sodium bromide was removed by filtration through Celite, the filtrate was extracted with EtOAc and water, and the organic layer was dried over MgSO₄. The solids of MgSO₄ were removed by filtration, and excess solvent was removed under vacuum. The product was identified by the appearance of new peaks on the NMR spectrum of 4-cyano-3-nitrobenzyl azide (8.4 ppm, s, 1 H; 8.2 ppm, d, 1 H; 8.0 ppm, d, 1 H; 4.8 ppm, s, 2 H).

4-cyano-3-nitrobenzylamine—The azide product was dissolved in 12 mL mixture of tetrahydrofuran (THF) and water (v/v 10:1). 1.8 g triphenylphosphine was added in small portions over 10 min, and the reaction was stirred at room temperature overnight. Excess solvent was removed under vacuum, the residue dissolved in dichloromethane and extracted with 0.2 M HCl aqueous solution. The aqueous phase was combined and cooled to 0 °C, 1.0 M NaOH was added until the solution showed pH = 10, the product was extracted with dichloromethane, washed with saturated NaCl (aq.) and dried over MgSO₄. The solids were removed by filtration. The product was identified by NMR (8.4 ppm, s, 1 H; 8.1 ppm, d, 1 H; 7.9 ppm, d, 1 H; 3.9 ppm, s, 2 H; 2.1 ppm, s, 2 H) and MS (177.9 *m/z* (ES⁺), 178.2 g/mol (calculated)).

4-chloro-N-(4-cyano-3-nitrobenzyl)-2-hydroxybenzamide—The amine product was dissolved in 6 mL solution of 1.0 g 4-chloro-2-hydroxybenzoic acid and 100 mg hydroxybenzotriazole (HOBt) in anhydrous THF. A 2 mL solution of 1.1 g N,N'-dicyclohexylcarbodiimide (DCC) in THF was added dropwise over several minutes, and the reaction was stirred at room temperature overnight. Solids were removed by filtration. The residue was applied to reverse phase HPLC (30–60% acetonitrile in water, 5 mL/min, 30 min) and excess solvent was removed by lyophilization.

Ethyl (5-Chloro-2-[(4-cyano-3-nitrobenzyl)amino]carbonyl)phenoxy) acetate—The amide product was dissolved in 8 mL solution of 300 mg K₂CO₃ in anhydrous acetone and 200 μ L ethyl bromoacetate was added. The solution was heated at 56 °C overnight. 1.0 M HCl was added until the solution showed pH = 1, and the mixture was extracted with EtOAc. The organic phase was combined, washed with saturated NaCl and dried over MgSO₄. The solids of MgSO₄ were removed by filtration. Excess solvent was removed under vacuum. The residue was applied to reverse phase HPLC (30–60% acetonitrile in water, 5 mL/min, 30 min). Excess solvent was removed by lyophilization.

(5-chloro-2-[(4-cyano-3-nitrobenzyl)amino]carbonyl)phenoxy) acetic acid—The product was dissolved in 7.5 mL ethanol and treated with 1.2 mL 2.0 M NaOH. The solution was stirred at room temperature for 4 hr. 1.0 M HCl was added until the solution showed pH = 7. Most solvent was removed under vacuum. The concentrated solution of product is adjusted to pH = 1, and a yellow precipitate appeared, which was dissolved and extracted by EtOAc. The organic phase was combined and washed with saturated NaCl and dried over MgSO₄. The solids of MgSO₄ were removed by filtration. Excess solvent was removed under vacuum. The residue was further applied to reverse phase HPLC (40–50% acetonitrile in water, 10 mL/min, 60 min). Excess solvent was removed by lyophilization. The final product was a white powder and was characterized by NMR (9.3 ppm, t, 1 H; 8.4 ppm, s, 1 H; 8.1 ppm, d, 1 H; 7.9 ppm, d, 1H; 7.8 ppm, d, 1H; 7.3 ppm, s, 1H; 7.2 ppm, d, 1H; 4.9 ppm, s, 2 H; 4.7 ppm, d, 2 H) and MS (390.0 *m/z* (ES⁺), 389.7 g/mol (calculated).

B. Protein expression and purification

The expression and purification was conducted as described before (6). The gene for WT *hALR2* was obtained from Dr. Alberto Podjarny in the pET-15b expression vector (Novagen). Mutations were performed using the Quikchange mutagenesis kits (Stratagene) with PCR primers purchased from Elim Biopharmaceuticals, Inc. All plasmids were transformed into BL21(DE3) *E. coli* strain (Novagen). Cells were incubated at 37 °C for 3 hr. Expression was then induced by adding IPTG (1 mM), followed by an additional 4-hr growth. The cells were then pelleted, flash frozen and stored at –20 °C. To purify the protein, the cell lysate was resuspended and sonicated. After removing cell debris, the lysate was applied to a Ni-NTA agarose column (Qiagen), and His-tagged protein was eluted using an imidazole gradient. The His-tag was cut off using thrombin from bovine plasma (Aldrich). The cleaved product was loaded onto 5 mL Hitrap Q HP anion exchange column (GE Healthcare) and purified by fast protein liquid chromatography (FPLC) with a gradient elution (0–120 mM NaCl, 5 mL/min, 12 min). The purity of the final cleaved product was confirmed by SDS polyacrylamide gel electrophoresis and mass spectrometry.

C. Kinetics measurements on *hALR2*

Kinetic experiments were performed using DL-glyceraldehyde as substrate and NADPH as cofactor in 20 mM, pH=7, hepes buffer at room temperature as previously described (6). Reaction rates were measured by following the decrease in NADPH adsorption at 340 nm over 5 min. The data were fit to the Michaelis-Menten equation to determine k_{cat} . Inhibition curves were determined at saturating substrate concentration. The slope of the decrease in

NADPH absorption was divided by blank, and fit into Eqn. 2 to obtain the apparent inhibition constant,

$$\frac{v_i}{v_0} = \frac{E - I - K_I + \sqrt{(E - I - K_I)^2 + 4E \cdot K_I}}{2E} \quad (2)$$

where E is the enzyme concentration, I is the inhibitor concentration, and K_I is the apparent inhibition constant (25, 26). All data were averaged from two parallel experiments.

D. X-ray crystallography

The hanging drop method was used to obtain crystals (EasyXtal Tool 24 culture plates, Qiagen). WT *hALR2* was co-crystallized with the oxidized form of coenzyme NADP⁺ (Sigma) and inhibitor **2** at room temperature (ratios of protein/inhibitor/coenzyme = 1/2/2). Hanging drops were made by the mixing mother liquor (0.06 M citric acid, 0.04 M Bis-tris propane, 12% (w/v) PEG 3350, pH 4.2) with holoenzyme solution (10 mg/mL, 20 mM hepes, pH 7.0). Crystals were observed after three days of equilibration. Cryofreezing was carried out through quick transfer into stabilization solution (25% PEG 3350), and then into cryo-protecting solution (55% PEG 3350), and finally into liquid nitrogen. Experiments were carried out at x-ray beamline 9–2 at the Stanford Synchrotron Radiation Light source (SSRL). Datasets were processed with HKL2000 and PHENIX software packages. Data collection and refinement statistics are provided in supplementary material (Table S1). The PDB code is 3T42.

E. IR measurements on *hALR2*

IR spectra were measured with a Bruker Vertex 70 FT-IR spectrometer at both room temperature and low temperatures. Samples were illuminated through a notch filter which transmitted light of 2000–2500 cm⁻¹ and spectra were collected with a liquid nitrogen cooled indium antimonide detector at 1 cm⁻¹ resolution. For room temperature measurements, the sample cell was made of sapphire windows separated by 75 μm and 100 μm Teflon spacers. Solutions of holoenzyme (ratio of protein/2/NADP⁺ = 1/1/2) were equilibrated for 1 hr at 4 °C, then flushed with an excess of 20 mM, pH=7, hepes buffer and concentrated to a final concentration of 2 mM. Variable temperature IR spectra were obtained using a temperature controlled water-jacketed cell from Lake Shore Cryotronics, Inc. (OH, USA). Ethylene glycol was added to the water bath to get temperatures below 0 °C. The samples were prepared by dissolving lyophilized powder of 4 mM holoenzyme solution in equal volume of 40% (w/w) trehalose/water. The use of trehalose ensures a fluid state below 0 °C, and also forms an optical-quality glass at cryogenic temperature. Five minutes of equilibration at each temperature was allowed before collecting data. To obtain a spectrum at 77K, the sample cell was made of sapphire windows separated by 30 μm and 60 μm Teflon spacers. The cell was filled with a solution of 4 mM WT *hALR2* holoenzyme or a mixture of 4 mM WT *hALR2* and 4 mM T113A holoenzyme in 40% trehalose buffer and immediately immersed in liquid nitrogen in a custom cryostat (6). All spectra were averaged on three parallel measurements, and fit using one or two Gaussian functions with Matlab.

F. Stark spectrum of inhibitor **2**

The sample cell was made of sapphire windows coated with 4.5 nm of Ni separated by a pair of 30 μm spacers, and attached by copper wires to a high voltage DC power supply (Trek Instruments Inc.) (6). The cell was filled with a solution of 50 mM inhibitor **2** in 2-methyl tetrahydrofuran (MeTHF) and immediately immersed in liquid nitrogen in a custom liquid

nitrogen cryostat. Applied fields of 0.7 ~1.6 MV/cm were synchronized with a home-built control unit. 128 scans with the field applied were alternated with 128 scans with no applied field, and the field-on-minus-field-off difference spectrum was obtained. The spectra were fit as described previously (5, 6, 21). The Stark tuning rate of the nitrile probe on **2** reported is the average of three separate experiments.

G. Molecular dynamics simulations

The crystallographic structure of the *h*ALR2/2/NADP⁺ complex was used in MD simulations. Amino acid mutations were made using Pymol software (27). The calculation strategy used here follows closely the approach developed in our lab for the ribonuclease S system containing nitrile probes (9). Molecular dynamics simulations were carried out in Gromacs 3.3.1 using the AMBER-'99 force field (28). Inhibitor **2** and the cofactor were parameterized using the Antechamber and Leap package with GAFF atom type and AM1BCC charges (29). More details about the parametrization of **2** can be found in supplementary material (Figure S1, Table S3). The protein was solvated with explicit SPC water molecules (30). Non-bonded cutoffs of 10 Å were used. Simulations were carried out with periodic boundary conditions and the calculation of long-range electrostatics was treated with the particle mesh Ewald model. Simulations were equilibrated (20 ps energy minimization, followed by 20 ps of heavy-atom position restrained refinement) and run using the Nose-Hoover thermostat and the Parinello-Raman Barostat. Replica exchange molecular dynamics (REMD, 4 ns) were performed with 24 replicas from 298 to 339.45 K, ordered in temperature with a spacing of 1.75 K per replica. Electric fields at the midpoint of the nitrile bond were calculated at every 2 fs step for all MD trajectories. Atomic positions were saved at every 2 ps step only for the 298 K trajectories. Therefore, in the 4 ns 298 K trajectories, 2×10^6 electric field data were recorded and 2×10^3 structures were saved. Structures were taken every 60 ps from the 298K trajectories of the REMD simulation over the last 3 ns, generating 50 unique starting structures to run 50 independent parallel 1 ns simulations (50×1 ns). In 50×1 ns simulation, 25×10^6 electric field data were recorded; structures were not saved due to limited hard drive space. Electric field histograms were plotted using 50×1 ns simulation dataset (the vertical axis represent the percentage of electric field counts within a bin of 1 MV/cm, based on all 25×10^6 electric fields data), and fit using one or two Gaussian functions. Correlation plots between electric field data and geometrical parameters were based on 4 ns 298 K trajectories from the REMD simulations, with 2×10^3 data points, each representing one electric field value and the corresponding structural data at that time point.

RESULTS AND DISCUSSION

Catalysis and Structure

All proteins studied in this work displayed normal catalytic activity and strong binding affinity toward inhibitor **2** (Table 1, column 2, 3). For example, WT *h*ALR2 showed $k_{\text{cat}} = 0.33 \text{ s}^{-1}$, close to the result reported earlier (6, 14). **2** bound to WT *h*ALR2 with apparent inhibition constant $K_I = 107 \text{ nM}$; compared to $2 \text{ }\mu\text{M}$ for inhibitor **1** to WT *h*ALR2 (6), the binding affinity has been increased by approximately 20-fold. The fact that **2** binds strongly to WT and mutant *h*ALR2 ensures the feasibility of using this inhibitor to carry the probe into the protein active site for IR experiments and for x-ray crystallography¹.

¹We attempted to co-crystallize WT *h*ALR2 with inhibitor **1** (Figure 1). Only crystals of the *h*ALR2/NADP⁺ complex were obtained, likely reflecting the much poorer binding of **1** than **2** at the active site. Likewise soaking native crystals of *h*ALR2/NADP⁺ complex in the presence of **1** failed.

*h*ALR2 holoenzyme folds as an eight-stranded α/β -barrel structure (Figure 1). The active site is a well resolved region located at the C-terminal loop (Figure 2), and **2** binds similarly to previously studied *h*ALR2/IDD-inhibitor/NADP⁺ complexes (11, 12). The carboxylate moiety of **2** is tightly anchored via hydrogen bond interactions with Y48, H110, and W111, as well as electrostatic interaction with cofactor NADP⁺. There is also hydrophobic π -stacking interaction between the amine moiety of **2** and the W111 side chain. The nitro functionality forms a hydrogen bond with the L300 backbone amide, which accounts for the enhanced binding affinity of **2**, consistent with earlier work on nitro-substituted IDD inhibitors (24). The nitrile probe is buried in the hydrophobic interior and well protected from close-range interaction with solvent molecules. Short contact exists between the nitrile group and the T113 side chain (N...O distance is 2.84 Å), which is a common binding mode when an inhibitor opens the specificity pocket of *h*ALR2 (e.g. similar effects are seen for zopolrestat, IDD594 and IDD388 (18, 11, 31)). For residue T113, no sign of multiple side chain occupancy was observed in the electron density map.

IR Spectroscopy

The vibrational Stark spectrum of inhibitor **2** is shown in Figure 3A. The magnitude of the Stark tuning rate of the nitrile probe on **2** is found to be 0.53 cm⁻¹/(MV/cm), which is somewhat smaller than what has been observed previously for nitrile groups on phenyl rings (e.g. inhibitor **1** has a Stark tuning rate of 0.77 cm⁻¹/(MV/cm)) (5, 6). This is likely due to the electron withdrawing property of the adjacent nitro group. Detailed analysis of the Stark spectrum can be found in supplementary material.

FTIR spectra of **2** bound to WT and mutant *h*ALR2 were taken to obtain information on electrostatic fields and/or hydrogen bond interactions in the vicinity of the nitrile probe. As seen in Figure 3B and Table 1, columns 4 and 5, when **2** is bound to WT *h*ALR2, two peaks separated by about 14 cm⁻¹ were observed in the nitrile region at room temperature. Based on the short contact observed in the crystallographic structure with the hydroxyl group of T113, it seems likely that the higher-energy, more intense band is due to the hydrogen bonded form. To test this idea, two single point mutations were examined, also shown in Figure 3B. T113A, in which the side chain hydroxyl group is removed, displays a single symmetric peak whose frequency is close to the lower-frequency peak of WT. T113S, which also has a side chain hydroxyl group, displays a major peak close to mutant T113A, with a small but reproducible shoulder on the high energy side. These results are consistent with a well-documented result for simple nitriles in hydrogen-bonding and non-hydrogen bonding solvents: the nitrile frequency typically shifts to higher wavenumbers upon accepting hydrogen bonds from hydroxylic solvent molecules (32–45). Qualitatively, Purcell and Drago (32) have argued that when nitriles form a complex with Lewis acids (including a hydrogen bond), the electron lone pair is alleviated from the nitrogen non-bonding orbital which overlaps with nitrile bonding orbitals, resulting in an increase in the nitrile force constant and a blue shift in the nitrile stretch frequency.

To better understand the origin of the two peaks of WT *h*ALR2, we obtained IR spectra as a function of temperature (Figure 3C, Table S2). The high-frequency (hydrogen bonded) peak shifts to higher frequencies and narrows with decreasing temperature. The blue shift of the hydrogen bonded peak could be explained by the strengthening of hydrogen bonds at low temperatures (9), with an accompanying decrease in bandwidth due to an increasingly more restrained system. The low-frequency (non-hydrogen bonded) peak becomes broader and more asymmetric as temperature is lowered, possibly suggesting that there may be more than one non-hydrogen bonded configuration². The area ratio of the high-frequency peak

²The center frequency of the non-hydrogen bonded peak first shifts slightly to the blue and then to the red.

over low-frequency peak increases as the temperature is lowered³, and at 77K essentially only a single high-frequency peak is observed. This suggests that the hydrogen bonded form is more favorable at low temperatures. We also measured the IR spectrum for mutant T113A at the same sample concentration at 77K: a single peak, which is 17 cm⁻¹ lower in frequency than the WT peak, is observed. This suggests that in the WT spectrum, the high frequency peak at 77K should be attributed to the hydrogen bonded form. The area ratio of the nitrile peak of WT over T113A at 77K is 1.7 ± 0.1. It is reasonable to assume that most population goes to the hydrogen bonded form at 77K for **2** bound to WT hALR2, so we can get an approximate value of the ratio of the integrated peak area for hydrogen bonded and non-hydrogen bonded nitrile species at 77K. The result is consistent with previous reports of simple model compounds: the integrated intensities for the hydrogen bonded forms are higher than the non-hydrogen bonded forms. For example, the –CN stretch for CH₃CN has twice the integrated intensity in H₂O as in the pure solvent (33), and for HCN, the CN stretching intensity increases by a factor of 1.4 upon forming a complex with HF (34). Assuming that the ratio of intensities does not change drastically with temperature, the relative populations of the hydrogen bonded and non-hydrogen bonded forms of **2** in WT hALR2 at room temperature can be estimated from IR absorption. The area ratio of the two peaks at room temperature is 1.9 ± 0.3⁴, from which we estimate a ratio of the two species of 1.1, which can be used later for comparisons with room temperature MD simulations. We can calculate the equilibrium constant, K_{eq}, of the equilibrium:

at various temperatures. Fitting the temperature dependence of K_{eq} to the van't Hoff equation:

$$R \times \ln K_{eq} = -\frac{\Delta H^\ominus}{T} + \Delta S^\ominus \quad (3)$$

where R is the molar gas constant, ΔH[⊖] is the standard enthalpy change, and ΔS[⊖] is the standard entropy change, we can extract thermodynamic parameters for the reaction above. A van't Hoff plot is shown in Figure 3D, giving ΔH[⊖] = -9.4 kJ mol⁻¹ and ΔS[⊖] = -34 J mol⁻¹ K⁻¹. These results will be discussed later.

MD simulations and electrostatics – Wild Type

In order to further investigate the molecular origins of these spectra, we have performed classical MD simulations. Classical MD applies Newton's laws of motion to a group of interacting atoms using a variety of force fields. In hydrogen bond interactions, both electrostatic interactions (dominant at long range) and exchange repulsion (short range) are important components. At the outset, we attempt to employ a very simple model to calculate IR absorption spectral shifts using the Stark tuning rate as the bridge between calculated changes in electric fields and IR shifts: we extract information on electric fields from the MD output, and from this obtain information on the structures, dynamics and populations that relate to room temperature IR results. In the particular case of hydrogen bonds to nitriles, quantum effects are generally considered to be more significant than electrostatics (32–45). As mentioned earlier, Purcell and Drago explained the observed blue-shifts of nitrile frequency upon formation of hydrogen bonds by loss of an electron lone pair from the non-bonding orbital of the nitrile triple bond based on molecular orbital calculations (32).

³The total integrated area increases by about 1.3 times.

⁴The value used here is obtained in 20 mM hepes buffer. The use of trehalose changed the area ratio at 25 °C slightly, see Table S2.

Reimers and Hall noted that when there is strong and directional solvent-solute interaction, such as a hydrogen bond or proton transfer, this interaction will be of primary importance, and the nitrile frequency becomes less sensitive to its electrostatic environments (35). Therefore we note at the outset that modeling the results as entirely electrostatic in origin will incorrectly assign the spectral shifts of the hydrogen bonded and non-hydrogen bonded populations. However, this anticipated discrepancy does not imply that the force field used in the simulations is wrong, since the energies of a nitrile in MD is not determined solely by electrostatics. In the MD force field, the quantum effect due to orbital overlap in hydrogen bond interactions is represented by the r^{-12} term in the Lennard-Jones parameters. Specifically, the parameters in our simulations were calibrated by Corcelli *et al.* against a DFT calculation of the interaction between acetonitrile and water in a linear hydrogen bonding geometry (44). The validity of the structural and population information then largely depends on the transferability of these parameters into a chemically different system (different donor, acceptor and hydrogen bond configurations), which can be tested by comparing simulated spectral features with experimental results. In our case, the most direct linkage between them is the population information. That is, if the populations that emerge from our analysis results are similar to those observed experimentally, this would suggest that the Lennard-Jones parameters are valid so that the energetics of a nitrile falls into a reasonable regime.

Classical MD simulations on the ns time scale were performed, and electric fields at every MD step were recorded giving a histogram of protein electric fields projected along the nitrile probe, as shown in Figure 4 and Table 2, columns 2 and 4. MD predicts a double-peaked field distribution for WT *h*ALR2, consistent with the two peaks observed in the room temperature IR spectrum (Figure 3B). For T113A, MD predicts a single peak, again consistent with the single peak observed for T113A. Although MD will not identify the two peaks in the WT spectrum, we can deduce their identity through the comparison with T113A: the single peak of T113A is close to the more positive field peak of WT *h*ALR2; therefore this peak is the non-hydrogen bonded peak, while the more negative field peak is the hydrogen bonded peak. More quantitatively, the MD simulations predict that in WT *h*ALR2, the population ratio of hydrogen bonded and non-hydrogen bonded is 1.5, which is quite close to the experimental value estimated above to be 1.1. As for T113S, MD predicts a double-peaked field distribution, and the area ratio of the more positive peak is larger compared to WT *h*ALR2.

With the basic confirmation that the simulations provide reasonable agreement for the populations and fields, we can exploit the MD trajectories to further investigate the microscopic interactions between individual atoms that lead to the field distributions in a given protein, something that is not possible from the experiments. Specifically, we extracted structural information from the MD output, and used this to establish correlations between computed electric fields and geometric parameters. Figure 5A shows the dependence of the electric field projected on the inhibitor –CN bond on the dihedral angle representing T113 side chain rotation for WT *h*ALR2. The relatively constant angle values indicate that the T113 side chain conformation does not change much during course of the simulation. As seen in Figure 5B, however, the two-distance correlation (the distance between T113 hydroxyl hydrogen atom and the nitrogen atom of the probe, the distance between the hydrogen atom and the oxygen atom of the T113 carbonyl group) displays a two-state behavior. To explain this, we propose a model of a hydrogen bond motif involving one donor and two acceptors. This is based on the observation of a short contact between the nitrile probe and T113 hydroxyl group (as seen in the low temperature x-ray structure), and also on the fact that threonine (serine also, see below) is frequently found to form an intra-residue hydrogen bond (11, 46). Dynamically, the hydroxyl group switches between hydrogen bonds to the nitrile probe and the amide carbonyl group of T113. Figures 5C and

D show the correlation between the computed electric fields and the angle and distance parameters of the hydrogen bond between the T113 hydroxyl group and the nitrile probe. In the field-angle correlation, the two-state behavior is seen again. All of the electric field data are sorted in Figure 5E by using geometrical criteria for hydrogen bond formation (angle $\leq 60^\circ$, distance $\leq 3.5 \text{ \AA}$, see figure caption, and Figure 5F which shows snapshots). This results in two distinguished peaks. The more negative field peak corresponds to hydrogen bonded nitrile species, the other to non-hydrogen bonded species. This is consistent with the observation that the single peak of T113A on the electric field histogram is close to the more positive field peak of WT *h*ALR2. As expected, the electrostatic model we employed predicts a red-shift in frequency of the nitrile probe upon hydrogen bond formation, failing to capture the blue-shift observed in IR experiments due to the dominating quantum effects in nitrile hydrogen bond formation. Finally, the calculated lifetimes for the two species are both on the picosecond time scale (hydrogen bonded 9.7 ps, non-hydrogen bonded 6.2 ps) (47). In summary, by analyzing the MD trajectories for WT *h*ALR2, we propose that the source of the two peaks in the protein electric field distribution projected on the probe –CN bond is the dynamical making and breaking of a hydrogen bond between the nitrile probe and T113 side chain hydroxyl group, which viewed from the nitrile IR band gives rise to the two peaks seen in the IR spectrum, and further that the T113 hydroxyl group is always hydrogen bonded, either to the inhibitor nitrile group or to its amide carbonyl group.

Following this analysis, the thermodynamic parameters obtained earlier actually correspond, in the context of the entire protein/inhibitor complex, to the equilibrium between two types of hydrogen bond, a hydrogen bond between T113 and its amide carbonyl group and between T113 and the inhibitor nitrile:

From other studies, hydrogen bond energies are typically about 10 to 30 of kJ mol^{-1} (48–53). The enthalpy change obtained from the van't Hoff plot in Figure 3D for the difference between a hydrogen bond with the nitrile as the acceptor and one with amide carbonyl as the acceptor, $\Delta H^\ominus = -9.4 \text{ kJ mol}^{-1}$, is reasonable in terms of order of magnitude. The negative sign indicates that nitrile nitrogen atom is a better hydrogen bond acceptor than the amide oxygen atom in this case, although previous studies, e.g. *ab initio* calculations (50) and Abraham's empirical hydrogen bond basicity scale (54) suggested the contrary. This discrepancy is likely due to an idiosyncratic behavior of this restrained system. The negative entropy change ($\Delta S^\ominus = -34 \text{ J mol}^{-1} \text{ K}^{-1}$) implies that the configuration on the left in Figure 5F is less disordered.

MD simulations and electrostatics – T113S

For T113S, a similar approach was carried out, and the results suggest both similarities and differences between this mutant and WT *h*ALR2. We first examine the dependence of electric fields on the S113 side chain rotation. As seen in Figure 6A, the dataset is composed of two populations, denoted state 1 and state 2. Comparing with threonine in WT *h*ALR2, in which the ©-methyl group largely blocks side chain rotation (Figure 5A), the serine side chain is more flexible in T113S, occupying two possible conformations, as illustrated in Figure 6D (state 1) and F (state 2). By sorting electric field data according to the dihedral angle parameter (Figure 6B), we find that the two populations contribute differently to the electric field distribution. The prediction of multiple side chain conformations for S113 is consistent with a recent paper on crystallographic studies of *h*ALR2 mutants bound with IDD inhibitors with halo substituents on phenyl para sites (31). The two side chain conformations of S113 in our system predicted by MD simulations resemble the two side chain conformations of S113 detected in structures of T113S/IDD383 or IDD594

complexes, and the predicted percentages of two configurations as depicted in Figure 6D and F are close to the measured fractional occupancies of S113 side chain in crystal structures of T113S/IDD383 or IDD594 complexes⁵. In state 1, there is a similar hydrogen bond motif as in WT *h*ALR2. In this case, the imidazole nitrogen atom in H306 acts as one hydrogen bond acceptor, while the other acceptor is the nitrogen atom on the probe nitrile, and the hydrogen bond donated by the S113 side chain hydroxyl group switches between them dynamically. If we use the same hydrogen bond criteria (angle $\leq 60^\circ$, distance ≤ 3.5 Å) to sort electric field data for state 1 (Figure 6C), the peak at a more negative electric field corresponds to the S113–OH...**2**–CN sub-species; the other correspond to the S113–OH...H306–imidazole sub-species. Snapshots for the two sub-species in state 1 are given in Figure 6D. In the case of state 2, there is also a similar hydrogen bond motif, in which the donor is the S113 side chain hydroxyl group, one acceptor is the nitrogen atom on the probe nitrile, but the other acceptor is the amide carbonyl oxygen atom of S113. Electric field data are sorted using the same criteria (angle $\leq 60^\circ$, distance ≤ 3.5 Å), and two separated peaks are seen again (Figure 6E). Snapshots for the S113–OH...**2**–CN species and S113–OH...S113–C=O species in state 2 are given in Figure 6F, which has a similar configuration as WT *h*ALR2 (*c.f.* Figure 5F). Overall, this could explain the IR spectrum of **2** bound to T113S - a major peak with a high-frequency tail, attributed to hydrogen bonding, but more complicated than WT: unlike WT *h*ALR2, in T113S, the serine residue displays two possible side chain conformations, each interpreted as a hydrogen bond making and breaking process, and state 1, the dominant configuration, supports a higher ratio of non-hydrogen bonded nitrile species.

Concluding comments on complications due to hydrogen bonds

As discussed earlier, many experiments reveal that the nitrile stretch frequency shifts to higher frequencies upon accepting a hydrogen bond. Using a purely electrostatic model, when the –CN bond interacts with a hydroxyl group in a configuration as in Figure 5F, the difference dipole moment of the nitrile bond $\Delta\mu_{CN}'$ (pointing from the nitrogen atom to carbon atom) is aligned along the electric field exerted by the hydroxyl dipole, and a red shift in energy is expected according to Eqn. 1. As a result, this purely electrostatic model predicts a frequency shift in the opposite direction to what is observed when a hydrogen bond is formed. For the same reason, when there is hydrogen bond interactions between the nitrile probe and surrounding environment, the idea of using the nitrile as a Stark probe to measure electrostatic fields in proteins based solely on Eqn. 1 is questionable: environment-induced frequency shifts may no longer respond linearly to electric fields, but are more dominated by the quantum mechanical nature of such interactions, and these effects may depend upon details of the geometry of a particular hydrogen bond (35, 45, 55). We recently presented a method to separate effects of hydrogen bonds on IR frequency shifts from environmental electrostatic contribution in the case of –SCN probes by correlating the ¹³C NMR chemical shift and the IR frequency (56). This approach is relatively simple to apply to –SCN probes as ¹³C can be directly introduced during labeling, but it would be more difficult to apply to a complex inhibitor such as **2** due to synthetic effort, though we are currently developing a synthetic strategy that may allow facile preparation of ¹³C labeled versions of aromatic nitrile-containing inhibitors. One interesting application would be using ¹³C labeled inhibitor **1** to acquire tandem IR and NMR measurements in the systems studied in part I of this series, where an x-ray structure could not be obtained, so that we can detect whether or not the nitrile probe accepts hydrogen bonds from nearby amino acid residues in the absence of crystallographic structures. If a comparison is made within a

⁵In our system, the predicted percentages of state 1 and state 2 are 72% and 28%, respectively. In the crystal structure of T113S/IDD383 complex, the percentages of the two corresponding configurations are 72% and 28%; and in the T113S/IDD594 complex, they are 68% and 32% (31).

series of mutants, pH changes or inhibitor changes, and their added electrostatic contribution is the goal, then a demonstration that a hydrogen bond is retained or lost by the NMR/IR correlation is very useful for vetting the data. It would also be interesting to measure $|\Delta\mu'_{CN}|$ for both hydrogen bonded and non-hydrogen bonded populations to see whether the Stark tuning rate is affected by a hydrogen bond. It has been shown in one case that hydrogen bonded nitrile probes show similar Stark tuning rate to non-hydrogen bonded ones (56). We attempted to measure the vibrational Stark effect of inhibitor **2** *in situ* in WT *h*ALR2, but the relatively low extinction coefficient and limited solubility of *h*ALR2 in buffered solution precluded this measurement.

While the interpretation of frequency shifts of electric fields with calculated shifts may be compromised when the nitrile is hydrogen bonded, in cases where the nitrile is not hydrogen bonded, we can compare changes in electric fields ($\Delta F_{\parallel}^{protein}$) measured by IR given the Stark tuning rate with predictions made by MD (Table 2, column 3, 5). It can be seen that the calculation strategy performs well for WT *h*ALR2 and T113S in terms of the direction, but not in magnitude. Treating the linewidth as due to inhomogeneous broadening from the distribution of local electric fields interacting with $\Delta\mu'_{CN}$, the simulation reproduces the observed trend for the three non-hydrogen bonded peaks (Table 2, column 4, 6), i.e. T113A shows the narrowest FWHM while WT *h*ALR2 shows the broadest FWHM. Thus, the basic computational strategy is qualitatively successful, but not quantitatively, likely due errors in the force field and/or insufficient sampling in the MD simulations. There are limited examples in the literature demonstrating the improvement of force fields in pursuit of precise structural feature assignment based on electric field calculations, but several important examples have been presented using the system of carbon monoxide photodissociation in myoglobin (57–60). A genuine QM/MM simulation might be able to better capture the properties of the hydrogen bonded populations. This system, which contains a hydrogen bonded and non-hydrogen bonded nitrile in the same macromolecular system, can serve as a test system for benchmarking advanced simulation methodology, and the independent predictions of the timescale for making and breaking hydrogen bonds can, in principle, be tested using 2D IR techniques (61). Such experiments are currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Aaron Fafarman and Lauren Webb for useful discussions on all aspects of this work. We thank Prof. Michael Van Zandt for useful discussions on inhibitor design. We also thank Dr. Sushant Malhotra for helpful discussions on inhibitor synthesis pathway, and Dr. Jorge Zuniga from the Brunger lab at Stanford for assisting us to set up robot screening trays to obtain the initial crystallization conditions.

Abbreviations

<i>h</i>ALR2	human aldose reductase
VSE	vibrational Stark effect
IR	infrared
IDD	Institute for Diabetes Discovery
FCC	flash column chromatography
DMF	dimethylformamide

MeTHF 2-methyltetrahydrofuran
MD molecular dynamics

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Figure 1.

Structural model of x-ray data for the *h*ALR2/2/NADP⁺ complex. The cofactor NADP⁺ is shown red, inhibitor **2** in yellow, and the nitrile probe is highlighted in green. The structure of inhibitor **1**(6) is shown in the upper left.

Figure 2.
Electron density map for the inhibitor binding pocket ($2F_{\text{obs}} - F_{\text{calc}}$ contoured at 1 sigma in blue mesh), identifying relevant residues involved in inhibitor binding.

Figure 3.

(A) Vibrational Stark spectrum of inhibitor **2** dissolved in MeTHF at 77K. The black curve is the difference spectrum (field on minus field off) scaled to sample an absorbance of 1 and an applied field strength of 1.0 MV/cm. The purple, blue and green curves represent, respectively, contributions from the zeroth, first and second derivative of the vibrational absorption spectrum of **2**. The red curve is the overall fit, and the second derivative contribution gives: $|\Delta\mu'_{CN}| = 0.53 \text{ cm}^{-1}/(\text{MV}/\text{cm})$. More detailed analysis of the Stark spectrum can be found in supplementary material. (B) Vibrational absorption spectra in the nitrile region of **2** in the active sites of WT and two mutants of hALR2 at room temperature. (C) Temperature dependence of IR absorption spectra of **2** bound to WT hALR2 in 40% trehalose buffer and compared with the low temperature absorbance of **2** bound to the T113A mutant. (D) van't Hoff plot derived from variable temperature IR spectra.

Figure 4. Calculated electric field histogram projected on the nitrile probe in WT and mutant *hALR2* bound to **2** extracted from MD simulations and using Eqn. 1 (*c.f.* Figure 3B).

Figure 5. MD trajectory analysis for WT *hALR2*

(A) Correlation of the calculated electric field projected on the inhibitor -CN bond with the dihedral angle formed by T113-C, -CA, -CB, and -OG1. Each data point represents one electric field value and the corresponding structural data at that time point; (B) correlation of two distances in the MD simulation: the distance between T113-HG1 and INH-N2, and the distance between T113-HG1 and T113-O (see inset panel A for labeling); (C) correlation of calculated electric field projected on the inhibitor -CN bond and the angle formed by T113-HG1, -OG1 and INH-N2; (D) correlation of calculated electric field projected on the inhibitor -CN bond and distance between T113-OG1 and INH-N2; (E) calculated electric fields projected on the inhibitor -CN bond sorted according to hydrogen bond geometrical criteria: the red solid curve represents the whole dataset (2001 data points from 4 ns REMD); the black dashed curve represents configurations in which the inhibitor nitrile is hydrogen bonded while the carbonyl group of T113 is non-hydrogen bonded (61%, angle $\leq 60^\circ$, distance ≤ 3.5 Å), and the blue dashed curve represents configurations in which the nitrile probe is non-hydrogen bonded while T113 carbonyl group is hydrogen bonded (39%); (F) detailed representation of the T113-OH...2-CN species (left) and T113-OH...T113-C=O species (right).

Figure 6. MD trajectory analysis of *hALR2* T113S

(A) Correlation of electric fields with the dihedral angle formed by S113-C, -CA, -CB, and -OG; (B) electric fields sorted according to serine side chain conformation. The blue curve represents full dataset (2001 data points). The yellow curve (71.8%) is the species called state 1. The pink curve represents (28.2%) state 2; (C) electric fields in state 1 sorted according to the hydrogen bond formation criteria. The light blue solid curve represents the entire data set (1436 data points); the red dashed curve represents sub-configurations in which the inhibitor nitrile is hydrogen bonded while the imidazole nitrogen atom of H306 is non-hydrogen bonded (23%, angle $\leq 60^\circ$, distance ≤ 3.5 Å); and the blue dashed curve represents sub-configurations in which the nitrile probe is non-hydrogen bonded while the imidazole nitrogen atom of H306 is hydrogen bonded (77%); (D) S113-OH...2-CN sub-species in state 1 (left) and S113-OH...H306-imidazole sub-species (right); (E) electric fields in state 2 sorted according to the hydrogen bond formation criteria. The peak solid curve represents the full dataset (565 data points). The red dashed curve represents sub-configurations in which the inhibitor nitrile is hydrogen bonded while the carbonyl group of S113 is non-hydrogen bonded (63%, angle $\leq 60^\circ$, distance ≤ 3.5 Å). The blue dashed curve represents sub-configurations in which the nitrile probe is non-hydrogen bonded while S113 carbonyl group is hydrogen bonded (37%); (F) S113-OH...2-CN sub-species in state 2 (left) and S113-OH...S113-C=O sub-species (right).

Scheme 1.

Synthetic pathway of **2**, (5-chloro-2-((4-cyano-3-nitrobenzyl)amino)carbonyl)phenoxy acetic acid.

Measured kinetics constants, nitrile vibrational frequency and full width at half maximum (FWHM) of inhibitor **2** in the specificity pocket of WT and mutant *h*ALR2 at room temperature.

Table 1

Sample	k_{cat} (s^{-1})	K_I (nM)	Frequency (cm^{-1})		FWHM (cm^{-1})	
buffer	---	---	2242.2 $\pm$ 0.1		10.6 $\pm$ 0.1	
T113A	0.20 $\pm$ 0.01	30 $\pm$ 1	2237.8 $\pm$ 0.1		5.8 $\pm$ 0.1	
WT	0.33 $\pm$ 0.01	107 $\pm$ 7	2238.2 $\pm$ 0.1	2252.3 $\pm$ 0.1	11.4 $\pm$ 0.4	9.5 $\pm$ 0.2
T113S	0.19 $\pm$ 0.01	32 $\pm$ 1	2238.8 $\pm$ 0.1	2247.4 $\pm$ 0.3	6.4 $\pm$ 0.1	8.2 $\pm$ 1.1

Table 2

Calculated peak position and FWHM for electric field distribution along the nitrile probe in WT and mutant *hALR2*. Measured and predicted changes in electric fields (non-hydrogen bonded peaks *vs.* mutant T113A) and FWHM. The measured $\Delta F_{\parallel}^{protein}$ and FWHM are calculated using the observed frequency shifts and the Stark tuning rate of **2** in MeTHF, $|\Delta\mu_{CN}'| = 0.53 \text{ cm}^{-1}/(\text{MV}/\text{cm})$.

Unit: MV/cm	MD			Experiment	
	Position	$\Delta F \rightarrow_{\parallel}^{protein}$	FWHM	$\Delta F \rightarrow_{\parallel}^{protein}$	FWHM
WT	-15.6	+0.5	23.6	+0.8	21.5
	-41.9	---	22.1	---	---
T113A	-16.1	---	15.2	---	10.9
T113S	-7.4	+8.7	20.8	+1.9	12.1
	-41.5	---	24.5	---	---