Electronic Supplementary Material (ESI) for Chemical Communications

SUPPORTING INFORMATION

Direct Spectroscopic Evidence for Binding of Anastrozole to the Iron Heme of Human Aromatase. Peering into the Mechanism of Aromatase Inhibition

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Experimental details

Cloning, expression and purification of aromatase

Human aromatase cDNA (NM_000103.2) was modified and cloned directly into a pCW Ori+ vector. Amino acids 1-39 at the N-terminus of the protein sequence were deleted and replaced by a short string of positively charged, hydrophilic amino acids (MAKKTSSKGR) as previously done by Hong and co-workers.[1] At the C-terminus a four-histidine tag was introduced to facilitate purification by affinity chromatography.

The expression of recombinant aromatase was carried out in *Escherichai coli* strain DH5α. Cells were resuspended in 100 mM potassium phosphate buffer pH 7.4 containing 20% glycerol, 0.1% Tween 20 and 1 mM β-mercaptoethanol and disrupted by sonication. For the purification of aromatase-anastrozole complex, a saturating amount of the inhibitor (1 μM) was added in all the buffer used during the purification. After ultracentrifugation, the supernatant containing the soluble protein was loaded onto a diethylaminoethyl ion-exchange column (DEAE sepharose Fast-Flow, GE healthcare) followed by a Nickel-ion affinity column (Chelating sepharose Fast-Flow, GE healthcare). The protein was concentrated using a 30kDa Ultracel centrifugal device (Amicon, Millipore) and stored at -20°C.

Sample characterization

UV-Visible measurements were performed with a diode array spectrophotometer (Agilent 8453E, Agilent Technologies). The spectra of purified aromatase (1 μM) in 100 mM potassium phosphate

buffer pH 7.4, 20% glycerol, 0.1% Tween 20, 1 mM β-mercaptoethanol were recorded in absence and presence of 1 μM anastrozole in a 1 cm pathlength cuvette.

The protein concentration of purified aromatase was estimated using reduced CO-difference spectra. The CO binding assay was performed at 30 °C by following the increase of the signal at 450 nm after complete reduction of rArom with 12.5 mM sodium dithionite and bubbling with CO. The iron (II)-CO complex amount was calculated by using an extinction coefficient at 450 nm of 91,000 M^{-1} cm^{-1}.[2]

X-band CW EPR spectra were recorded at 77K on a Bruker EMX spectrometer (microwave frequency 9.75 GHz) equipped with a cylindrical cavity. A microwave power of 10 mW, modulation amplitude of 0.3 mT and a modulation frequency of 100 KHz was used.

Pulse EPR experiments were performed on an ELEXYS 580 Bruker spectrometer (at the microwave frequency of 9.76 GHz) equipped with a liquid-helium cryostat from Oxford Inc. All pulse EPR experiments were performed at 7 K.

Electron-spin-echo (ESE) detected EPR experiments were carried out with the pulse sequence: $\pi/2-\ \tau-\ \pi-\ \tau-$echo, with microwave pulse lengths $t_{\pi/2}$ = 16 ns and t_{π} = 32 ns and a τ value of 160 ns.

Hyperfine Sublevel Correlation (HYSCORE) experiments[3] were carried out with the pulse sequence $\pi/2-\ \tau-\pi/2-t_1-\pi-t_2-\pi/2-\tau-$ echo with the microwave pulse length $t_{\pi/2}$ = 16 ns and t_{π} = 32 ns. The time intervals t_1 and t_2 were varied in steps of 16 ns starting from 96 ns to 4704 ns. A τ value of 176ns was chosen. A four-step phase cycle was used for eliminating unwanted echoes. The time traces of the HYSCORE spectra were baseline corrected with a third-order polynomial, apodized with a Hamming window and zero filled. After two-dimensional Fourier transformation, the absolute value spectra were calculated. The HYSCORE spectra were simulated using the Easyspin program. [4] The same τ value was chosen for the simulation as for the experiment. The simulation of the aromatase-anastrozole complex (Figure S3) were carried out considering the three spin system S=1/2, I=1, I=1.

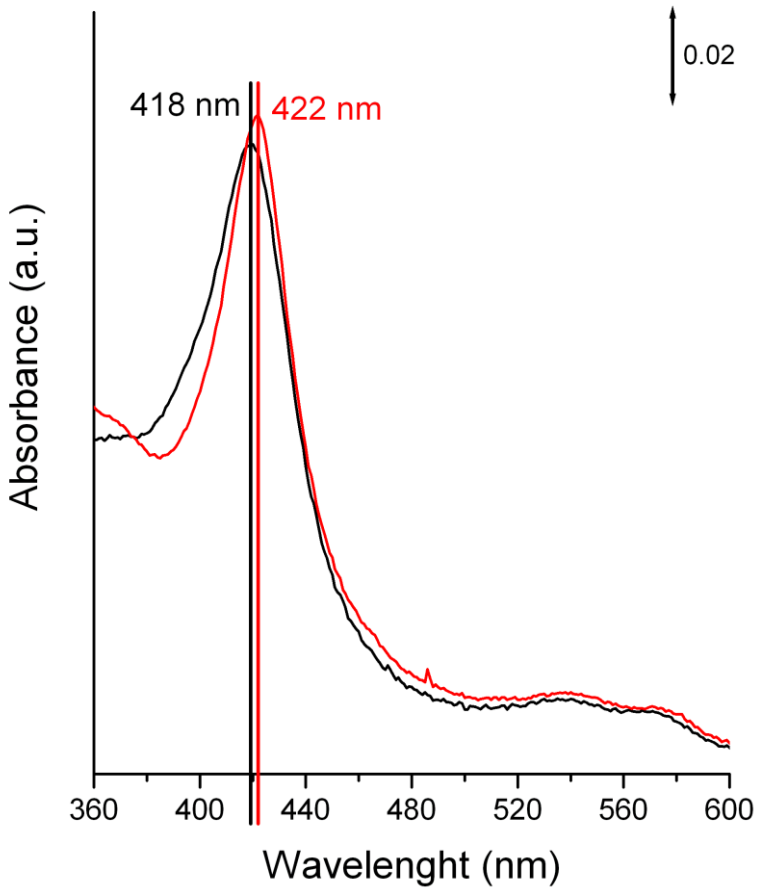

Figure S1. UV-Vis spectra of aromatase (black line) and aromatase co-purified with a saturatine amount of anastrozole (red line).

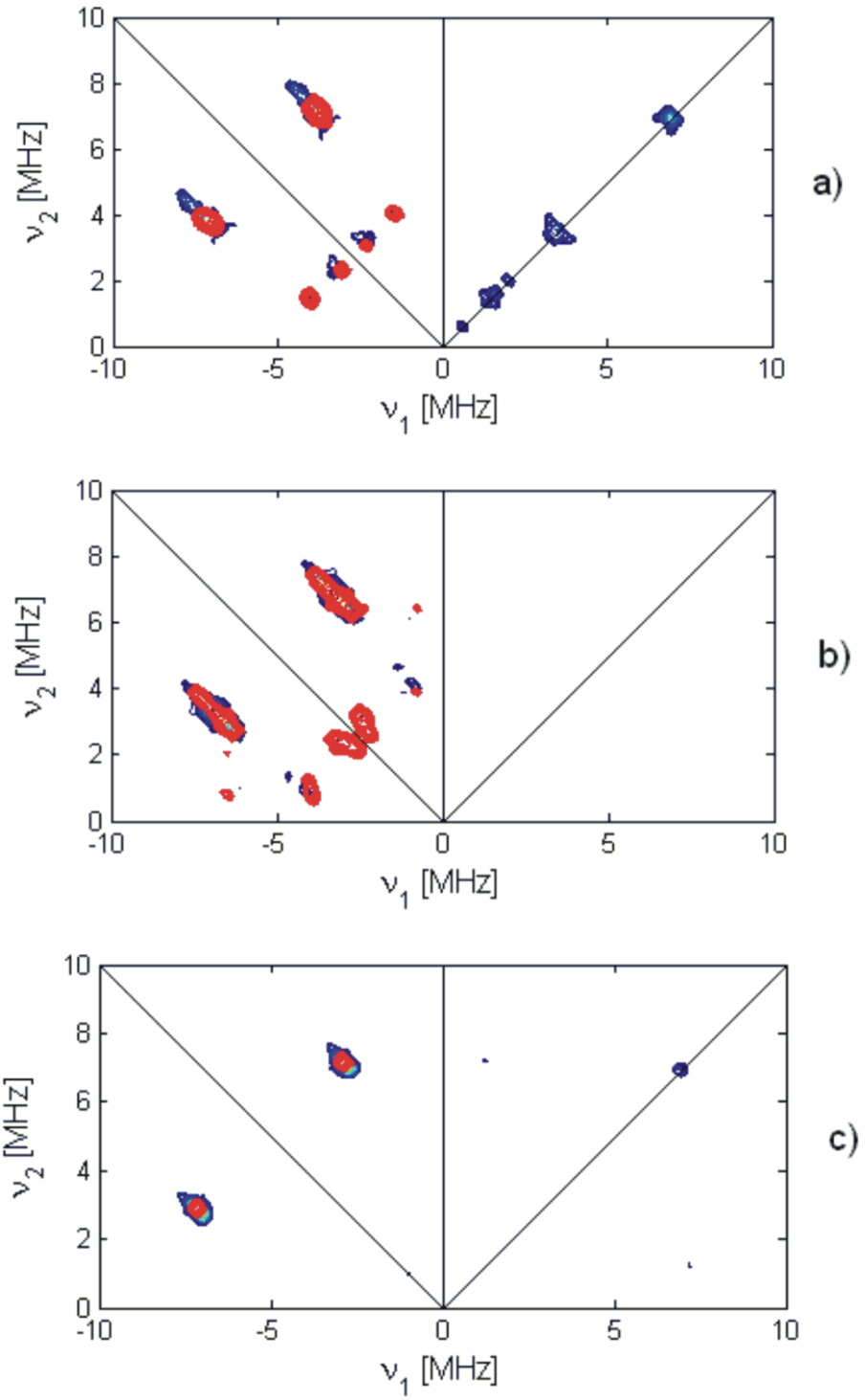

Figure S2. Experimental (blue) and simulated (red) ^{14}N HYSCORE spectra of aromatase taken at the field positions corresponding to (a) g_z (280.0 mT), (b) g_y (309.5 mT) and (c) g_x (365.5 mT) features. Both experimental and simulated HYSCORE spectra are taken at τ value 176 ns. The simulated parameters are listed in Table 1.

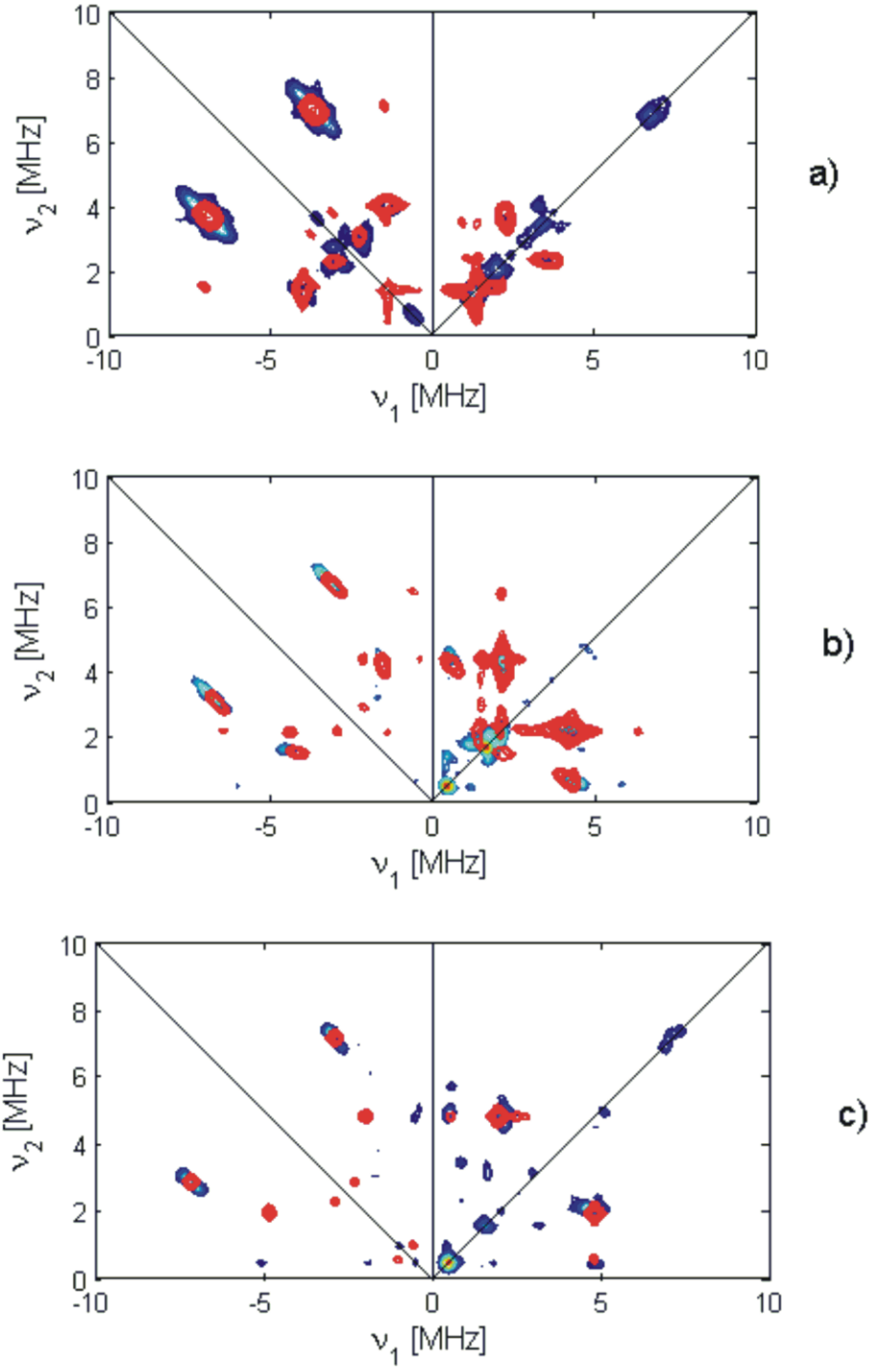

Figure S3. Experimental (blue line) and simulated (red line) ^{14}N HYSCORE spectra of aromatase co-purified with a saturating amount of anastrozole taken at the field positions corresponding to (a) g_z (280.0 mT), (b) g_y (309.5 mT) and (c) g_x (365.5 mT) features. Both experimental and simulated HYSCORE spectra are taken at τ value 176 ns. The simulated parameters are listed in Table 1.

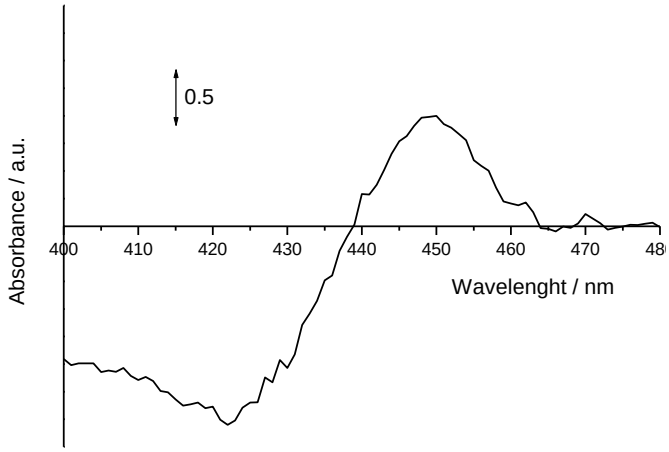

Figure S4 UV-Vis difference spectrum of reduced human aromatase in complex with anastrozole in the presence and in the absence of CO.

The UV-Vis difference spectrum of reduced human aromatase in complex with anastrozole in the presence and in the absence of CO reported in Figure S4, shows a negative peak at 422 nm and a positive peak at 450 nm demonstrating the displacement of the inhibitor by CO. Such a shift is typical of P450s in the presence of inhibitory nitrogen containing ligands [5] Importantly, it is known from the literature that coordination of histidine residues, created for example by engineering cytochrome P450 BM3, is essentially irreversible [6] and no such shift is observed. This observation, together with the large distance of all histidine residues from the heme (Table S1), allow excluding the possibility that the observed ^{14}N couplings observed in the HYSCORE spectrum of the anastrozole-aromatase complex (Figure 2) can arise from an endogenous histidine.

Table S1. Distances between the Nε atoms of the 13 residues present in aromatase and the iron atom. Values obtained from analysis of the 3D crystal structure of human aromatase (PDB 3EQM) by the software UCSF Chimera

Residue	Distance from the iron atom (Å)
His10	N-terminal >30
His62	24
His105	24
His109	23
His111	22
His128	20
His171	21
His325	25
His402	13
His459	31
His475	17
His480	15
His503	C-terminal >30

References

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