SUPPORTING INFORMATION

Do Ligand Binding and Solvent Exclusion Alter the Electrostatic Character Within the Oxyanion Hole of an Enzymatic Active Site?

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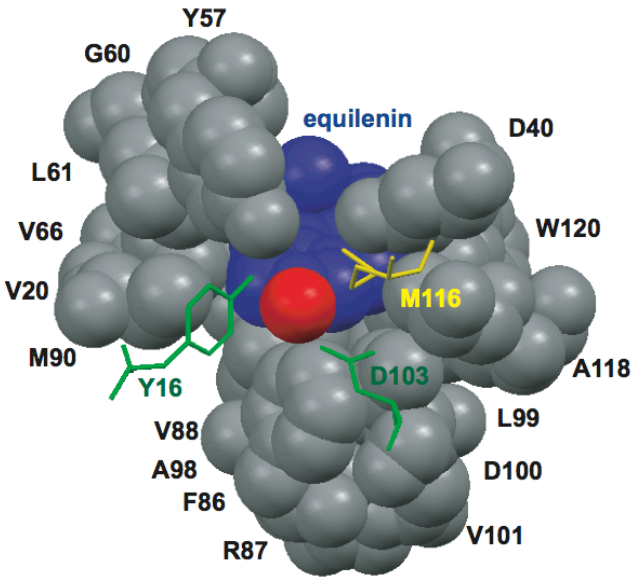
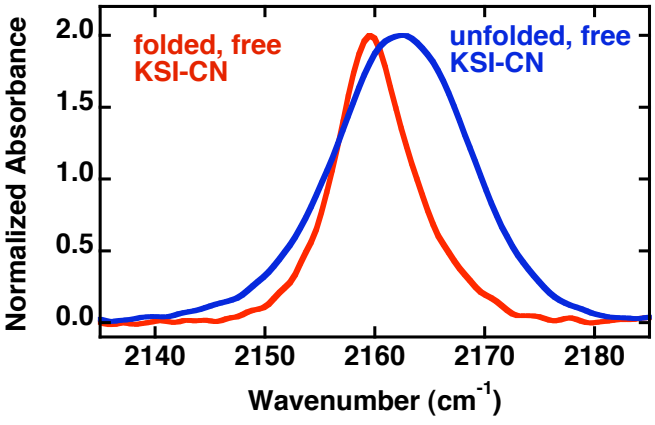
I. Figure S1



Space-filling x-ray structural model of equilenin bound to the KSI active site (PDB 1OH0), showing the dense packing of side-chains (grey) around the bound steroid (blue) and the location of M116 (yellow), residue at which the –CN probe was inserted, proximal to H-bond donors Y16 and D103 (green) and the equilenin oxygen (red). Other groups that surround the M116 side chain have been omitted for clarity.

II. Figure S2



IR absorption spectra of free, folded KSI-CN (peak center 2159.6 cm^{-1}, FWHM = 8 cm^{-1}) and free, unfolded KSI-CN in 5.3 M urea (peak center 2162.5 cm^{-1}, FWHM = 14 cm^{-1}).

III. Experimental Methods

A. Materials

All reagents were of the highest purity commercially available (≥ 97%). 4-F-3-CH_3-phenol, 2-naphthol, potassium cyanide (KCN), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich, and equilenin and 19-nortestosterone were from Steraloids. All buffers and solutions were prepared with reagent grade chemicals or better.

B. KSI Mutagenesis

The plasmid encoding *Pseudomonas putida* KSI was a gift from Kwan Yong Choi. QuikChange site-directed mutagenesis (Stratagene) was used to introduce the D40N, C69S, C81S, C97S, and M116C mutations (see text for discussion of these mutations), which were confirmed by sequencing miniprep DNA from DH5-alpha cells on an ABI3100 capillary sequencer.

C. KSI Expression and Purification

KSI was expressed and purified as previously described.[1] Final purity was >99% as estimated from a Coomassie-stained SDS-PAGE gel. Protein concentration was determined using the calculated molar extinction coefficient in 6M guanidinium chloride.[2]

D. Cyanylation of KSI

A single nitrile group was incorporated into KSI by labeling the unique thiol of M116C using previously described methods, with minor modifications.[3] Briefly, ~1mL of a ~20 mg/mL solution of KSI in 40 mM potassium phosphate (KP_i) (pH 7.2), 1 mM EDTA, and 2 mM DTT was passed over a 5 mL HiTrap Desalting Column (GE Healthcare) and eluted in 400 mM potassium phosphate (pH 7.2), 1 mM EDTA. This step was necessary to remove DTT and increase the buffering capacity to prevent pH spikes upon addition of excess KCN (see below) that result in cleavage of the protein backbone. KSI-containing fractions were pooled (total volume ~2.5 mL) and diluted into 3 volumes of 8 M urea in 400 mM KP_i, 1 mM EDTA (final urea concentration 6 M) to denature the enzyme. Initial work suggested that M116C was unsusceptible to labeling in folded KSI, presumably due to the close packing of groups around that position.

Two molar equivalents of DTNB from a freshly prepared solution (~20 mM in 100 mM KP_i, pH 7.5) were added to the denatured KSI solution and allowed to react for 1 hour at room temperature. The reaction was followed by monitoring the increase in absorbance at 412 nm until completion (less than one hour). Quantitative cyanylation of M116C was achieved by

adding 100 molar equivalents of KCN from a freshly prepared solution (~300 mM in water) to the KSI/DTNB solution and following the absorbance at 412 nm until completion (~ 1 hour). KSI was refolded by diluting the reaction mix into 9 volumes 0.4 M KP_i, 1 mM EDTA (final urea concentration 0.6 M) and incubating on ice for 1 hour. The refolded-KSI solution was then centrifuged 10 minutes at 20,000 rpm, concentrated to ~1 mL using Amicon Ultra centrifugal filter devices, and run over a 100 mL Superose-12 gel filtration column using 40 mM KP_i, pH 7.2, 1 mM EDTA.

Quantitative cyanylation of KSI was confirmed by LC-MS, which indicated intact protein masses of 14,460 Da for unlabeled D40N/C69S/C81S/C97S/M116C KSI (theoretical mass 14,459) and 14,485 Da for cyanylated KSI (theoretical mass 14,484). The observed 25 Da increase in KSI MW after reaction is consistent with conversion of M116C-SH to M116C-SCN. Specific cyanylation of M116C was confirmed by labeling in 40 mM KP_i, condition in which addition of 100 equivalents of KCN results in a spike to pH 9 and hydrolysis of the peptide bond N-terminal to the modified cysteine.[4] The single 12,568 Da intact mass observed by LC-MS for KSI cyanylated under these modified conditions corresponded to the expected mass for residues 1-115 of D40N/C69S/C81S/C97S/M116C KSI from cleavage between residues 115 and 116, confirming specific cyanylation of M116C.

E. IR Absorption Spectra of KSI-CN

Infrared absorption spectra of free and ligand-bound KSI-CN were obtained in liquid solution at room temperature using previously described methods.[5] Samples contained ~1.5 mM enzyme and 1 to 5 molar equivalents of ligands, sufficient to ensure >95% binding of KSI-CN based on the nanomolar to low micromolar affinities of the ligands studied (data not shown). Samples were loaded into a liquid infrared cell (~20 μL volume) constructed of two sapphire windows separated by two Teflon spacers. Spacers of different thickness (75 and 100 μm) were used to minimize fringes due to reflections between the two windows. A solution of 40 mM KP_i was used to acquire a background spectrum, as uncyanylated KSI absorbs minimally between 2100-2200 cm^{-1}. Spectra were obtained using a Bruker Vertex 70 FTIR spectrometer with a nitrogen-cooled indium antimonide detector and 1 cm^{-1} resolution and employing a 4-5 μm broad band-pass interference filter (Spectrogon US, Inc.). Baseline imperfections arising from the significant absorption of water in the 2100-2200 cm^{-1} region were corrected by fitting a polynomial through points away from the nitrile absorption. Reported peak frequencies and linewidths are averages of 3 or more replicates using independently prepared samples and are calculated using the OPUS software package from Bruker instruments, which employs an algorithm based on calculation of the second derivative of the spectrum. The standard deviation for all samples was ±0.1 cm^{-1} (peak position) and ±0.2-0.6 cm^{-1} (FWHM peak width).

IV. References

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