Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Krebs Cycle Metabolon: Structural Evidence of Substrate Channeling Revealed by Cross-Linking and Mass Spectrometry**

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Materials: All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Fresh beef heart was purchased from local slaughter house.

Preparation of Intact Mitochondria: Mitochondria were isolated from beef heart cell according to Pallotti's procedure with slight modifications.[1] Beef heart cut into small cubes was blended by a Waring laboratory blender in chilled isolation buffer (0.01 M Tris-HCl buffer, 0.25 M sucrose, 0.2 mM EDTA and 1 μM PMSF, pH 7.8). Meat suspension was centrifuged at 1200 x g for 20 min, and the supernatant (filtered through two layers of cheesecloth) was centrifuged at 26000 x g for 15 min. Pellet was homogenized in isolation buffer using a glass-Teflon homogenizer and centrifuged again at 26000 x g for 15 min. Supernatant was discarded and pellet resuspended in isolation buffer was centrifuged at 12000 x g for 30 min. Mitochondria pellet was homogenized in 20 mL of 0.01 M phosphate buffer containing 0.25 M sucrose (pH 7.8) and stored in small aliquots at -80 °C before use.

In-vivo Cross-linking of the Krebs Cycle Metabolon: Mitochondria aliquot was diluted in 10 mM PBS (pH 7.4) to a final protein concentration of 2 mg/mL. 3.26 mg of DSG dissolved in 50 μL of DMF was added to the mitochondria suspension to a final concentration of 1 mM. Approximate DSG/protein molar ratio was 50:1.

As a native control, 50 µL of DMF containing no DSG was also added to a separate mitochondria suspension. Cross-linking was incubated at room temperature for 30 min under gentle shaking and quenched by adding 100 uL of 2 M Tris buffer (pH 8.3).

Isolation of Cross-linked Matrix Protein Complexes: Cross-linked mitochondria were collected from suspensions centrifuged at 26000 x g for 30 min and resuspended in 10 mL of lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, and 0.5% sodium cholate, pH 7.4). Membrane disruption was done by homogenizing mitochondria suspension in a glass-glass homogenizer on ice and incubated at 4 °C for 30 min under gentle shaking. Lysed mitochondria were further sonicated by an ultra-sonication probe (FB505, Fisher Scientific) in ice bath for 2 min (5 sec on pulses and 15 sec off pulses). Membrane fractions were centrifuged down at 5000 x g for 30 min. Unwanted large fractions and pre-aggregated proteins were removed by precipitating with 35% ammonium sulfate at 4 °C for at least 1 h under gentle stirring and centrifugation at 5000 x g for 15 min. Yellowish supernatant was then dialyzed in cellulose ester dialysis membrane (Spectra Por ® Biotech) with a molecular mass cutoff at 100 kDa against 2 L of 50 mM Tris-HCl buffer (pH 7.4) at 4 °C overnight and applied to a pre-packed Sephadex™ G-25M column (GE Healthcare) to remove excessive salts and detergents. To separate

proteins in dialyzed supernatant, reducing SDS-PAGE was performed onto a 4%-20% gradient gel (Thermo Scientific) according to the protocol provided by manufacturer.

In-gel Digestion: Gel bands of interest were excised then de-stained twice in 1 ml of 50% methanol with 50 mM ammonium bicarbonate at room temperature while being gently vortexed for about 1 h. The gel slices were re-hydrated in 1 ml of 50 mM ammonium bicarbonate for 30 min at room temperature then the gel bands/spots were cut into several pieces. The gel pieces were dehydrated in 1 ml of 100% acetonitrile for 30 min at room temperature with gentle shaking. The acetonitrile was carefully removed away from the gel pieces with a pipette tip prior to proteolytic digestion. 10 to 20 µL of sequence-grade modified trypsin (20 ng/µL, Promega) in 50 mM ammonium bicarbonate was added and adsorbed into the gel pieces and incubated overnight at 37^0C. The digestion was quenched by the addition of 20 µL ml of 1% formic acid. This solution was allowed to stand and peptides that dissolved in the 1% formic solution were extracted and collected. Further extraction of peptides from the gel material was performed twice by the addition of 50% acetonitrile with 1% formic acid and sonicated at 37°C for 20 min, and these solutions were also collected and combined. A final complete dehydration of the gel

pieces was accomplished by addition of 20 μL of 100% acetonitrile and incubation at 37°C for 20 min. The combined supernatant solutions of extracted peptides were combined and dried in a vacuum centrifuge (Speed-Vac). The peptides were reconstituted in 100 μL of 5% acetonitrile with 0.1% formic acid for LC-MS/MS analysis.

Mass Spectrometric Instrumentation: Peptides were analyzed using a nano-LC-MS/MS system comprised of a nano-LC pump (Eksigent) and a LTQ-FT mass spectrometer (ThermoElectron Corporation). The LTQ-FT is a hybrid mass spectrometer with a linear ion trap used typically for MS/MS fragmentation (i.e. peptide sequence) and a Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometer used primarily for primary MS accurate mass measurement of peptide molecular ions. The LTQ-FT is equipped with a nanospray ion source (ThermoElectron Corporation). Approximately 5 to 20 femto-moles of tryptic digest or phosphopeptide-enriched samples were dissolved in 5% acetonitrile with 0.1% formic acid and injected onto a C18 nanobore LC column for nano-LC-MS/MS and identification of peptides. The nanobore column was homemade (C18 (Waters Corporation); 3 um particle; column: 75um ID x 100 mm length) Atlantis dC18, 3 μm × 75 μm × 100 mm (Waters Corporation). A linear gradient LC profile was used to separate and elute peptides, consisting of 5 to 70% solvent B in 78 min with a

flow rate of 350 nL/minute (solvent B: 80% acetonitrile with 0.1% formic acid; solvent A: 5% acetonitrile with 0.1% formic acid). The LTQ-FT mass spectrometer was operated in the data-dependent acquisition mode controlled by *Xcalibur 1.4* software, in which the "top 10" most intense peaks observed in an FT primary scan (i.e. MS survey spectrum) are determined by the computer on-the-fly and each peak is subsequently trapped for MS/MS analysis and peptide fragmentation (sequencing by collision-induced dissociation) in the LTQ linear ion trap portion of the instrument. Spectra in the FT-ICR were acquired from *m*/*z* 400 to 1700 at 50000 resolving power with about 3 ppm mass accuracy. The LTQ linear ion trap was operated with the following parameters: precursor activation time 30 ms and activation *Q* at 0.25; collision energy was set at 35%; dynamic exclusion width was set at low mass of 0.1 Da and high mass at 2.1 Da with one repeat count and duration of 10 s.

Mascot Database Searches: LTQ-FT MS raw data files were processed to peak lists with *BioworksBrowser 3.2* software (ThermoElectron Corporation). Processing parameters used to generate peak lists were as follows: precursor mass 401-5500 Da; grouping was enabled allowing 5 intermediate MS/MS scans; precursor mass tolerance 5 ppm, minimum ion count in MS/MS was set to 15, and minimum group count was set to 1. Resulting DTA files from each data acquisition file were merged

and the data file was searched for identified proteins against the NCBI or custom databases, using MASCOT search engine (Matrix Science Ltd.; version 2.2.1; in-house licensed). Searches were done with tryptic specificity, allowing two missed cleavages, or "non-specific cleavage" and a mass error tolerance of 5 ppm in MS spectra (i.e. FT-ICR data) and 0.5 Da for MS/MS ions (i.e. LTQ linear ion trap). Identified peptides were generally accepted only when the MASCOT ion score value exceeded 20.

Identification of Cross-linked Peptides: Mass spectrometric raw files were analysed in *Thermo Xcalibur* and peptide peaks of interest were picked manually. Mass lists were screened using *FindPept* tool (http://web.expasy.org/findpept/) against enzyme sequences obtained from UniProtKB/Swiss-Prot (mMDH: Q32LG3; CS: Q29RK1; ACON: P20004) to identify non-cross-linked peptides (MS tolerance = 6 ppm). A theoretical mass database of potential inter-protein cross-links was built up using a spreadsheet by combining two peptides, which were identified in native individual enzymes but missed in cross-linked enzyme complex. Additional peptide peaks only found in cross-linked spectra were screened against the mass database. Cross-link candidates were selected by following rules: (1) trypsin will not cut at C-terminus of modified lysines or lysines with proline on the right (C-terminus); (2) up to two missed cleavages are allowed; (3) peptide length is 3 to 30 amino acids;

(4) each cross-linked peptide has at least two lysines (one for cross-linking and one at C-terminal); (5) mass signals present in at least duplicate experiments; (6) MS tolerance = 20 ppm. Throughout the whole search process, custom-specified modifications were applied and the respective mass variations (to residues) were summarized in Table S1.

Hybrid Protein Docking: Global docking and local docking were done by Cluspro server (http://cluspro.bu.edu/)[2] and Rosetta server (http://rosie.rosettacommons.org/)[3], respectively. In global docking, cross-linked lysines summarized in Figure S2 were set as attracting residue. Prior to local docking, all model candidates were screened by *Xwalk* software suite[4] to filter out false positives of cross-link candidates. Maximum Euclidean distance limit was set to 25 Å, which is a combination of DSG spacer arm length (7.7 Å), lysine side chain length (6 Å × 2) and backbone flexibility. In addition to Euclidean distance limit, solvent accessible surface (SAS) distance was also set at 30 Å, to mimic molecular flexibility of DSG when cross-linking two residues without penetrating protein surface. Solvent radius was 1.4 Å by default and set to 2 Å for SAS distance calculation.[5] Rotamers were removed and only the distance of Cβ-Cβ between two lysines was calculated. After local docking, 10 models of lowest interface energy were screened

again by *Xwalk* for picking up the one containing most cross-links. Euclidean distances within final complex were measured by *Xwalk* and interfacial residues were determined when measured distance was less than 20 Å. The whole process for the hybrid docking method is illustrated in Figure S3.

Simulation of Surface Electrostatic Potential: Prior to simulation, .pdb files of docking models were converted to .pqr files by *PDB2PQR* server (nbcr-222.ucsd.edu/pdb2pqr_1.8) to assign charges and protonation at pH 7.8.[6] Surface ESP calculation was done by *Delphi*. [7]

Graphic Preparation: All figures of protein structures and visualization of surface ESP were made in *Chimera* obtained from www.cgl.ucsf.edu/chimera/ .[8]

References

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Supporting Figures and Tables

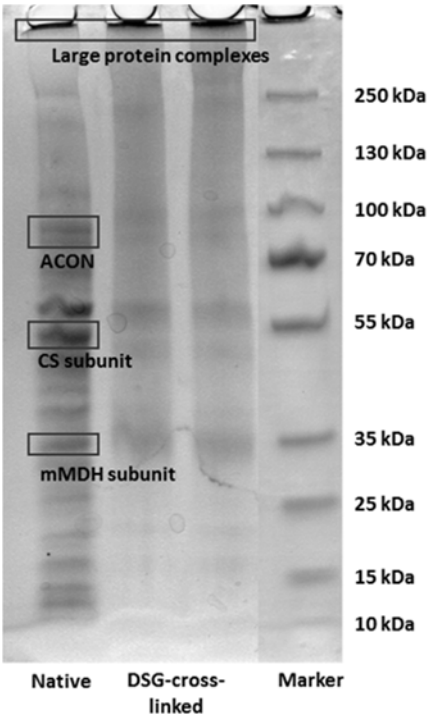

Figure S1. Reducing SDS-PAGE of cross-linked matrix proteins isolated from beef mitochondria. Bands in black squares were cut for digestion and mass spectrometric analysis.

Table S1. Custom-specific modifications for cross-link identification.

Modification	**Residue**	**Mass variation (Th)**
Acetylation	Lysine	+ 42.011
Oxidation	Methionine Tryptophan	+ 15.995
Trimethylation	Lysine	+ 42.047
Phosphorylation	Proline	+ 79.966
Carboxyamidomethylation	Cysteine	+ 57.02
Propioamide	Cysteine	+ 71.037
Mono-link (DSG)	Lysine	+ 114.032

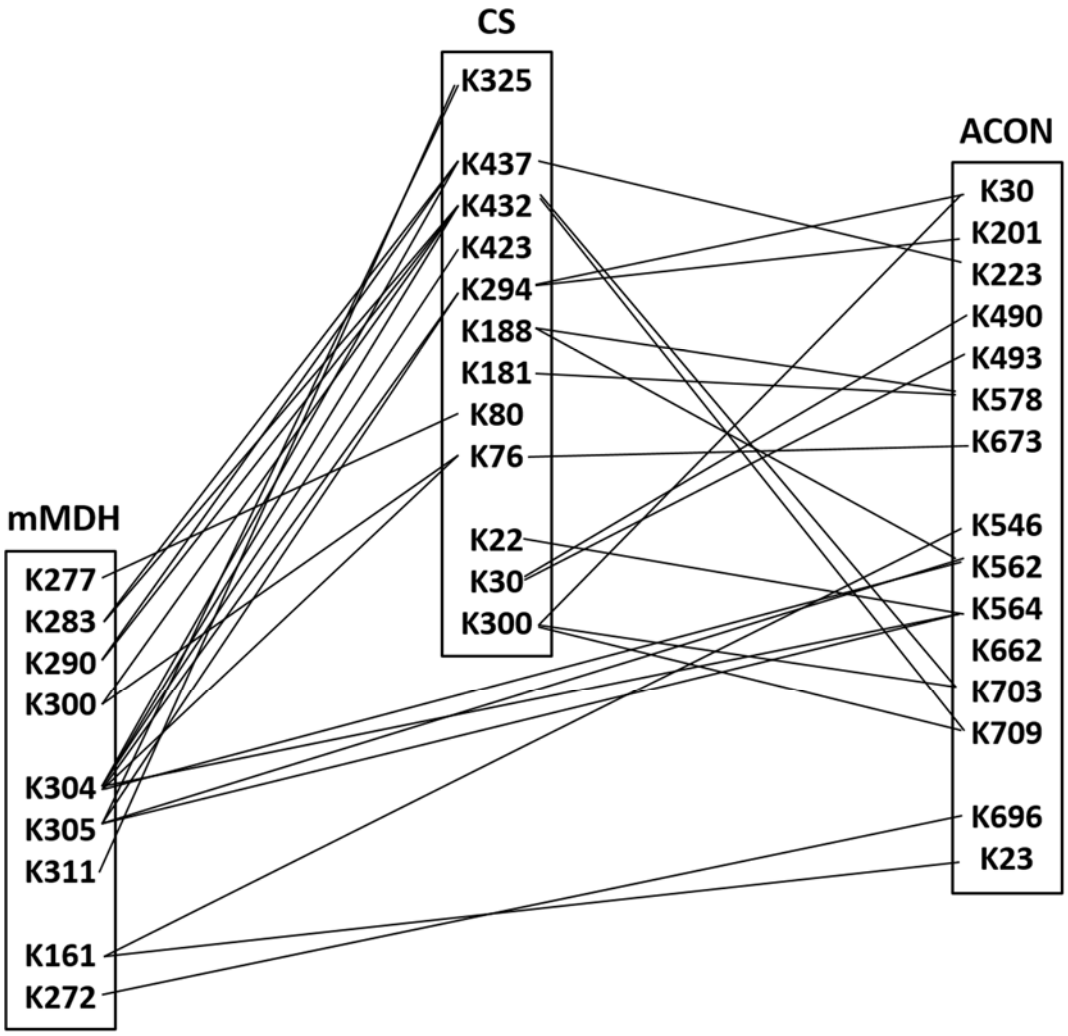

Figure S2. Network of cross-link candidates in mMDH-CS-ACON complex.

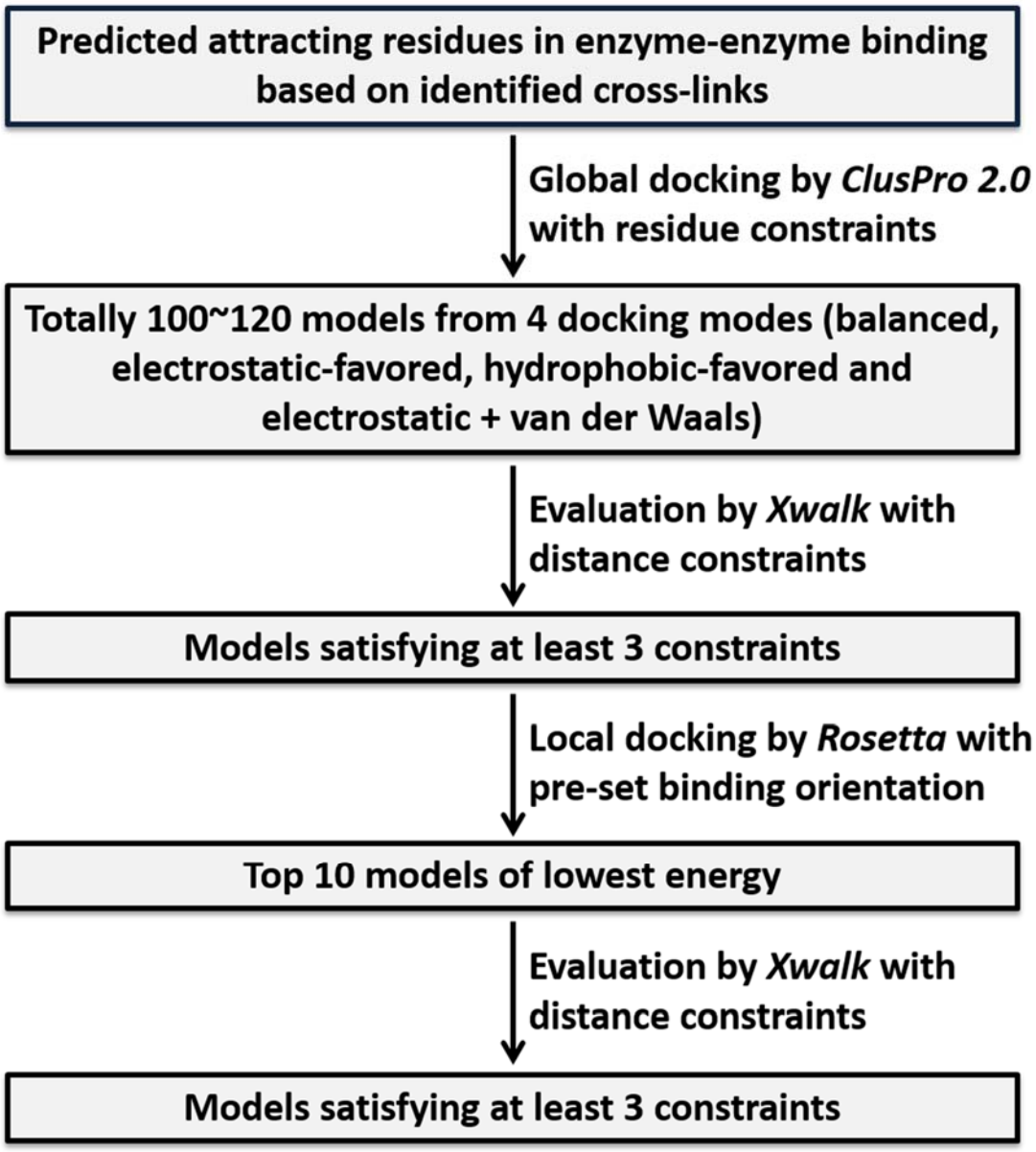

Figure S3. Scheme of protein docking with distance constraints.