Central Science
Supporting Information:
Refining the Structural Model of a Hetero-hexameric Protein Complex:
Surface Induced Dissociation and Ion Mobility Provide Key Connectivity
and Topology Information

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Figure S-7. RMSD of backbone atoms over simulation time for trimer model candidates. A). Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); B) Simulation in vacuum.

Figure S-8. RMSD of backbone atoms over simulation time for trimer model candidates in periodic water boxes at three repeats represented by three different colors. Trimer model candidates A) 3hht, B) 1ahj, C) 1ugs, D) 1ugq.

Figure S-9. Collisional cross-section for trimer model candidates during simulation compared with the experimental value. Atomic coordinates are subjected to CCS calculation every 2 ns. A). Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); B) Simulation in vacuum.

Figure S-10. RMSD of backbone atoms over simulation time for TNH hexamer model candidates. Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); Hexamer 7 and 8 failed simulation due to instability.

Figure S-11. Collisional cross-section for TNH hexamer model candidates during simulation compared with the experimental value. Atomic coordinates are subjected to CCS calculation every 2 ns for simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl). Hexamer 7 and 8 failed simulation due to instability. Hexamer 5 and Hexamer 10 do not have β-β and γ-γ interaction between the two trimers, thus were eliminated.

Figure S-12. Interfacial strengths predicted in SID/IM experiments match with interfacial area calculated by PDBePISA from the three possible TNH hexamer structures energy minimized and equilibrated in vacuum for 10ns.

Figure S-13. MS/MS spectra of crosslinked peptides and their validation on the atomic structures of proposed TNH hexamers. MS/MS spectra of BS^3 (11.4 Å spacer arm) crosslinked A) α N-terminus to α N-terminus B) α N-terminus to β N-terminus. Although AR is short, only β has AR on its N-terminus. MS/MS spectra of BS^2G (7.7 Å spacer arm) crosslinked C) α N-terminus to α N-terminus D) α N-terminus to β N-terminus. C) The proposed hexamer structure 1 has α N-terminus to α N-terminus distance of 7 Å. D) The proposed hexamer structure 3 has α N-terminus to β N-terminus distance of 7.5 Å. The detected crosslinked peptides add confidence to our proposed hexamer structures.

Scheme S-1. A) The flow chart of the surface mapping experiment. B) Labeling reagent PGO and its reaction with arginine rendering a mass increase of 134.0368 or 116.0262.

Supplemental methods.

Measurement of Catalytic activity of TNH in the presence of methanol.

Modification of mobcal.f.

An example of αβγ.mfj is as following:

TNH expression and purification.
Table S-1. SiD/IM experiment, trap DC bias and trap SiD lens voltages at different SiD acceleration voltages. (Unit: V)

| Trap SiD ΔV | 30  | 40  | 50  | 60  | 70  | 80  | 90  | 100 | 110 | 120 | 130 | 140 | 150 | 160 | 170 | 180 |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| trap DC bias| 83  | 93  | 103 | 113 | 123 | 133 | 143 | 153 | 163 | 173 | 183 | 193 | 203 | 213 | 223 | 233 |
| 1. Entrance 1| -80 | -70 | -65 | -60 | -50 | -40 | -35 | -20 | -10 | 0   | 10  | 20  | 30  | 40  | 50  | 60  |
| 2. Entrance 2| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105| -105|
| 3. Front Top | -150| -150| -150| -150| -150| -150| -150| -150| -150| -150| -150| -150| -150| -150| -150|
| 4. Front Bottom| -69 | -60 | -50 | -40 | -30 | -20 | -10 | 0   | 10  | 20  | 30  | 40  | 50  | 60  | 70  | 80  |
| 5. Mid Bottom | -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125|
| 6. Surface   | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 | -95 |
| 7. Rear Top  | -270| -270| -270| -270| -270| -270| -270| -270| -270| -270| -270| -270| -270| -270| -270|
| 8. Rear Bottom| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125| -125|
| 9. Exit 1    | -130| -130| -140| -140| -140| -140| -140| -140| -140| -140| -140| -140| -140| -140| -140|
| 10. Exit 2   | -115| -115| -115| -115| -115| -115| -115| -115| -115| -115| -115| -115| -115| -115| -115|

Table S-2. Experimentally determined CCS of subunits and subcomplexes, radius of subunits

|       | α   | β   | γ   | αβ  | αβγ | 6mer |
|-------|-----|-----|-----|-----|-----|------|
| CCS (Å) MeOH | 1870±40 | 1110±30 | n.d. | 2450±70 | 2890±70 | 4860±70^a |
| CCS(Å) SID | 1880±40 | 1110±30 | 1210±30 | 2440±60 | 2730±160 |
| r(Å)^b      | 23.0 | 17.4 | 18.3 | Linear | ?     | ?     |

^a The CCS of hexamer is measured from 0.1 M AmAc aqueous solution at charge states 16-19.

^b Radius of a spherical model r=(CCS/n)^0.5-r_H^, and r_H=1.4Å.

^c The γ-subunit is only present in low abundance and not reproducibly produced under these conditions.

Standard deviations were calculated from data obtained on four different days.
Table S-3. GDH exposed arginine information and labeling results. (Related to Figure S-5)

| Res. No. | Hexamer (A-F are subunits) | Monomer | PGO (mM) labeling results and repeats |
|----------|----------------------------|---------|--------------------------------------|
|          | SASA ratio (%) | Exposed? | 0.5 | 1 | 2 | 4 | 7 | 0.5 | 1 | 2 | 4 | 7 |
| A | B | C | D | E | F | % Exposed? | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 3 | 86 | 100 | 76 | 72 | 82 | 75 | Y | 86 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 19 | 48 | 50 | 51 | 48 | 50 | 48 | Y | 48 | Y | N | Y | Y | N | Y | N | Y | Y | Y | Y |
| 35 | 66 | 69 | 62 | 67 | 68 | 64 | Y | 79 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 42 | 34 | 37 | 35 | 33 | 38 | 34 | Y | 34 | Y | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC |
| 44 | 62 | 67 | 66 | 64 | 65 | 64 | Y | 62 | Y | NC | NC | NC | NC | NC | NC | NC | NC | NC | NC |
| 66 | 45 | 46 | 42 | 45 | 42 | 42 | Y | 51 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 67 | 26 | 26 | 26 | 26 | 24 | 27 | N | 26 | N | N | N | N | N | N | N | N | N | N | N |
| 79 | 0 | 0 | 0 | 0 | 0 | 0 | N | 0 | N | N | N | N | N | N | N | N | N | N | N |
| 86 | 28 | 29 | 18 | 20 | 18 | 18 | N | 18 | N | N | N | N | N | N | N | N | N | N | N |
| 94 | 3 | 5 | 5 | 2 | 2 | 4 | N | 3 | N | N | N | N | N | N | N | N | N | N |
| 146 | 28 | 32 | 26 | 29 | 29 | 30 | N | 28 | N | N | N | N | N | N | N | N | N | N | N |
| 147 | 35 | 39 | 38 | 40 | 35 | 38 | Y | 35 | Y | N | Y | Y | Y | N | Y | Y | Y | Y | Y |
| 174 | 42 | 41 | 37 | 41 | 44 | 42 | Y | 46 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 211 | 11 | 12 | 13 | 12 | 11 | N | 11 | N | N | N | N | N | N | N | N | N | N | N | N |
| 217 | 19 | 16 | 18 | 18 | 16 | 18 | N | 19 | N | N | N | N | N | N | N | N | N | N | N |
| 261 | 28 | 26 | 25 | 28 | 28 | 27 | N | 28 | N | N | N | N | N | NC | N | N | N | N | N |
| 265 | 54 | 49 | 52 | 52 | 56 | 52 | Y | 54 | Y | Y | Y | Y | Y | NC | Y | Y | Y | Y | Y |
| 338 | 61 | 64 | 60 | 60 | 60 | 58 | Y | 61 | Y | N | N | N | N | N | N | Y | Y | N | Y |
| 363 | 33 | 35 | 37 | 39 | 35 | 32 | Y | 33 | Y | N | N | N | N | N | N | N | N | N | N |
| 396 | 5 | 4 | 5 | 5 | 7 | 6 | N | 71 | Y | N | N | N | N | N | N | N | N | N |
| 403 | 35 | 32 | 35 | 34 | 35 | 37 | Y | 38 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 419 | 59 | 46 | 64 | 47 | 51 | 47 | Y | 64 | Y | N | N | N | N | Y | N | N | N | N |
| 439 | 15 | 35 | 25 | 24 | 20 | 24 | Y | 74 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 459 | 48 | 47 | 47 | 50 | 47 | 50 | Y | 49 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| 462 | 44 | 45 | 46 | 46 | 46 | 46 | Y | 44 | Y | N | N | Y | Y | Y | N | N | Y | Y |
| 466 | 56 | 52 | 51 | 52 | 55 | 54 | Y | 56 | Y | N | Y | Y | Y | Y | N | Y | Y | Y | Y |
| 478 | 0 | 0 | 0 | 0 | 1 | 1 | N | 0 | N | N | N | N | N | N | N | N | N | N |
| 491 | 50 | 37 | 56 | 56 | 55 | 45 | Y | 50 | Y | Y | Y | Y | Y | Y | Y | Y | Y | Y |
| No. of labeled R that >30% SASA | 8 | 12 | 13 | 13 | 14 | 8 | 13 | 14 | 14 | 15 |
| No. of labeled R that <30% SASA | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 2 |
| No. of R that are not labeled | 19 | 15 | 14 | 13 | 10 | 19 | 14 | 13 | 12 | 10 |
| Res. No. | SASA %<sup>a</sup> | Exp? | PGO(mM) | Res. No. | SASA %<sup>a</sup> | Exp? | PGO(mM) |
|---------|-----------------|------|---------|---------|-----------------|------|---------|
| α12     | 49              | Y    | NC NC NC NC | α14     | 79              | Y    | NC NC NC NC |
| α15     | 64              | Y    | NC NC NC NC | β2      | 69              | Y    | N N N N N  |
| α19     | 38              | Y    | NC NC N N  | β27     | 64              | Y    | Y Y Y Y Y  |
| a41     | 58              | Y    | Y Y Y Y Y  | β33     | 37              | Y    | NC NC NC NC |
| a49     | 35              | Y    | N N N N Y  | β53     | 7               | N    | N N N N Y  |
| a53     | 40              | Y    | Y Y Y Y Y  | β58     | 24              | N    | N N N N N  |
| a61     | 50              | Y    | N N Y Y Y  | β72     | 24              | N    | N N N N N  |
| a73     | 80              | Y    | Y Y Y Y Y  | γ3       | 62              | Y    | Y Y Y Y Y  |
| a88     | 42              | Y    | Y Y Y Y Y  | γ4<sup>b</sup> | 61          | Y    | N N N N N  |
| a126    | 2               | N    | N N N N N  | γ12     | 41              | Y    | N N Y Y Y  |
| a128    | 14              | N    | N N N N N  | γ14     | 48              | Y    | Y Y Y Y Y  |
| a131    | 30              | N    | N N N N N  | γ23     | 9               | N    | N N N N N  |
| a134    | 61              | Y    | N N N N N  | γ26     | 78              | Y    | N N N N N  |
| a151    | 38              | Y    | Y Y Y Y Y  | γ29     | 30              | N    | N N N N N  |
| a160    | 6               | N    | NC N N N N  | γ50<sup>b</sup> | 83          | Y    | N N N N N  |
| a166    | 60              | Y    | Y Y Y Y Y  | γ51     | 74              | Y    | N N N N Y  |
| a167    | 41              | Y    | Y Y Y Y Y  | γ52     | 81              | Y    | Y Y Y Y Y  |
| a182    | 58              | Y    | Y Y Y Y Y  | γ56     | 62              | Y    | Y Y Y Y Y  |
| a185    | 27              | N    | N N N N N  | γ64     | 11              | N    | NC NC N N  |
| α95     | 45              | Y    | Y Y Y Y Y  | γ95     | 45              | Y    | Y Y Y Y Y  |

a. Due to the dynamic nature of molecules in solution, the % SASA is the biggest number chosen from 2 ns, 4 ns, 6 ns, 8 ns, 10 ns, 12 ns and 14 ns simulation results.

b. Arginines on γ4 and γ50 are not labeled, may due to steric effect when the PGO labeling of the adjacent arginines on γ3 and γ52, respectively.
Table S-5. Information about TNH homologs and TNH similarity (Taken from protein data bank)

| PDB ID | Cofactor | Binding motif | Organism             | Identity     |
|--------|----------|---------------|----------------------|--------------|
| 3hht   | Co(III)  | α C116 C119 C121 | Geobacillus pallidus | TNH α to α 55% |
|        |          |               |                      | TNH β to β 31% |
|        |          |               |                      | TNH γ to β 40% |
| 1ahj   | Fe (III) | α C110 C113 C115 | Rhodococcus sp.      | TNH α to α 51% |
|        |          |               |                      | TNH β to β 33% |
|        |          |               |                      | TNH γ to β 43% |
| 1ugs   | Co       | α C108 C111 C113 | Pseudonocardia thermophila | TNH α to α 46% |
|        |          |               |                      | TNH β to β 36% |
|        |          |               |                      | TNH γ to β 39% |
| 1ugq   | Apo      |               | Pseudonocardia thermophila | TNH α to α 46% |
|        |          |               |                      | TNH β to β 36% |
|        |          |               |                      | TNH γ to β 39% |

Function: The four TNH homologs are nitrile hydratase that convert nitriles to corresponding amides. They have different activity towards nitriles with different sizes and functional groups.

Sequence alignment of TNH subunits to α and β subunits in homologs:

TNH α to 3hht α

| Score | Expect | Method | Compositional matrix adjust. | Identities | Positives | Gaps |
|-------|--------|--------|------------------------------|------------|-----------|------|
| 189 bits(480) | 2e-64 | | 101/184(55%) | 128/184(69%) | 2/184(1%) |

Query 11 ARVRLERERVIAGLVTDQDITLHHLNLSRAPFNGRLVALVARWTVSPDFDLLLLGEPAA 70
Sbjct 27 ARAKALELSLLIEKGLSADAIERNVIKHYEHELGMNQAKVAKWTDPARKQLLEDSET 86

Query 71 ALREMGLD8LADDDEHEL1V1ANTPVH1N1V1C1SC1CY1F1V1L1G1PSW1YS1K1S1D1Y1R1V1R1V1 130
Sbjct 87 VLR6LGY6L--Q6EH1R1V1ENTDTH1N1V1C1SC1CY1F1W1PLL1P1PS1W1K1EP1Y1R1V1V1 144

Query 131 REPRAVLAEEFGTVLPAEV0VUVR1V1D1AS1A1E1AY1MV1PP1R1P1AG1TE1G1L1D1E1EGL1AAR1VT1R1AG1L1G1 190
Sbjct 145 KEPQV1LKE1FG1L1D1PS1V1R1V1D1S1S1E1IR1F1V1L1P1Q1R1P1EG11TE1E1E1AL1K1L1V1R1D1S1M1G1 204

Query 191 TAPV | 194
Sbjct 205 VAKI 208

TNH β to 3hht β (residues 4-73)
### TNH γ to 3hht β (residues 141-228)

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 38.5 bits | 3e-09  | Compositional matrix adjust. | 22/70 (31%) | 34/70 (48%) | 2/70 (2%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 141-228  | LNDIGGTFGYGSIPIDGEAEPPHPWRHDEAREVFALAHLAVAGVTAS---ELRDAERVP G+ ++D+G G+G+E+H+DHE ++G G+ +E+R E+ |

**Subject**

| Residues | Sequence |
|----------|----------|
| 60       | IHVGGMDGFKNVM/KEEEDIYTHDWREIgLALGLVAGCMAQGLGM/KAFDEFGRIGELMR |

### TNH α to 1ahj α

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 65.9 bits | 6e-19  | Compositional matrix adjust. | 36/89 (40%) | 46/89 (51%) | 2/89 (2%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 4-89     | RFAARLWLE-SGTHLVLIDLWECEYLEPA |

**Subject**

| Residues | Sequence |
|----------|----------|
| 91       | RF A+LW6 V IDLWE Y+EP + |

### TNH β to 1ahj β (residues 4-89)

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 163 bits | 2e-54  | Compositional matrix adjust. | 88/172 (51%) | 113/172 (65%) | 8/172 (4%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 4-89     | GLTD---=EQLDTELHNLRSAPTFNGARL2ARVTSPDFDRLLLLGEPAAALREIKGLDGL |

**Subject**

| Residues | Sequence |
|----------|----------|
| 80       | GLV D E E+S P GA LVARANT P+FR LLL +AA+ +G G |

### TNH γ to 1ahj β (residues 123-212)

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 50.8 bits | 1e-13  | Compositional matrix adjust. | 28/86 (33%) | 48/86 (55%) | 2/86 (2%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 123-212  | LNDIGGTFGYGSIPIDGEAEPPHPWRHDEAREVFALAHLAVAGVTAS---ELRDAERVP G+ ++D+G G+G+E+H+DHE ++G G+ +E+R E+ |

**Subject**

| Residues | Sequence |
|----------|----------|
| 60       | VHDLGQVQFGQKVPTDADIGPTTFAEHEHLPYSLMFAGVGLGAFSDEVRYVVERME |

### TNH γ to 1ahj β (residues 123-212)

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 50.8 bits | 1e-13  | Compositional matrix adjust. | 28/86 (33%) | 48/86 (55%) | 2/86 (2%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 123-212  | GLTD---=EQLDTELHNLRSAPTFNGARL2ARVTSPDFDRLLLLGEPAAALREIKGLDGL |

**Subject**

| Residues | Sequence |
|----------|----------|
| 80       | GLV D E E+S P GA LVARANT P+FR LLL +AA+ +G G |

### TNH β to 1ahj β (residues 4-89)

| Score | Expect | Method     | Identities | Positives | Gaps |
|-------|--------|------------|------------|-----------|------|
| 50.8 bits | 1e-13  | Compositional matrix adjust. | 28/86 (33%) | 48/86 (55%) | 2/86 (2%) |

**Query**

| Residues | Sequence |
|----------|----------|
| 123-212  | LNDIGGTFGYGSIPIDGEAEPPHPWRHDEAREVFALAHLAVAGVTAS---ELRDAERVP G+ ++D+G G+G+E+H+DHE ++G G+ +E+R E+ |

**Subject**

| Residues | Sequence |
|----------|----------|
| 60       | VHDLGQVQFGQKVPTDADIGPTTFAEHEHLPYSLMFAGVGLGAFSDEVRYVVERME |

### TNH γ to 1ahj β (residues 123-212)
| Score     | Expect | Method                           | Identities | Positives | Gaps   |
|-----------|--------|---------------------------------|------------|-----------|--------|
| 63.9 bits | 2e-18  | Compositional matrix adjust.    | 39/90(43%) | 50/90(55%) | 3/90(3%) |

**Query 5**
FSVGDPPVRVRAVPDPHHTVPRY/RGHLNHVTV-TPQQPPCPDPDDVARRDPPFRVLPVTV
FVG VVR P H R+ P Y G + S + P P D + R+ P Y V

**Sbjct 123**
FGVQRVPRDVEVPNGHIREMPAYCRGRVSTISHTTKEWPFPAIIGHGRNDOGEPEPYHV

**Query 64**
RAAFDLWGSST--LVLDDLWCEYLEEPAA
+F A + L G S T V++DL+E YLPEAA

**Sbjct 183**
KFAAEELFGSDTDDGSVVLDFEGYLEPEAA 212

**TNH α to 1ugs α**

| Score     | Expect | Method                           | Identities | Positives | Gaps   |
|-----------|--------|---------------------------------|------------|-----------|--------|
| 177 bits  | 3e-60  | Compositional matrix adjust.    | 89/194(46%) | 125/194(64%) | 3/194(1%) |

**Query 1**
STEHSLPLPAARVRLLEERVIAAGLVTDEQLDTLIEHNSRATPFNGARLVARAVTSPDF
S E + I ARV+ LE + I G++ T + D + E + P GA++V +ANT P+F

**Sbjct 8**
SDDEEQKIEITAVXKLESNHLLEQGLTTSIDRMADIEYNVGHLEKGVVVKWNTDEF

**Query 61**
RDLLEGLEAALRMELGDGLLADDDHELRLVVANTFTVWVWVCTLCSCYPVLLLPSWY
+ LL + A + F+E+ G L + D + V NT VH+VWVCTLCSC P + LG P++4

**Sbjct 68**
KKRRLLADGTEACKLIGIGLQGD--MMPVENTDEVHHVWVCTLCSCPWPLGPLNWF

**Query 121**
KSDAYVRARVRPRARVL--EGFETYLPAEVDVRVIAAAAMYWRPRAATGTCGLDEEGL
K YR+R+REPR+L EFG +P ++VND+S+E R++VLP+RPAGT+G EE L

**Sbjct 126**
KEQQYRSRVRPRQQLKEEFFGPEVPSXIEKVDSSSEMRFVVLQPQPAGTDGMLEE

**Query 180**
AARVTRAGLIGTP 193
A VTR + IG P

**Sbjct 186**
ATLVRESMGVEP 199

**TNH β to 1ugs β (residues 6-69)**

| Score     | Expect | Method                           | Identities | Positives | Gaps   |
|-----------|--------|---------------------------------|------------|-----------|--------|
| 38.1 bits | 4e-09  | Compositional matrix adjust.    | 24/66(36%) | 32/66(48%) | 2/66(3%) |

**Query 5**
DIGITFGYGSIPMDGAEPPHPWRHDEWAFVAVLGLAMVAGTIASELRAAERVPPNDY
D+GGT G G I EP +R +WE FA+ AG E R E++ P +Y

**Sbjct 6**
DVGTDGLGPINRPAEPEV--FRAEVEKVFAMFPATFRAQFMGLDEFRGBGIEQPPAEX

**Query 65**
LAASYY 70
L + YY

**Sbjct 64**
LESPYY 69

**TNH γ to 1ugs β (residues 138-224)**

| Score     | Expect | Method                           | Identities | Positives | Gaps   |
|-----------|--------|---------------------------------|------------|-----------|--------|
| 50.4 bits | 2e-13  | Compositional matrix adjust.    | 35/90(39%) | 41/90(45%) | 8/90(8%) |

**Query 4**
RSVGDPPVRVRAVPDPHHTVPRY/RGHLNHVTV-TPQQPPCPDPDDVARRDPPFRVLP--YY
+F GD VR A P H R RYRG G VV PD + P +Y

**Sbjct 138**
KFKEGDVRRRSAAPKGHHARRYVRRKTVVHHNGAYIPDTAG---NLGECPEHYL

**Query 62**
TVRFARDDLW--SGTH.LVLDDLWCEYLEYE
TVRF A++LLW 6 V D WE Y+E

**Sbjct 195**
TVRFQAELWGPESSDFNSYVYDCUEPYIE 224
### TNH α to 1ugq α

| Score     | Expect | Method                          | Identities | Positives | Gaps          |
|-----------|--------|---------------------------------|------------|-----------|---------------|
| 181 bits(459) | 1e-61  | Compositional matrix adjust.    | 90/194(46%) | 126/194(64%) | 3/194(1%)    |

**Query 1**

S E + I ARV+ LE +I G++T +D + E + P GA++V +AWT P+F

**Subjct 8**

SDEEQKEITARVKALESNLIQILTTSMIDRMAYNEYENEGVGILGAKLVKAVDTPEF

**Query 61**

RLDGLGPAALHLEMRMLGDLADDEHLRWANPPTVHVNPVVTCLCSCPYPVLLGPSPNY

+ LL + A +E+F+ G +L +D + V NT VHV+VVTCLCSCP +LG P+v+

**Subjct 68**

KKLALADDGECKELIGGDLQGED---MMVENTDEVHVH+VVTCLCSCP+PVGLP+NNWF

**Query 121**

KSYARVRARVREPRAVLA-EFGTYLPAEVDVRVUASAEARYMVPRRPRAGTE6ELDEEGL

K YR+RVRREPR +L EF G +P+++ +D+S+E R++VLP+RPAGT+G EE L

**Subjct 126**

KEPQYRSRIVREPRQLKKEEGFESVSPEKEIKVH+SS+RFPVVLQPRAGTDG+EEEL

**Query 180**

A VTR +IG P

**Subjct 186**

ATLVTRESKIGVEP 199

### TNH β to 1ugq β (residues 6-69)

| Score     | Expect | Method                          | Identities | Positives | Gaps          |
|-----------|--------|---------------------------------|------------|-----------|---------------|
| 38.1 bits(87) | 4e-09  | Compositional matrix adjust.    | 24/66(36%) | 32/66(48%) | 2/66(3%)     |

**Query 5**

DIGGTGFPGSIPMDGAEPFHPRWHDWARFYAVLVGLAMAGVTVTASELRAEERVPPNDY

D+G+T G G I EP +R +WE FA+ AG E R E++ P +Y

**Subjct 6**

DVGITDGLPGIPRPADEPV---FRAWKEKAFAMFPATFRAGFMGLDEFRGIIEQ+WPAEY

**Query 65**

LAASYY 70

L + YY

**Subjct 64**

LESPYY 69

### TNH γ to 1ugq β (residues 138 to 224)

| Score     | Expect | Method                          | Identities | Positives | Gaps          |
|-----------|--------|---------------------------------|------------|-----------|---------------|
| 50.4 bits(119) | 2e-13  | Compositional matrix adjust.    | 35/90(39%) | 41/90(45%) | 8/90(8%)     |

**Query 4**

RFSVGDPRVRPAPDPHHPTRPVRYVRGHLGVTYQVQPPCLPDDVARRRDPFRLP--+VY

+F GD VR A P H R RV+RG G VV PD + P +Y

**Subjct 138**

KFKEGDVVFSTSPKGARRARYVRGKGTGTVVHKH+GAYIPDTAG---NGLGECPEHL+Y

**Query 62**

TVRFAARDLW---SSGTHVLILDVESYCLE 88

TVRF A+LP 5 V D WE Y+E

**Subjct 195**

TVRFTAQELWGPSDPNSVYDDCNEPYIE 224
Figure S-1. Dissociation of TNH hexamer with ammonium acetate. Increasing ionic strength from F) 0.1M ammonium acetate to A) 1M ammonium acetate does not induce the dissociation of TNH hexamer.

Figure S-2. Catalytic activity of TNH in the presence of methanol. The plot shows that TNH hexamer largely remains intact and folded up to 60% methanol disruption.
Figure S-3. SID/IM/SID The energy resolved transfer-SID. Plots show SID dissociation of (A) +8 and (B) +9 TNH αβγ trimer generated in the trap-SID and separated in the ion mobility cell.

Figure S-4. IM/MS plot of the 50% methanol perturbed TNH solution. The intensity of spots shows the normalized abundance of the ion species in a square root scale. Folded α and β subunits (narrow drift time distribution) were readily observed and used for the CCS calculation.
Figure S-5. Phenylglyoxal labeling of glutamate dehydrogenase. Plots show sensitivity and specificity of phenylglyoxal surface labeling on glutamate dehydrogenase hexamer.

Figure S-6. Higher concentration of the labeling reagent slightly disrupts the structure of TNH hexamer to subcomplexes. As the concentration of the labeling reagent increases, the percentage of the hexamer decreases (dashed line), the percentage of the αβγ trimer (solid dark line) and αβ dimer (solid purple line) increase.
Figure S-7. RMSD of backbone atoms over simulation time for trimer model candidates. A). Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); B) Simulation in vacuum
Figure S-8. RMSD of backbone atoms over simulation time for trimer model candidates in periodic water boxes at three repeats represented by three different colors. Trimer model candidates A) 3hht, B) 1ahj, C) 1ugs, D) 1ugq.
Figure S-9. Collisional cross-section for trimer model candidates during simulation compared with the experimental value. Atomic coordinates are subjected to CCS calculation every 2 ns. A). Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); B) Simulation in vacuum
Figure S-10. RMSD of backbone atoms over simulation time for TNH hexamer model candidates. Simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl); Hexamer 7 and 8 failed simulation due to instability.
Figure S-11. Collisional cross-section for TNH hexamer model candidates during simulation compared with the experimental value. Atomic coordinates are subjected to CCS calculation every 2 ns for simulation in a periodic water box (a layer of water 10 Å on edge and charge balanced by 0.15M NaCl). Hexamer 7 and 8 failed simulation due to instability. Hexamer 5 and Hexamer 10 do not have β-β and γ-γ interaction between the two trimers, thus were eliminated.
Figure S-12. Interfacial strengths predicted in SID/IM experiments match with interfacial area calculated by PDBePISA from the three possible TNH hexamer structures energy minimized and equilibrated in vacuum for 10ns.
Figure S-13. MS/MS spectra of crosslinked peptides and their validation on the atomic structures of proposed TNH hexamers. MS/MS spectra of BS۳ (11.4 Å spacer arm) crosslinked A) α N-terminus to α N-terminus B) α N-terminus to β N-terminus. Although AR is short, only β has AR on its N-terminus. MS/MS spectra of BS۲G (7.7 Å spacer arm) crosslinked C) α N-terminus to α N-terminus D) α N-terminus to β N-terminus. C) The proposed hexamer structure 1 has α N-terminus to α N-terminus distance of 7 Å. D) The proposed hexamer structure 3 has α N-terminus to β N-terminus distance of 7.5 Å. The detected crosslinked peptides add confidence to our proposed hexamer structures.
Scheme S-1. A) The flow chart of the surface mapping experiment. B) Labeling reagent PGO and its reaction with arginine rendering a mass increase of 134.0368 or 116.0262.

Supplemental methods
Measurement of Catalytic activity of TNH in the presence of methanol

TNH was incubated for 10 minutes in 50 mM potassium phosphate pH 7.4 with various concentrations of methanol from 0 to 60% (v/v) after which the enzyme was tested for activity in a solution of 5 mM toyocamycin (Berry and Associates, Dexter, Michigan) and 50 mM potassium phosphate pH 6.5 and a final concentration of 2 nM TNH. These reactions were quenched after reacting for 1 minute by mixing of 20 µL of the reaction solution into 4 µL of 30%
(w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation, and the product sangivamycin was quantified using HPLC as previously described\textsuperscript{1}.

Modification of mobcal.f

Remove the trajectory method (TJM) by placing a ‘c’ in front of the Line#338.

Insert at Line#671 and Line#2682 the following lines describing the radius of coarse-grained sphere $\alpha$, $\beta$ and $\gamma$ subunits.

```
c     alpha (in TNH)
c
if(imass(iatom).eq.100) then
  itest=1
  xmass(iatom)=21190.3d0
  eolj(iatom)=1.35d-3*x
  rolj(iatom)=23d0*1.0d-10
  rhs(iatom)=23d0*1.0d-10
endif
c
c     beta (in TNH)
c
if(imass(iatom).eq.101) then
  itest=1
  xmass(iatom)=9974.4d0
  eolj(iatom)=1.35d-3*x
  rolj(iatom)=17.4d0*1.0d-10
  rhs(iatom)=17.4d0*1.0d-10
endif
```
gamma (in TNH)

if(imass(iatom).eq.102) then
  itest=1
  xmass(iatom)=11444.1d0
  eolj(iatom)=1.35d-3*xe
  rolj(iatom)=18.3d0*1.0d-10
  rhs(iatom)=18.3d0*1.0d-10
endif

An example of αβγ.mfj is as following:

```
1
3
ang
none
1.0000
  0  0  0  100  .10000
  0  26.5  0  101  .10000
  0  20.7  21  102  .10000
3
```

TNH expression and purification

The genes encoding the three subunits of toyocamycin nitrile hydratase were cloned as follows for heterologous co-expression in *E. coli* BL21 (DE3): the alpha subunit was cloned into the Ndel and XhoI sites of pACYCDuet-1, the gamma subunit was cloned into the Ncol and HindIII sites of pACYCDuet-1, and the beta subunit was cloned into the Ndel and XhoI sites of pET-29a. During initial purifications, the yield of the gamma subunit relative to the other two was found to decrease over the course of the multiple purification steps. To increase the yield of the gamma subunit and thus the replete heterohexamer at the beginning of the purification, a second copy of the gene encoding the gamma subunit was cloned in the Ncol and HindIII site of pETDUET-1.

Expression plasmids were introduced into *E. coli* with electroporation and plated on lennox broth agar containing 34 µg/mL of kanamycin and chloramphenicol and 100 µg/mL of ampicillin, which all further cultures shared. A single colony was used to inoculate a 0.1 L overnight starter culture which was spread evenly among six 2.5 L fernbach flasks containing 1 L of lennox broth. These were grown at 37 °C to an OD600 nm of 0.5 at which point expression was induced with the addition of isopropyl β-D-thiogalactopyranoside to 0.1 mM and CoCl$_2$ to a final concentration of 0.05 mM. After eight hours, these were spun down at 4,000 xg, and cell paste was frozen with liquid N$_2$ and stored at -80 °C.

All purification steps were carried out at 4 °C. The cell paste was resuspended in 20 mM Tris HCl pH 8.0 and 1 mM phenylmethylsulfonyl fluoride and disrupted on ice with sonication at 50% for 10 min with 30 sec bursts and 30 sec rests. Insoluble material was removed with centrifugation at 18,000 xg for 30 min. Clarified lysate was loaded onto a Q-sepharose column (2.6 x 12.5 cm) pre-equilibrated with 20 mM Tris HCl pH 8.0 and eluted over a 0.7 L gradient to the same buffer with 0.5 M NaCl. Fractions containing TNH as determined by SDS-PAGE and an orange color were pooled and brought to 1 M (NH$_4$)$_2$SO$_4$ with the addition of solid (NH$_4$)$_2$SO$_4$ over 20 min on ice with gentle stirring. This was loaded onto a butyl-sepharose column (2.6 x 12.5 cm) pre-equilibrated in 20 mM Tris HCl pH 8.0 and 1 M (NH$_4$)$_2$SO$_4$ and eluted with a 0.7 L gradient to the same buffer lacking (NH$_4$)$_2$SO$_4$ and with 10% ethylene glycol (v/v). Fractions containing TNH were pooled and concentrated by addition of solid (NH$_4$)$_2$SO$_4$ to 60% saturation over 20 min on ice with gentle stirring followed by centrifugation at 18,000 xg for 30 min. The precipitated pellet was resuspended in 1 mL of 25 mM HEPES•NaOH pH 7.5 and loaded onto a HiPrep 16/60 Sephacryl S200 column (GE Healthcare) pre-equilibrated in the same buffer. Fractions containing TNH were pooled and concentrated with Amicon Vivaspin Turbo 10K (polyethersulfone membrane) centrifugal concentrators and flash frozen with liquid N$_2$ and stored at -80 °C.

References
1. Nelp, M. T., Astashkin, A. V., Breci, L. A., McCarty, R. M., and Bandarian, V. (2014) The Alpha Subunit of Nitrile Hydratase Is Sufficient for Catalytic Activity and Post-Translational Modification, *Biochemistry* 53, 3990-3994.