Supplementary Information

Rational Thermostabilisation of Four-Helix Bundle Dimeric *De Novo* Proteins

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GLY A 3 70.3 0.51 LYS A 4 137.2 0.64 LEU A 5 5.4 0.03 ASN A 6 71.2 0.42 LYS A 7 152.3 0.70 LEU A 8 41.1 0.22 VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 HIS A 11 81.0 0.63 LUYS A 166 0.31 LEU A 162.0 0.50 LUN A 14 102.1 0.11 LEU A 162.0 0.50 LEU A 162.0 0.03 GLU A 165 0.02 GLN A 166 0.24 ASN A 22 1.00 GLN A 265 0.02 </th <th>Amino acids</th> <th>Chain</th> <th>Residues</th> <th>ASA</th> <th>ASA ratio</th> <th>Amino acids</th> <th>Chain</th> <th>Residues</th> <th>ASA</th> <th>ASA ratio</th>	Amino acids	Chain	Residues	ASA	ASA ratio	Amino acids	Chain	Residues	ASA	ASA ratio
LYS A 4 137.2 0.64 LEU A 5 5.4 0.03 ASN A 6 71.2 0.42 LYS A 7 152.3 0.70 LEU A 8 41.1 0.22 VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 MS A 13 66.6 0.31 LEU A 12 0.50 0.08 LEU A 14 102.1 0.51 LEU A 14 102.1 0.51 GLN A 17 110.0 0.55 MSE A 64 1.0 0.00 GLN A 16 5.9 0.03 GLN A 26 1.72 0.64 KWSE A 64 1.0 0.00 GLW A 26 <td< th=""><th>GLY</th><th>Α</th><th>3</th><th>70.3</th><th>0.51</th><th>ASP</th><th>Α</th><th>53</th><th>71.8</th><th>0.49</th></td<>	GLY	Α	3	70.3	0.51	ASP	Α	53	71.8	0.49
LEU A 5 5.4 0.03 ASN A 6 71.2 0.42 LYS A 7 152.3 0.70 LEU A 8 41.1 0.22 VAL A 9 120 0.68 GLU A 10 102.8 0.53 ILE A 11 81.0 0.41 HIS A 11 66.6 0.31 LVX A 13 66.6 0.31 LEU A 15 34.8 0.19 LEU A 16 5.9 0.03 GLN A 18 101.3 0.50 GLN A 18 101.3 0.50 GLN A 24 137.2 0.69 AGG A 22 41.5 0.24 HIS A 24 137.2 0.58 GLN A 27 <	LYS	Α	4	137.2	0.64	ASP	Α	54	98.9	0.60
ASN A 6 71.2 0.42 LYS A 7 152.3 0.70 LEU A 8 41.1 0.22 VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 HIS A 11 80.0 0.41 HIS A 12 0.5 0.00 LEU A 15 0.48 0.49 LEU A 15 34.8 0.49 LEU A 16 59 0.03 GLN A 17 110.0 0.55 LYS A 16 59 0.33 GLN A 166 96.0 0.49 LYS A 21 10.7 0.43 LYS A 24 15 0.22 HIS A 22 41.5 0.26 HIS A 23 10	LEU	А	5	5.4	0.03	GLY	Α	55	40.1	0.45
LVS A 7 152.3 0.70 LEU A 8 41.1 0.22 VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 HE A 12 0.50 0.08 JYS A 13 66.6 0.31 LEU A 12 0.50 0.00 LEU A 16 5.9 0.03 GLU A 14 102.1 0.51 LEU A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 17 10.0 0.52 MSE A 68 4.64 0.00 JEU A 12 111.0 0.52 MSE A 24 137.2 0.69 ASN A 23 10.7 0.44 MSE A 24	ASN	А	6	71.2	0.42	LYS	Α	56	115.2	0.55
LEU A 8 41.1 0.22 VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 III B10 0.41 0.51 IIIE A 11 81.0 0.41 IIIS A 11 66.6 0.31 GLU A 14 102.1 0.51 GLU A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 16 0.40 GLN A 17 10.0 0.55 GLN A 18 0.01 0.49 GLN A 66 96.0 0.49 GLN A 66 96.0 0.49 GLN A 67 1.2 0.01 MSE A 66 96.0 0.43 GLN A 26 57 0.03	LYS	А	7	152.3	0.70	LEU	Α	57	32.0	0.18
VAL A 9 12.0 0.08 GLU A 10 102.8 0.53 HIS A 11 81.0 0.41 LE A 12 0.5 0.00 LVS A 13 66.6 0.31 LEU A 14 10.21 0.51 GLU A 14 10.21 0.51 LEU A 16 5.9 0.03 GLN A 17 10.0 0.55 GLN A 17 10.0 0.55 GLN A 17 10.0 0.52 ASN A 20 75.1 0.43 JYS A 21 111.0 0.52 ASN A 22 41.5 0.24 HIS A 24 137.2 0.69 HIS A 22 137.2 0.58 GLN A 27	LEU	Α	8	41.1	0.22	GLN	Α	58	88.4	0.46
GLU A 10 102.8 0.53 HIS A 11 81.0 0.41 LE A 12 0.5 0.00 LYS A 13 66.6 0.31 GU A 14 102.1 0.51 GUN A 14 102.1 0.51 GUN A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 GLN A 12 0.11 MSE A 66 96.0 0.49 PHE A 67 1.2 0.01 MSE A 66 96.0 0.44 VX A 22 41.5 0.22 MSE A 68 4.6 0.02 MSE A 23 10.7 0.04 MSE A 68.0 0.44 VA 24 137.2	VAL	А	9	12.0	0.08	ASN	Α	59	108.1	0.63
HIS A 11 81.0 0.41 LLE A 12 0.5 0.00 LVS A 13 66.6 0.31 GLU A 14 102.1 0.51 LEU A 15 34.8 0.19 LEU A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 LEU A 18 101.3 0.50 LEU A 12 0.11 MSE A 68 4.6 0.02 ASN A 20 75.1 0.43 LYS A 21 111.0 0.52 MSE A 68 4.6 0.02 ASN A 22 41.5 0.24 HIS A 24 137.2 0.69 ARG A 27 55.3	GLU	Α	10	102.8	0.53	MSE	Α	60	21.1	0.11
LE A 12 0.5 0.00 LYS A 13 66.6 0.31 GLU A 14 102.1 0.51 LEU A 15 34.8 0.19 LEU A 16 5.9 0.03 GLN A 17 110.0 0.55 MSE A 66 96.0 0.49 PHE A 67 1.2 0.01 GLN A 18 101.3 0.50 MSE A 66 46.6 0.02 LEU A 19 4.5 0.02 ASN A 22 41.5 0.24 HIS A 24 137.2 0.68 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 GLN A 23 <	HIS	А	11	81.0	0.41	ILE	Α	61	15.7	0.09
LYS A 13 6666 0.31 GLU A 14 102.1 0.51 GLN A 14 102.1 0.51 GLN A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 GLN A 18 10.3 0.50 GLN A 19 4.5 0.02 ASN A 20 75.1 0.43 UYS A 21 111.0 0.52 ASN A 22 10.7 0.04 HIS A 22 137.2 0.69 HIS A 22 137.2 0.69 HIS A 22 137.2 0.68 GLN A 27 55.3 0.28 GLN A 28 28.6 0.32 MSE A 30	ILE	Α	12	0.5	0.00	HIS	Α	62	91.8	0.48
GLU A 14 102.1 0.51 LEU A 15 34.8 0.19 GLN A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 LEU A 19 4.5 0.02 JKN A 20 75.1 0.43 ASN A 20 75.1 0.43 MSE A 68 4.6 0.02 HIS A 22 41.5 0.24 HIS A 22 41.5 0.24 HIS A 24 137.2 0.68 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLN A 28 28.6 0.32 GLN A 30 4.8 0.03 MSE A 33	LYS	Α	13	66.6	0.31	GLU	Α	63	59.7	0.32
LEU A 15 34.8 0.19 LEU A 16 5.9 0.03 GLN A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 MR 19 4.5 0.02 LEU A 19 4.5 0.02 ASN A 22 41.5 0.24 MSE A 69 7.3.8 0.43 UYA A 22 41.5 0.24 HIS A 22 41.5 0.24 HIS A 26 5.7 0.03 GLN A 26 5.7 0.03 MSE A 30 4.8	GLU	Α	14	102.1	0.51	MSE	Α	64	1.0	0.00
LEU A 16 5.9 0.03 GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 LEU A 19 4.5 0.02 ASN A 20 75.1 0.43 LYS A 21 111.0 0.52 ASN A 22 41.5 0.24 TRP A 23 10.7 0.04 HIS A 24 137.2 0.69 HIS A 26 5.7 0.03 GLN A 27 36.3 0.22 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 MIS A 31 94.1 0.46 GLN A 276 36.3 0.22 BLEU A 30 0.30 35.3 HIS A 33	LEU	Α	15	34.8	0.19	GLN	Α	65	80.1	0.40
GLN A 17 110.0 0.55 GLN A 18 101.3 0.50 LEU A 19 4.5 0.02 ASN A 20 75.1 0.43 LYS A 21 111.0 0.52 ASN A 22 41.5 0.24 TRP A 23 10.7 0.04 HIS A 24 137.2 0.69 ARG A 25 137.2 0.68 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLN A 30 4.8 0.03 MSE A 31	LEU	Α	16	5.9	0.03	GLN	Α	66	96.0	0.49
GLN A 18 101.3 0.50 LEU A 19 4.5 0.02 ASN A 20 75.1 0.43 LYS A 21 111.0 0.52 ASN A 22 41.5 0.24 TRP A 23 10.7 0.04 HIS A 24 137.2 0.68 ARG A 25 137.2 0.68 GLN A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 MSE A 30 4.8 0.03 MSE A 31 94.1 0.46 ASP A 80 0.53 HIS A 33 9.8 0.05 MSE A 33 9.8 0.52 GLN A 36 69.4	GLN	Α	17	110.0	0.55	PHE	Α	67	1.2	0.01
LEU A 19 4.5 0.02 ASN A 20 75.1 0.43 LYS A 21 1111.0 0.52 ASN A 22 41.5 0.24 HIS A 22 41.5 0.24 HIS A 24 137.2 0.69 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 SER A 77 50.4 0.42 GLV A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 80 89.6 0.53 THR A 81 22.4 0.10 MSE A 33 9.8 0.05 GLN A 36	GLN	Α	18	101.3	0.50	MSE	Α	68	4.6	0.02
ASN A 20 75.1 0.43 LYS A 21 111.0 0.52 ASN A 22 41.5 0.24 TRP A 23 10.7 0.04 HIS A 24 137.2 0.69 HIS A 25 137.2 0.58 GLN A 26 5.7 0.03 GLY A 28 6.032 GLY A 28 6.032 GLY A 28 0.60 JEU A 30 4.8 0.03 HIS A 31 9.41 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 MSE A 37 9.2 0.04 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 <t< td=""><td>LEU</td><td>Α</td><td>19</td><td>4.5</td><td>0.02</td><td>ASN</td><td>Α</td><td>69</td><td>73.8</td><td>0.43</td></t<>	LEU	Α	19	4.5	0.02	ASN	Α	69	73.8	0.43
LYSA21111.00.52ASNA2241.50.24TRPA2310.70.04HISA24137.20.69ARGA25137.20.58HISA265.70.03GLNA2755.30.28GLYA2944.50.26JEUA304.80.03HISA2944.50.26LEUA304.80.03MSEA3194.10.46ASPA3288.00.53MSEA339.80.05ASNA3422.10.17GLNA3669.40.36MSEA379.20.04GLNA39109.60.58LEUA440.0MSEA39109.6GLNA3661.1GLNA440.0GLNA4417.8GLNA4553.0PHEA440.0GLNA46HISA46GLNA46GLNA46GLNA46GLNA46GLNA46GLNA46GLNA46GLNA46GLN	ASN	Α	20	75.1	0.43	GLN	Α	70	86.0	0.44
ASN A 22 41.5 0.24 TRP A 23 10.7 0.04 HIS A 24 137.2 0.69 ARG A 25 137.2 0.58 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 MSE A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 MSE A 37 9.2 0.04 GLN A 36 69.4 0.36 GLN A 38 71.3 0.39 GLN A 39 109.6 0.58 GLN A 39	LYS	Α	21	111.0	0.52	VAL	Α	71	4.0	0.03
TRP A 23 10.7 0.04 HIS A 24 137.2 0.69 ARG A 25 137.2 0.58 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 73 106.2 0.42 GLV A 26 5.7 0.03 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 MSE A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLN A 39 109.6 0.58 LEU A 40	ASN	Α	22	41.5	0.24	ASP	Α	72	36.3	0.22
HIS A 24 137.2 0.69 ARG A 25 137.2 0.58 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 ASN A 29 44.5 0.26 JHIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MISE A 33 9.8 0.05 MSE A 33 9.8 0.05 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 38 71.3 0.39 GLU A 42 <	TRP	Α	23	10.7	0.04	ASN	Α	73	106.2	0.62
ARG A 25 137.2 0.58 HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 43	HIS	Α	24	137.2	0.69	HIS	Α	74	19.9	0.11
HIS A 26 5.7 0.03 GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MISE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 39 109.6 0.58 GLU A 38 71.3 0.39 GLU A 43 66.1 0.03 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 <	ARG	Α	25	137.2	0.58	LEU	Α	75	7.7	0.04
GLN A 27 55.3 0.28 GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 22.1 0.17 GLN A 34 28.1 0.17 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLN A 38 71.3 0.39 GLN A 42 82.5 0.43 GLN A 42 82.5 0.43 GLN A 45 <th< td=""><td>HIS</td><td>Α</td><td>26</td><td>5.7</td><td>0.03</td><td>GLN</td><td>Α</td><td>76</td><td>84.2</td><td>0.45</td></th<>	HIS	Α	26	5.7	0.03	GLN	Α	76	84.2	0.45
GLY A 28 28.6 0.32 ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 <th< td=""><td>GLN</td><td>Α</td><td>27</td><td>55.3</td><td>0.28</td><td>SER</td><td>Α</td><td>77</td><td>50.4</td><td>0.42</td></th<>	GLN	Α	27	55.3	0.28	SER	Α	77	50.4	0.42
ASN A 29 44.5 0.26 LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLN A 38 71.3 0.39 GLU A 38 71.3 0.39 GLN A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLN A 44 0.0 0.00 GLN A 44 0.0 0.00 GLN A 44	GLY	Α	28	28.6	0.32	GLU	Α	78	0.0	0.00
LEU A 30 4.8 0.03 HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLN A 38 71.3 0.39 GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLN A 42 82.5 0.43 GLN A 45 53.0 0.26 PHE A 44 <	ASN	A	29	44.5	0.26	SER	Α	79	10.2	0.08
HIS A 31 94.1 0.46 ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 47 98.2 0.59 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLN A 42 82.5 0.43 GLN A 44 0.0 0.00 GLN A 45 <t< td=""><td>LEU</td><td>A</td><td>30</td><td>4.8</td><td>0.03</td><td>ASP</td><td>A</td><td>80</td><td>89.6</td><td>0.53</td></t<>	LEU	A	30	4.8	0.03	ASP	A	80	89.6	0.53
ASP A 32 88.0 0.53 MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLN A 38 71.3 0.39 GLU A 38 71.3 0.39 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 46 <t< td=""><td>HIS</td><td>A</td><td>31</td><td>94.1</td><td>0.46</td><td>THR</td><td>A</td><td>81</td><td>22.4</td><td>0.15</td></t<>	HIS	A	31	94.1	0.46	THR	A	81	22.4	0.15
MSE A 33 9.8 0.05 ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 44 0.0 0.00 GLN A 48 38.1 0.19 HIS A 48 <t< td=""><td>ASP</td><td>A</td><td>32</td><td>88.0</td><td>0.53</td><td>VAL</td><td>A</td><td>82</td><td>0.1</td><td>0.00</td></t<>	ASP	A	32	88.0	0.53	VAL	A	82	0.1	0.00
ASN A 34 28.1 0.17 GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLU A 38 71.3 0.39 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 ASN A 93 40.7 0.20 ASN A 94 85.8 0.52 GLN A 46 123.7 0.62 PHE A 46 123.7 0.62 PHE A 46	MSE	A	33	9.8	0.05	HIS	A	83	89.3	0.45
GLN A 35 110.8 0.58 GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 MSE A 37 9.2 0.04 GLN A 38 71.3 0.39 GLU A 38 71.3 0.39 GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 46 123.7 0.62	ASN	A	34	28.1	0.17	HIS	A	84	103.1	0.53
GLN A 36 69.4 0.36 MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLU A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 HIS A 95	GLN	A	35	110.8	0.58	PHE	A	85	24.6	0.11
MSE A 37 9.2 0.04 GLU A 38 71.3 0.39 GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 HIS A 49 122.6 0.62 GLN A 49 126.1 0.63 GLN A 49	GLN	A	36	69.4	0.36	HIS	Α	86	12.9	0.07
GLU A 38 71.3 0.39 GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 <td>MSE</td> <td>A</td> <td>37</td> <td>9.2</td> <td>0.04</td> <td>ASN</td> <td>A</td> <td>87</td> <td>98.2</td> <td>0.59</td>	MSE	A	37	9.2	0.04	ASN	A	87	98.2	0.59
GLN A 39 109.6 0.58 LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLN A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 HIS A 48 38.1 0.19 HIS A 48 38.1 0.19 HIS A 49 122.6 0.62 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 <td>GLU</td> <td>A</td> <td>38</td> <td>71.3</td> <td>0.39</td> <td>LYS</td> <td>A</td> <td>88</td> <td>87.1</td> <td>0.41</td>	GLU	A	38	71.3	0.39	LYS	A	88	87.1	0.41
LEU A 40 1.8 0.01 PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 HIS A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 50 56.9 0.64 Wall A 100 19.5 0.12 HIS A 101 150.9 0.62	GLN	A	39	109.6	0.58	LEU	A	89	6.1	0.03
PHE A 41 17.8 0.08 GLN A 42 82.5 0.43 GLU A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 MSE A 49 122.6 0.62 GLN A 49 122.6 0.62 HIS A 99 40.4 0.22 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 50 56.9 0.64 WAL A 100 19.5 0.12 HIS A 101 150.9 0.62	LEU	A	40	1.8	0.01	GLN	A	90	78.5	0.40
GLN A 42 82.5 0.43 GLU A 43 66.1 0.35 PHE A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 MSE A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 50 56.9 0.64 ASN A 99 40.4 0.22 GLN A 51 25.6 0.16 HIS A 99 40.4 0.22 GLN A 51 25.6 0.164 HIS A 101 150.9 0.62	PHE	A	41	17.8	0.08	GLU	A	91	118.2	0.61
GLU A 43 66.1 0.35 PHE A 443 0.0 0.00 GLN A 444 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 50 56.9 0.64 ASN A 99 40.4 0.22 VAL A 100 19.5 0.12 HIS A 101 150.9 0.62	GLN	A	42	82.5	0.43	LEU	A	92	15.0	0.08
PHE A 44 0.0 0.00 GLN A 44 0.0 0.00 GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 Other HIS A 100 19.5 0.12 HIS A 101 150.9 0.62	GLU	A	43	66.1	0.35	MSE	A	93	40.7	0.20
GLN A 45 53.0 0.26 HIS A 46 123.7 0.62 PHE A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 Old A 100 19.5 0.12 HIS A 101 150.9 0.62	PHE	A	44	0.0	0.00	ASN	A	94	85.8	0.52
HIS A 46 123.7 0.62 PHE A 46 123.7 0.62 PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 ASN A 51 25.6 0.16 HIS A 101 150.9 0.62	GLN	A	45	53.0	0.26	ASN	A	95	72.0	0.42
PHE A 47 39.6 0.19 MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 ASN A 51 25.6 0.16	HIS	A	46	123.7	0.62	PHE	A	96	2.6	0.01
MSE A 48 38.1 0.19 GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 ASN A 51 25.6 0.16	PHE	A	47	39.6	0.19	HIS	A	97	83.7	0.43
GLN A 49 122.6 0.62 GLY A 50 56.9 0.64 ASN A 51 25.6 0.16	MSE	A	48	38.1	0.19	HIS	A	98	126.1	0.63
GLY A 50 56.9 0.64 VAL A 100 19.5 0.12 ASN A 51 25.6 0.16 HIS A 101 150.9 0.62	GLN	A	49	122.6	0.62	LEU	A	99	40.4	0.22
ASN A 51 25.6 0.16 HIS A 101 150.9 0.62	GLY	A	50	56.9	0.64	VAL	A	100	19.5	0.12
	ASN	A	51	25.6	0.16	HIS	A	101	150.9	0.62

Table S1. Accessible surface areas (ASA) of amino acid residues in the WA20 structure. The data (chain A) of the crystal structure of WA20 (PDB ID: 3VJF)¹⁶ were calculated by the program AREAIMOL^{22,23} in the CCP4 suite²⁴. Red letters represent hydrophilic residues with small ASA ratio (ASA ratio ≤ 0.11). (ASA ratio: ratio of ASA to calculated GXG (Gly-Xaa-Gly) value.). The target residues for mutations selected in this study are shown in bold letters.

Amino acids	Chain	Residues	ASA	ASA ratio	Amino acids	Chain	Residues	ASA	ASA ratio
LEU	В	5	166.3	0.72	HIS	В	62	100.9	0.52
ASN	В	6	8.0	0.04	GLU	В	63	75.9	0.40
LYS	В	7	98.7	0.48	MSE	В	64	4.7	0.02
LEU	В	8	63.8	0.34	GLN	В	65	46.1	0.23
VAL	В	9	3.5	0.02	GLN	В	66	95.5	0.49
GLU	В	10	126.5	0.65	PHE	В	67	1.6	0.01
HIS	В	11	92.2	0.47	MSE	В	68	5.6	0.03
ILE	В	12	2.1	0.01	ASN	В	69	61.5	0.36
LYS	В	13	65.6	0.30	GLN	В	70	96.5	0.48
GLU	В	14	100.8	0.51	VAL	В	71	1.6	0.01
LEU	В	15	44.2	0.24	ASP	В	72	31.4	0.19
LEU	В	16	15.5	0.08	ASN	В	73	103.5	0.61
GLN	В	17	109.8	0.55	HIS	В	74	9.7	0.05
GLN	В	18	121.3	0.60	LEU	В	75	7.5	0.04
LEU	В	19	8.5	0.05	GLN	В	76	82.4	0.45
ASN	В	20	56.1	0.34	SER	В	77	46.7	0.35
LYS	В	21	153.4	0.70	GLU	В	78	0.0	0.00
ASN	в	22	61.0	0.35	SER	В	79	2.3	0.02
TRP	В	23	2.7	0.01	ASP	В	80	84.9	0.51
HIS	В	24	110.8	0.54	THR	В	81	17.4	0.12
ARG	B	25	172.9	0.69	VAI	B	82	0.1	0.00
HIS	В	26	3.2	0.02	HIS	В	83	69.9	0.35
GLN	В	27	64.4	0.34	HIS	B	84	93.6	0.48
GLY	B	28	30.2	0.33	PHF	B	85	13.5	0.06
ASN	B	29	51.1	0.30	HIS	B	86	14.1	0.07
	B	30	56	0.00	ASN	B	87	90.2	0.54
HIS	B	31	85.9	0.05	LYS	B	88	76.4	0.36
	B	32	66.5	0.40	LEU	B	89	6.9	0.00
MSE	B	33	7.0	0.10	GLN	B	90	82.7	0.01
	B	34	27.4	0.04	GLU	B	90	103.7	0.42
GLN	B	35	87.3	0.17	I FU	B	92	14.9	0.02
GLN	B	36	42.4	0.40	MSE	B	02	14.5	0.00
MSF	R	37	-+2.4 8.8	0.22	ASN	R	93	+J.0 83.4	0.22
GLU	R	20	78 /	0.04		R	04	81 F	0.01
GLN	R	30	109.3	0.44		B	90	56	0.47
	R	40	0.2	0.00		B	90 Q7	0.0 00 8	0.03
	R	/1	0.2 22.1	0.00		R	08	100.0	0.47
GLN	P	41	23.1	0.11		P	90 90	122.3	0.01
	P	42	201.0	0.40		P	39 100	43.0	0.20
	P	43	0.1	0.21		P	100	160.4	0.22
	P	44	0.1	0.00	GIU	D	101	100.4	00.0
	D	40	09.0	0.40					
HIS	В	46	145.7	0.74					
PHE	В	47	105.2	0.47					
MSE	В	48	112.3	0.47					
LYS	В	56	186.6	0.71					
LEU	В	57	52.5	0.28					
GLN	В	58	104.5	0.53					
ASN	В	59	101.9	0.60					
MSE	В	60	54.7	0.27					
ILE	В	61	13.4	0.07					

Table S1 (continued). Accessible surface areas (ASA) of amino acid residues in the WA20 structure. The data (chain B) of the crystal structure of WA20 (PDB ID: 3VJF)¹⁶ were calculated by the program AREAIMOL^{22,23} in the CCP4 suite²⁴. Red letters represent hydrophilic residues with small ASA ratio (ASA ratio \leq 0.11). (ASA ratio: ratio of ASA to calculated GXG (Gly-Xaa-Gly) value.) The target residues for mutations selected in this study are shown in bold letters. 3

Primer name	Sequence $(5' \rightarrow 3')$						
T7 promoter primer	TAATACGACTCACTATAGGG						
T7 terminator primer	GCTAGTTATTGCTCAGCGG						
WA20_N22A_Fw	TGCAACAGTTGAACAAA <mark>GCC</mark> TGGCACAGGC						
WA20_N22E_Fw	TGCAACAGTTGAACAAA <mark>GAA</mark> TGGCACAGGC						
WA20_N22K_Rv	GCCTGTGCCACTTTTGTTCAACTGTTGCAGCAATTC						
WA20_N22L_Rv	GCCTGTGCCA <mark>GAG</mark> TTTGTTCAACTGTTGCAGCAATTC						
WA20_H86K_Fw	GACACCGTGCATCATTTCAAGAACAAATTGCAGGAGC						
WA20_H86S_Fw	GACACCGTGCATCATTTCAGCAACAAATTGCAGGAGC						
WA20_H74V_S79F_Rv	GAAATGATGCACGGTGTC <mark>GAA</mark> CTCGCTCTGCAG <mark>AAC</mark> GTTGTCGACTTGGTTCATGAACTG						

Table S2. Oligonucleotide primer sequences used in this study.

Red letters correspond to the codons for amino acid substitutions.



Figure S1. Potential target residues on the interface of α-helices in the WA20 structure.

Two target residues (N22 and N34) on the interface of α -helices were chosen to potentially enhance helix-helix interactions. The target residues are shown as sticks. Chains A and B of the crystal structure of WA20 (PDB: 3VJF)¹⁶ are shown in magenta and cyan, respectively. The images were created using open-source PyMOL, version 2.4 (https://github.com/schrodinger/pymol-open-source).



Figure S2. Correlation of experimental $T_{\rm m}$ and RMSD calculated by high-temperature MD simulations for WA20 mutants.

MD simulations under high temperature condition (600 K, 10 ns) were performed for WA20 and the thermostabilised mutants reported in a previous study²⁰. Root mean square deviations (RMSD) between initial structures and structures after high-temperature MD simulations were calculated to evaluate the degrees of protein unfolding. MD simulations were performed ten times for each protein. There is a strong negative correlation between RMSD and experimentally determined $T_{\rm m}$ of WA20 and the mutants (r = -0.966, $p = 5.461 \times 10^{-5}$).



Figure S3. RMSD of the WA20 mutant structures calculated by high-temperature MD simulations. MD simulations under high temperature condition (600 K, 10 ns) were performed for WA20 and all of the possible single mutants of WA20 generated by *in silico* saturation mutagenesis at the four target residue sites. Root mean square deviations (RMSDs) between the initial structures and the structures after high-temperature MD simulations were calculated to evaluate the degrees of protein unfolding. MD simulations were performed ten times for each mutant. Blue bars represent the original WA20. Red bars of small RSMD values represent the mutants selected for experimental evaluations in this study.





Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the WA20 mutants were performed for the eluted fractions by immobilised metal ion affinity chromatography (IMAC) with TALON metal affinity resin (Takara Bio, Otsu, Japan). The protein bands surrounded in the black square show purified WA20 and the mutants (molecular mass of a monomer of WA20 and its mutants: ~12.5 kDa). Protein molecular weight markers (broad) (Takara Bio) are shown in left. Proteins were stained with Coomassie brilliant blue.

Abbreviations: M, molecular weight markers; P, pellet fraction; L, lysate; F, flow-through fraction; W, wash fractions; E, eluted fractions.



Figure S5. Circular dichroism (CD) spectra of the WA20 mutants. CD spectra were measured at 25 °C.



Figure S6. Thermal denaturation curves of (A, B, C) WA20 mutants and (D) SUWA mutants. The *Y*-axis represents the normalised value of $[\theta]_{222 \text{ nm}}$. The experimental data were fitted to a two-state model with ΔC_p fixed to zero using CDpal³³. The T_m values of the WA20 mutants and the SUWA mutants are shown in Table 1 and Table 2, respectively. The graphs were created using CDpal³³, version 2.18 (https://github.com/PINT-NMR/CDpal/).





SUWA_N22E SUWA_H86K

Figure S7. SDS-PAGE analysis of SUWA and the SUWA mutants.

SDS-PAGE analysis of SUWA and the SUWA mutants were performed for the eluted fractions by IMAC with TALON metal affinity resin (Takara Bio). The protein bands surrounded in the black square show purified SUWA and the mutants (molecular mass of a monomer of SUWA and its mutants: ~12.6 kDa). Protein molecular weight markers (broad) (Takara Bio) are shown in left. Proteins were stained with Coomassie brilliant blue.

Abbreviations: M, molecular weight markers; L, lysate; F, flow-through fraction; E, eluted fractions.



Figure S8. Circular dichroism (CD) spectra of the SUWA mutants CD spectra were measured at 30 °C.



Figure S9. Size exclusion chromatography (SEC) analyses of ROWA and ROSA dimers.

SEC profiles of IMAC-purified samples of ROWA (left) and ROSA (right) with a Superdex 75 increase 10/300 GL column (Cytiva) are shown in top panels. The isolated fractions of the dimer peaks of ROWA and ROSA were reanalysed immediately after the SEC purification (0 day, middle panels) and after keeping them at 20 °C for 7 days (bottom panels). These results suggest that the dimers did not change to the other oligomers, i.e., the oligomeric states do not exchange one another on a timescale of a week. The *Y*-axis represents the intensity of UV absorbance ($A_{280 \text{ nm}}$).



Figure S10. Guinier plots of the SAXS data of the ROWA and ROSA samples.

Forward scattering intensity, $I(q \rightarrow 0)$, and radius of gyration, R_g , were estimated by Guinier approximation³⁹ using AUTORG in ATSAS³⁸ with SAngler³⁶. However, the Guinier approximation was not applied to the data of the ROSA (iii+iv) sample because the linearity of the Guinier plot of ROSA (iii+iv) was not good enough for a linear approximation probably due to polydispersity or aggregation.



Figure S11. Plots of the scattering curves calculated from the DAMMIN models fitting to the experimental SAXS data.

The concentration-normalised SAXS intensity, I(q)/c, of the ROWA and ROSA samples (black open circle) and that optimised by the DAMMIN procedure (red line). The χ^2 value represents the degree of fitting between the experimental data and the data calculated from the DAMMIN model.



Figure S12. Model structures of the thermostabilised mutants of WA20 and SUWA.

(A) Model structures of the N22A and N22E mutants of WA20 and SUWA. The left panels show the structures of WA20 and SUWA and the middle and right panels show the model structures of the mutants. Chains A and B of WA20 are shown in magenta and cyan, respectively. Chains A and B of SUWA are shown in gray and yellow, respectively. The target and mutated residues are shown as green sticks. The interatomic distances between oxygen of E22 and nitrogen of R25 (Å) are shown with dashed lines. (B) Model structures of the H86K mutants of WA20 and SUWA. Chains A and B of WA20 are shown in magenta and cyan, respectively. Chains A and B of SUWA are shown in gray and yellow, respectively. The target and mutated residues are shown as green sticks. The interatomic distances between oxygen of D72 and nitrogen of H86 or K86 (Å) are shown with dashed lines. The model structures were constructed from the crystal structures of WA20 (PDB ID: 3VJF) or SUWA (PDB ID: 6KOS) and optimised by MD simulation at 300 K for 1 ns. The images were created using open-source PyMOL, version 2.4 (https://github.com/schrodinger/pymol-open-source).

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Figure S13. Model structures of the destabilised mutants of WA20.

The left panels show the structure of WA20. The middle and right panels show the model structures of the N22K and N22Lmutants, respectively. Chains A and B of WA20 are shown in magenta and cyan, respectively. The target and mutated residues are shown as green sticks in the upper panels. In the lower panels, all atoms including hydrogens are shown as spheres, and colors are the same as the upper panels. The model structures were constructed from the crystal structure of WA20 (PDB ID: 3VJF) and optimised by MD simulation at 300 K for 1 ns. The images were created using open-source PyMOL, version 2.4 (https://github.com/schrodinger/pymol-open-source).

WA20

MYGKLNKLVE HIKELLQQLN KNWHRHQGNL HDMNQQMEQL FQEFQHFMQG NQDDGKLQNM IHEMQQFMNQ VDNHLQSESD TVHHFHNKLQ ELMNNFHHLV HR (Molecular mass of a monomer: 12547)

ROWA (N22A/H86K)

MYGKLNKLVE HIKELLQQLN K**A**WHRHQGNL HDMNQQMEQL FQEFQHFMQG NQDDGKLQNM IHEMQQFMNQ VDNHLQSESD TVHHF**K**NKLQ ELMNNFHHLV HR

(Molecular mass of a monomer: 12495)

SUWA (H26L/G28S/N34L/V71L/E78L)

MYGKLNKLVE HIKELLQQLN KNWHRLQSNL HDMLQQMEQL FQEFQHFMQG NQDDGKLQNM IHEMQQFMNQ LDNHLQSLSD TVHHFHNKLQ ELMNNFHHLV HR (Molecular mass of a monomer: 12550)

ROSA (N22E/H26L/G28S/N34L/V71L/E78L/H86K)

MYGKLNKLVE HIKELLQQLN KEWHRLQSNL HDMLQQMEQL FQEFQHFMQG NQDDGKLQNM IHEMQQFMNQ LDNHLQSLSD TVHHFKNKLQ ELMNNFHHLV HR (Molecular mass of a monomer: 12556)

(Molecular mass of a monomer: 12556)

Figure S14. Amino acid sequences of WA20, ROWA, SUWA, and ROSA.

Red letters represent the stabilising mutations found in this study. Blue letters represent the stabilising mutations reported in a previous study²⁰.