Hairpin structure of a biarsenal –tetracysteine motif determined by NMR

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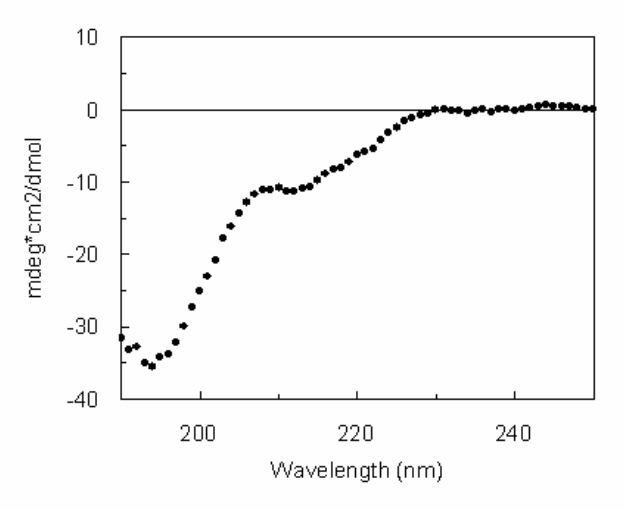

Figure S1: CD spectrum recorded at 277K for 6 μM concentration of the peptide bound to the ReAsH, pH 7.4. The CD spectrum was recorded with 0.05 mm optical pathway and was baseline corrected using a buffer solution.

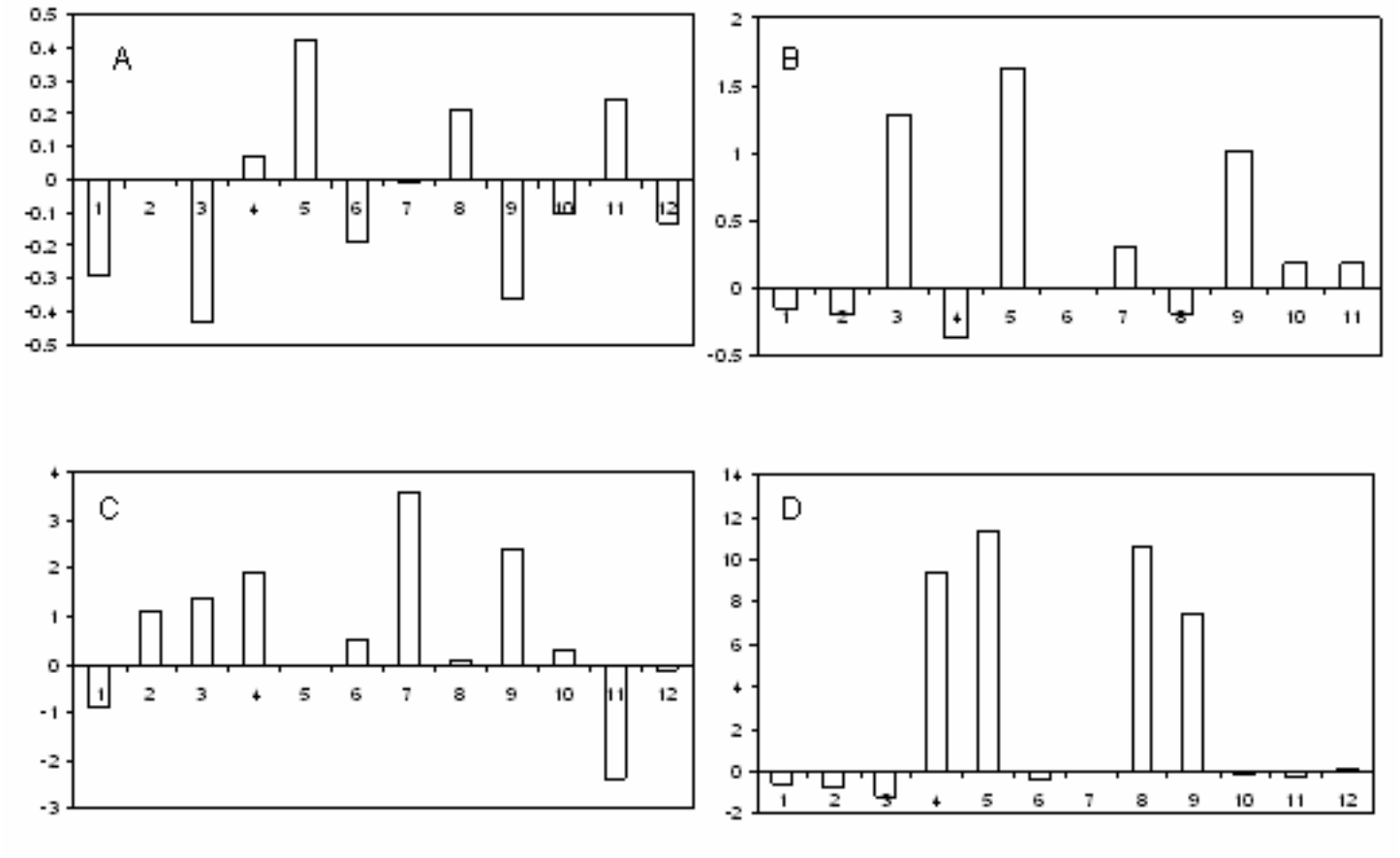

Figure S2: Secondary chemical shifts (ppm) for proton and carbon resonances against residue number were calculated according to the method of Wishart and Sykes[1]. (A) H^{α} secondary chemical shifts (B) H^{N} secondary chemical shifts (C) C^{α} secondary chemical shifts and (D) C^{β} secondary chemical shifts.

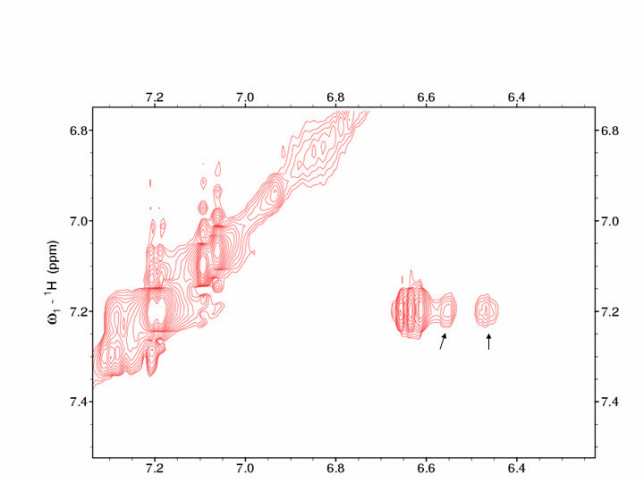

Figure S3. Partial 500 MHz NOESY spectrum with the mixing time of 300 ms. Arrows show the crosspeaks between the 16(12) proton of ReAsH and aromatic protons of F1.

***Table S1*:** Observed chemical shifts (ppm) for the 12-residue peptide FLNCCPGCCMEP bound to the ReAsH.

Residue	**H^N**	**H^α**	**H^β**	**H^γ**	**H^δ**	**H^ε**	**H^ζ**	**C^α**	**C^β**	**C^γ**	**C^δ**	**C^ε**	**C^ζ**
Phe1	8.15	4.34	3.25 2.74	-	6.47	6.55	6.55	56.8	39.0	-	129.8	130.7	129.8
Leu2	8.03	4.32	1.56 1.46	1.51	0.81 0.85	-	-	56.2	41.6	27.0	24.2	-	-
Asn3	9.66	4.31	2.89 2.76	-	7.79 7.09	-	-	54.5	37.7	-	-	-	-
Cys4	7.86	4.61	3.17 3.17	-	-	-	-	60.1	37.4	-	-	-	-
Cys5	9.85	4.96	3.31 3.07	-	-	-	-	-	39.4	-	-	-	-
Pro6	-	4.23	2.24 1.86	1.96 2.10	3.79 3.66	-	-	63.8	31.7	34.7	50.7	-	-
Gly7	8.60	4.11 3.80	-	-	-	-	-	45.7	-	-	-	-	-
Cys8	8.04	4.75	2.89 3.07	-	-	-	-	58.3	38.6	-	-	-	-
Cys9	9.25	4.18	3.41 3.46	-	-	-	-	60.6	35.4	-	-	-	-
Met10	8.47	4.38	1.96 2.07	2.42 2.42	-	2.05	-	55.7	32.7	31.9	-	16.4	-
Glu11	8.54	4.57	1.92 2.03	2.21 2.41	-	-	-	54.2	29.6	35.9	-	-	-
Pro12	-	4.29	2.25 1.89	n.d[a]	3.66 3.79	-	-	63.2	32.2	n.d[a]	50.7	-	-
ReAsH	H15/H11[b] 6.64	H16/H12 7.20	C15/C11 131.7	C16/C12 129.2									
Succinyl	(H1)	(H2)	(C1)	(C2)									
group	2.10	1.98	27.5	27.3									

[a]not determined.

[b]the resorufin resonances are degenerate due to the symmetry of the molecule, which was not broken by its asymmetric environment.

Materials and Methods

ReAsH-tetracysteine complexes. Peptides were synthesized by standard Fmoc solid-phase techniques using a Pioneer (Perseptive Biosciences) or a Symphony peptide synthesizer (Protein Technologies, Inc). TGR-resin (Novagen) bound-peptides were acylated on the N-terminus by reaction with 5 equivalents of NHS-PEG^{TM} (MW=245, 333, 509 or 685; Quanta BioDesign Ltd), or 50 equivalents of succinic anhydride or chlorosulfonic acid in DMF containing excess DIEA. Peptide was cleaved from the support with TFA-EDT-triisopropylsilane-H_2O and precipitated with cold ether-hexanes. Crude peptide was reacted with excess ReAsH-EDT_2 in DMSO containing 4-methylmorpholine for at least 1 hr at room temperature before purification and analysis by LC-MS (Agilent 1100 with Ion trap) on C18 columns (analytical or semi-prep; Phenomenex) with acetonitrile-H_2O-0.05% TFA gradient and lyophilization. For NMR, the ReAsH-tetracysteine complex was dissolved in a minimum of 50 mM potassium phosphate pH 7.2, filtered (0.2 μm centrifugal; Corning) and concentration determined by dilution in 0.1N NaOH (ε_{578} = 63000 $M^{-1}cm^{-1}$).

Absorbance, fluorescence and stability to dithiol of ReAsH-tetracysteine complexes. Purified ReAsH-tetracysteine stock solutions were in 50% aqueous acetonitrile containing 0.05% TFA. Extinction coefficients were determined in 10 mM K.MOPS pH 7.2 by comparison with the subsequent absorbance at 578.5 nm on adding 1N-NaOH to give a final concentration of 0.1N[2]. The extinction coefficient of ReAsH-EDT_2 in 0.1N NaOH is 63000 $M^{-1}cm^{-1}$.

Fluorescence spectra were measured in 100 mM KMOPS pH 7.2 using a Fluorolog (Horiba Jovin Yvon) fluorimeter. Rhodamine 101 in EtOH was used as a standard (ϕ = 1.0)[3] to determine fluorescent quantum yield. Relative stabilities of the ReAsH-tetracysteine complexes were measured by following the decrease in fluorescence (excitation 590 nm emission 610 nm) on adding 0.5 mM EDT (stock freshly prepared in DMSO) to the complex in 5 mM 2-mercaptoethanesulfonate 100 mM KMOPS pH 7.2. Apparent rate constants were determined by fitting to a single or double exponential.

Circular dichroism. Circular Dichroism spectra were recorded on a JASCO J-720 CD spectrometer at 277K. Wavelengths between 190 nm and 220 nm were recorded, using a bandwidth of 0.2 nm. A quartz cuvette with an optical path length of 0.05 mm was used, requiring approximately 100 µl of sample. 5 µl of the sample was diluted to a concentration of 6 µM for the CD experiment. The background signal was subtracted from the peptide spectrum.

NMR experiments. All NMR experiments were carried out at a temperature of 278K on a Bruker Avance spectrometer equipped with a cryoprobe and operating at a Larmor frequency of 500 MHz, a Varian Inova NMR spectrometer equipped with a triple resonance probe head operating at a Larmor frequency of 600 MHz and a Bruker Avance spectrometer equipped with a double resonance probe head operating at a Larmor frequency of 400 MHz.

NOESY spectra[4], TOCSY spectra[5] and DQF-COSY spectra[6] were recorded at proton frequencies of 500 and 600 MHz. The NOESY spectra were performed with a mixing time of 150 - 500 ms while the TOCSY spectra used 20, 30, 60 and 80 ms. Data were typically collected as 2048×512 data point matrices with 32-64 scans. In order to simplify the assignment and find the resonances of the four hydrogen atoms of ReAsH, a natural abundance carbon filtered HSQC was recorded on the sample. The water signal was suppressed with the WATERGATE or excitation sculpting methods[7, 8]. Diffusion coefficients were measured using the pulse field gradient spin echo experiment (PFG-LED)[9] with a fixed diffusion time and a pulsed field gradient increasing linearly over 32 steps. A measured diffusion coefficient could be related to a molecular weight via the Stokes-Einstein relationship according to a scaling law calibrated for unstructured peptides [10].

Structure calculation. The spectra were processed with NMRpipe[11] and the processing included zero-filling to 4096×2048 data points and multiplication of a shifted sine bell function prior to Fourier transformation. 2D-spectral analysis, peak picking and crosspeak integration were performed in Sparky 3.113[12]. CYANA 2.0[13, 14] was used to convert the intensity of the NOESY crosspeaks into upper distances constrains.

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