Supporting Information

Disulfide-Depleted Selenoconopeptides: Simplified Oxidative Folding of Cysteine-Rich Peptides

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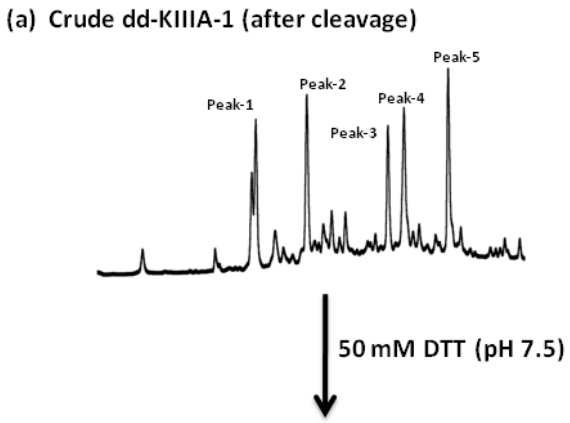
EXPERIMENTAL PROCEDURES

Peptide and selenopeptide analogs were synthesized on a solid support using standard Fmoc (N-(9-flourenyl)methoxycarbonyl) chemistry, as described elsewhere. [1-3] The cysteine residues were protected with S-trityl groups. Fmoc-protected selenocysteine residues with side chains protected with selenium-p-methoxybenzyl groups were purchased from Chem-Impex International, Wood Dale, IL, USA). Peptide-resin was treated for 3 h with reagent K (trifluoroacetic acid (TFA)/thioanisole/phenol/water (90:2.5:7.5:2.5, v/v/v/v) and 1.3 equivalents DTNP [2,2′-dithiobis(5-nitropyridine)]). After the selenoconopeptide analogs were washed with methyl *tert*-butyl ether, they were treated for 1 h with DTT (dithiothreitol; 100 mM), Tris (tris(hydroxymethyl)aminomethane; 0.1M), EDTA (ethylenediaminetetraacetic acid; 1 mM), pH 7.5 at room temperature. This reaction was quenched by acidification with 8% formic acid. The peptides were purified using reverse-phase HPLC separations. Using a semi-preparative C_{18} HPLC column (Vydac), the peptide analogs were eluted at 5 mL/min flow rate using a linear gradient of acetonitrile: 5% to 35% solvent B over 35 min, where solvent A was 0.1% TFA in water and solvent B was 0.1%TFA, v/v, in 90% aqueous acetonitrile. The elution was monitored by measuring the absorbance at 220 nm. Oxidative folding was performed at room temperature in a buffer solution containing Tris-HCl (0.1 M, pH 7.5), EDTA (1 mM), a mixture of oxidized and reduced glutathione (1mM GSSG and 1 mM GSH). Final concentration of the peptide analogs in the folding reaction solution was 20 μM. After 1 h, the reaction was quenched by acidification. The oxidized peptide analogs were purified by C_{18} semi-preparative HPLC. The folded peptides were quantified by measuring UV absorbance at 280 nm. Chemical identity of each analog was confirmed by mass spectrometry analysis (Table S1).

Electrophysiological experiments were carried out identically as described elsewhere. [4] Oocytes expressing mammalian Na_Vs were prepared and placed in a chamber containing ND96 and two-electrode voltage clamped with a holding potential of –80 mV. Next, the membrane potential was stepped to a value between -20 and 0 mV for 50 ms every 20 sec. Three μL of the peptide solution was applied, and the bath was manually stirred for about 5 seconds. All recordings were done at room temperature (~21 °C).

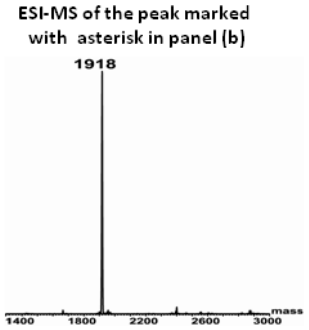
Table S1. Molecular masses of the folded selenoconopeptide analogs and their linear forms used for glutathione-based oxidation. The observed mass of the peptides were derived using MALDI (reflectron) mass spectrometry. HPLC retention times are based on reversed-phase separation using analytical C_{18} HPLC column (Vydac). the analogs were eluted at 1 mL/min flow rate using a linear gradient of acetonitrile: 5% to 35% buffer B over 35 min, where buffer A was 0.1% TFA in water and buffer B was 0.1%TFA, v/v, in 90% aqueous acetonitrile. The elution was monitored by measuring the absorbance at 220 nm.

Name of the peptide	**Observed (calculated) mass of the linear peptide $[M+H]^+$ Da (M Da)**	**Observed (calculated) mass of the folded peptide $[M+H]^+$ Da (M Da)**	**HPLC retention time of the folded species (min)**
dd-KIIIA-1	1919.63 (1920.69)	1917.67 (1916.69)	18.2
dd-KIIIA-2	1919.68 (1920.69)	1917.69 (1916.69)	18.5
dd-KIIIA[K7A]	1862.50(1863.63)	1860.63 (1859.63)	18.5
dd-KIIIA[K7F]	1938.62 (1939.66)	1936.63 (1935.66)	26.4
dd-KIIIA[K7S]	1878.53 (1879.63)	1876.62 (1875.63)	18.5
dd-KIIIA[K7T]	1892.82 (1893.64)	1890.82 (1889.64)	22.3
dd-KIIIA[K7D]	1906.61 (1907.62)	1904.52 (1903.62)	19.4
dd-KIIIA[K7G]	1848.59 (1849.61)	1846.34 (1845.61)	19.4
dd-KIIIA[K7L]	1904.65 (1905.68)	1902.50 (1901.68)	23.1
dd-KIIIA[K7V]	1890.65 (1891.66)	1888.47 (1887.66)	24.7
dd-KIIIA[K7Dap]	1877.75 (1878.62)	1875.72 (1874.62)	22.8



Mass spectrometric analysis of the fractions collected from crude dd-KIIIA-1 and possible interpretations

Elution peak	MALDI-MS $[M+H]^+$ Da	ESI-MS M Da	Possible interpretation
Peak-1	1917.7	1916.3	Folded dd-KIIIA-1
Peak-2	3833.2	3831.9	Dimeric dd-KIIIA-1
Peak-3	3971.6	3969.2	unknown
Peak-4	1916.5, 2072.3 & 2226.5	2225.4	Two 5-thionitropyridyl containing dd-KIIIA-1
Peak-5	1917.0, 2071.9 & 2228.3	2225.9	Two 5-thionitropyridyl containing dd-KIIIA-1



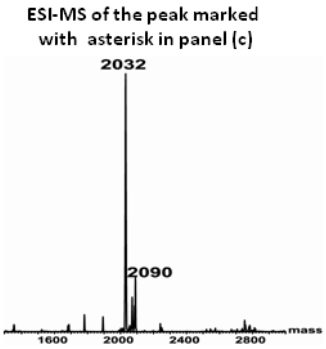


Figure S1. Elution profiles and mass spectra of deprotected, reduced and alkylated dd-KIIIA-1. (a) Crude peptide was obtained by deprotection from resin using enriched reagent K. Mass spectral analysis and corresponding interpretations of the fractions collected from elution profiles are described in the table. (b) Reduced peptide obtained by thiolysis of crude peptide using dithiothretol (DTT). Observed mass of the reduced peptide is 2 Da less than the predicted mass suggesting the presence of preformed diselenide bridge in the reduced peptide. (c) Alkylation of reduced peptide using iodoacetamide (alkylation reaction was carried by incubating 5 nmol of peptide in 100 mM iodoacetamide containing 100 mM Tris.Hcl (pH 7.5) for 45 min in dark). Observed mass shift of linear peptide by alkylation is 114 Da, consistent with presence of two alkylation sites in the reduced peptide further supporting the presence of the diselenide bridge in the linear peptide that was subjected for folding experiment. Molecular masses of the by-product,

minor peaks suggested a presence of a fully reduced form, and another species with two alkylation sites, and also the fully oxidized peptide (please note that peptides containing diselenide and free thiols can undergo a complete oxidation in 10 min at pH 8.0 in the absence of any oxidizing agents[5], thus under our experimental conditions thiols do compete for alkylation and oxidation). An independent alkylation experiment using N-ethylmaleimide yielded a product with a mass of 2167.3 Da, further confirming the presence of two alkylation sites in reduced peptide that was subjected for folding studies.

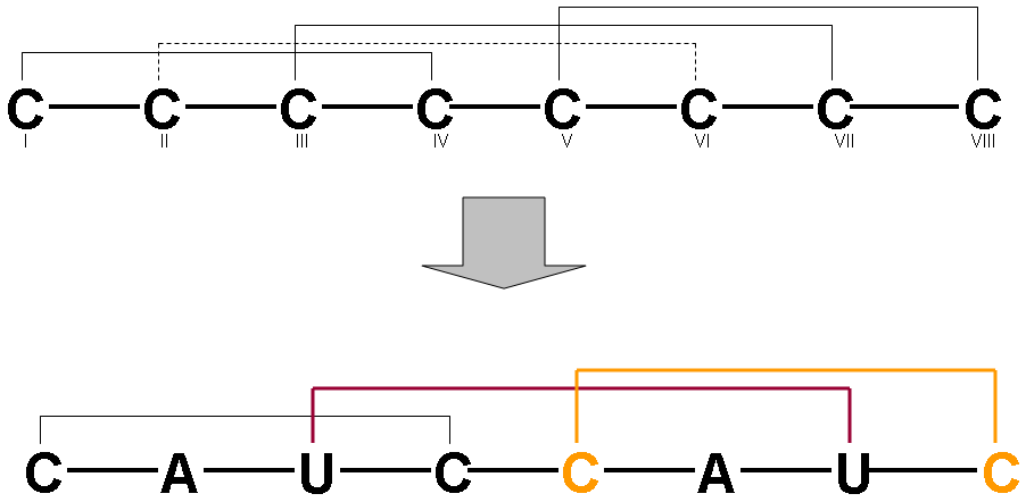


Figure S2. Application of the disulfide-deficient selenopeptide strategy to peptides containing four disulfide bridges. Although such peptides may fold quite efficiently during the direct oxidation method, functional characterization of SAR analogs often requires unambiguous determination of disulfide connectivity in the synthesized peptides. To accomplish disulfide mapping for multiple analogs might be technically challenging and time-consuming task. In order to apply the disulfide-deficient selenopeptide technology, it is prerequisite to identify non-critical disulfide bridges. In a model peptide shown here, a disulfide bridge CysII-CysVI was removed by replacing a pair of Cys residues with Ala. Next, a third disulfide bridge, CysIII-CysVII is replaced by a diselenide bridge (red), whereas CysV and CysVIII are $^{15}N/^{13}C$ labeled (yellow). Such configuration allows to map disulfide crosslinks by NMR, as described previously [3, 6, 7]. Converting of a four disulfide bridge peptide shown here into bioactive disulfide-depleted selenopeptide analogs may accelerate their structure-function studies.

REFERENCES

1. Fuller, E.; Green, B. R.; Catlin, P.; Buczek, O.; Nielsen, J. S.; Olivera, B. M.; Bulaj, G. Oxidative folding of conotoxins sharing an identical disulfide bridging framework. *Febs J* 2005, 272, 1727-38.
2. Bulaj, G.; West, P. J.; Garrett, J. E.; Watkins, M.; Zhang, M. M.; Norton, R. S.; Smith, B. J.; Yoshikami, D.; Olivera, B. M. Novel conotoxins from Conus striatus and Conus kinoshitai selectively block TTX-resistant sodium channels. *Biochemistry* 2005, 44, 7259-65.
3. Walewska, A.; Zhang, M. M.; Skalicky, J. J.; Yoshikami, D.; Olivera, B. M.; Bulaj, G. Integrated oxidative folding of cysteine/selenocysteine containing peptides: improving chemical synthesis of conotoxins. *Angew Chem Int Ed Engl* 2009, 48, 2221-4.
4. Zhang, M. M.; Green, B. R.; Catlin, P.; Fiedler, B.; Azam, L.; Chadwick, A.; Terlau, H.; McArthur, J. R.; French, R. J.; Gulyas, J.; Rivier, J. E.; Smith, B. J.; Norton, R. S.; Olivera, B. M.; Yoshikami, D.; Bulaj, G. Structure/function characterization of micro-conotoxin KIIIA, an analgesic, nearly irreversible blocker of mammalian neuronal sodium channels. *J Biol Chem* 2007, 282, 30699-706.
5. Muttenthaler, M.; Nevin, S. T.; Grishin, A. A.; Ngo, S. T.; Choy, P. T.; Daly, N. L.; Hu, S. H.; Armishaw, C. J.; Wang, C. I.; Lewis, R. J.; Martin, J. L.; Noakes, P. G.; Craik, D. J.; Adams, D. J.; Alewood, P. F. Solving the alpha-conotoxin folding problem: efficient selenium-directed on-resin generation of more potent and stable nicotinic acetylcholine receptor antagonists. *J Am Chem Soc* 132, 3514-22.
6. Walewska, A.; Skalicky, J. J.; Davis, D. R.; Zhang, M. M.; Lopez-Vera, E.; Watkins, M.; Han, T. S.; Yoshikami, D.; Olivera, B. M.; Bulaj, G. NMR-Based Mapping of Disulfide Bridges in Cysteine-Rich Peptides: Application to the μ-Conotoxin SxIIIA. *J Am Chem Soc* 2008, 130, 14280-14286.
7. Gowd, K. H.; Yarotskyy, V.; Elmslie, K. S.; Skalicky, J. J.; Olivera, B. M.; Bulaj, G. Site-Specific Effects of Diselenide Bridges on the Oxidative Folding of a Cystine Knot Peptide, omega-Selenoconotoxin GVIA. *Biochemistry* 49, 2741-52.