

N-Methyl Scanning Mutagenesis Generates Protease-Resistant G Protein Ligands with Improved Affinity and Selectivity

Stephen V. Fiacco and Richard W. Roberts*^[a]

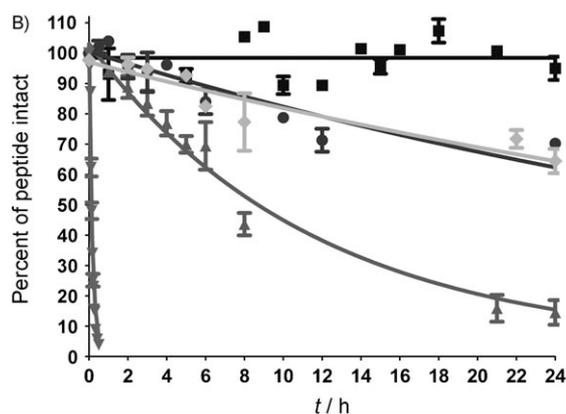
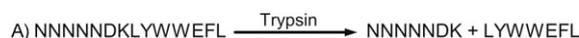
Peptide ligands often suffer from poor bioavailability, in part because of the proteolytic degradation *in vivo*. Previous work has shown that homo-oligomers of *N*-methyl Phenyl alanine are highly resistant to proteolysis from either chymotrypsin or proteinase K.^[1] This work also showed that oligopeptides made with a mixture of Phe and *N*-methyl Phe retain some protease resistance. Similarly, peptoids, polymers of *N*-alkyl glycine, show resistance to proteolysis^[2] and may enhance affinity and specificity when substituted for proline.^[3] Unfortunately, movement of the α -carbon side chain to the amide nitrogen atom often disrupts peptide function. We were interested in strategies that enhance proteolytic stability while preserving the function of the underlying peptide. Toward that end, we have explored the effect of inserting single *N*-methyl amino acids into functional peptides, a method we refer to as *N*-methyl scanning mutagenesis. Our hope was to find one or more substitutions that enhance protease resistance while retaining binding affinity.

We chose the G-protein-binding core-motif peptide DKLYWWEFL, which binds the heterotrimeric inhibitory G protein G α i1*GDP with good affinity ($K_d = 200$ nM), as our model system.^[4] Our strategy was to systematically construct nine variants of this peptide bearing a single *N*-methyl analogue of the natural residue at each position. One challenge we faced was that previous synthetic methods for incorporating *N*-methyl residues involved long multistep reactions per coupling or resulted in low yields.^[5,6] The synthetic difficulty is the result of coupling the secondary amine of an *N*-methyl peptide.^[7] This coupling must go to near completion, in a short period of time to avoid diketopiperazine formation.^[6] To couple *N*-methyl residues we followed a strategy similar to Giralt and co-workers,^[6] but used the additive pair *O*-(7-azabenzotriazol-1-yl)-*N,N,N,N'*-tetramethyluronium hexafluorophosphate (HATU)/1-hydroxy-7-azabenzotriazole (HOAt). Coupling each *N*-methyl amino acid goes to near completion in 20 min in one step with no preactivation for all residues we tested. As a result, we were able to construct the *N*-methyl peptides reliably with a final yield of 20–25% (Table 1).

To investigate the effect of *N*-methylation on protease activity, the half-life was measured for trypsin cleavage for *N*-methyl substitutions were examined at the P2, P1, P1', and P2' positions (*N*-Me-D, *N*-Me-K, *N*-Me-L8, and *N*-Me-Y, respectively; Figure 1). Inserting *N*-methyl residues at these positions results

Table 1. *N*-methyl peptides examined. Peptides were synthesized with and without biotin conjugation at the N terminus. Additionally, five N-terminal Asn residues were added to enhance peptide solubility.

Compound	Sequence
<i>N</i> -Me-D	NNNNN(N-MeD)KLYWWEFL
<i>N</i> -Me-K	NNNNND(N-MeK)LYWWEFL
<i>N</i> -Me-L8	NNNNNDK(N-MeL)YWWEFL
<i>N</i> -Me-Y	NNNNNDKL(N-MeY)WWEFL
<i>N</i> -Me-W10	NNNNNDKLY(N-MeW)EFL
<i>N</i> -Me-W11	NNNNNDKLYW(N-MeW)EFL
<i>N</i> -Me-E	NNNNNDKLYWW(N-MeE)FL
<i>N</i> -Me-F	NNNNNDKLYWWE(N-MeF)L
<i>N</i> -Me-L14	NNNNNDKLYWWEF(N-MeL)



Peptide	Half life	Increase
NNNNNDKLYWWEFL	6.6 min	–
<i>N</i> -Me-L8	>5 days	>1000 x
<i>N</i> -Me-Y	42 h 9 min	383 x
<i>N</i> -Me-K	34 h 36 min	313 x
<i>N</i> -Me-D	7 h 58 min	72 x

Figure 1. Peptide stability to proteolysis. A) Site of trypsin digestion. B) Proteolysis of unmodified peptide (▼), *N*-Me-L8 (■), *N*-Me-K (●), *N*-Me-Y (◆), and *N*-Me-D (▲) by trypsin. C) Experimentally determined half life data.

in a dramatic increase in proteolysis resistance, ranging from 72 to >1000-fold (Figure 1C). To our surprise, the site of *N*-methyl incorporation does not need to be at the scissile bond or at the trypsin recognition site in order to confer this stability. Indeed, our observations indicate that inserting a single *N*-methyl residue substantially reduces proteolysis in a four-residue window.

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Supporting information for this article is available on the WWW under <http://www.chembiochem.org> or from the author.

We next worked to determine if our *N*-methyl insertions altered the peptide binding affinity to $G\alpha 1^*GDP$. While *N*-methyl amino acid incorporation generally reduced binding, we observed increased binding affinity for *N*-Me-K. We quantitated this increase through equilibrium competition with the R6A peptide (MSQTKRLDDQLYWWEYL; $K_d = 60$ nM).^[8] This analysis indicated that *N*-Me-K enhanced binding by 2.5-fold, a $\Delta\Delta G$ of -0.50 kcal mol⁻¹ (Figure 2).

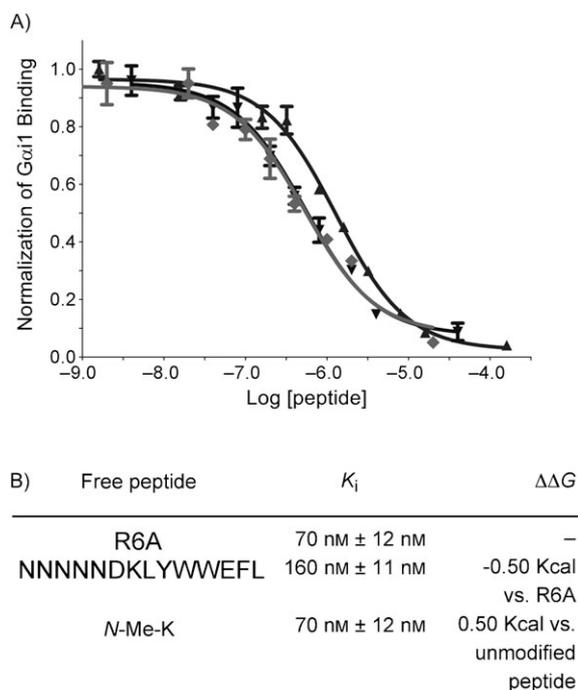


Figure 2. Relative peptide affinities. A) Radiolabeled $G\alpha 1$ was incubated with immobilized, biotinylated R6A and varying concentrations of either R6A (\blacktriangledown), unmodified peptide (\blacktriangle), or *N*-Me-K (\blacklozenge) for 3 h. at 4 °C. The radiolabel bound was normalized to that which bound in the absence of competitor. The plotted values represent the mean of three experimental values and the error bars correspond to the standard deviation. B) Experimentally determined binding data is shown above. The error is calculated based on the standard error of the mean (SEM) for the log IC50 using GraphPad 5.0.

The crystal structure of R6A in complex with $G\alpha 1^*GDP$ (S. Sprang, A. Adhikari, personal communication) provides insight into our binding data. There, *N*-methyl substitutions would disrupt many of the peptide–protein interactions or the fold of the bound peptide. From the crystal structure of a similar sequence, we observe that a hydrogen bond is present from the backbone of V (equivalent to L in our peptide) to $G\alpha 1^*GDP$.^[9] This would indicate that *N*-methyl incorporation at this position would disrupt binding to $G\alpha 1^*GDP$, which we observed. Additional insight may be inferred by the results of other peptides selected by mRNA display to bind to $G\alpha 1^*GDP$, for which we have observed a significant conservation of the EFL residues.^[10] Any mutations are subtle, E to D or F to Y, for example. Therefore, it would be suspected that *N*-methyl incorporation at any of these positions would be deleterious to peptide binding. This was experimentally confirmed here for our peptide binding to $G\alpha 1^*GDP$.

The Q residue in R6A (equivalent to K in our peptide) adopts phi and psi angles of -60 and 128 , respectively. Previous work indicated that peptoids have a limited number of energetically favorable conformations.^[11] It is likely that the incorporation of an *N*-methyl amino acids results in a conformation similar to an optimal orientation for $G\alpha 1$ binding. This leads us to a conformational hypothesis for *N*-methylation. Assuming no unfavorable steric interactions, we would predict that *N*-methyl amino acid incorporation will be favorable or neutral when Phi, Psi angles are similar to those found in peptoids.^[11] This heuristic should provide a good starting point for *N*-methyl amino acid modifications that improve peptide stability without impairing function. Further structural analysis could be beneficial and will be carried out in future work.

Finally, we wanted to see if *N*-methyl incorporation had altered the specificity of our peptides. Members of the peptide library were tested for binding against nine members of the G-protein family known to have various affinities to R6A. Five of the nine *N*-methyl peptide variants showed a dramatic increase in specificity (see the Supporting Information). One variant showed specific affinity for $G\alpha 12$ over $G\alpha 1$ which the original selection targeted (Figure 3). This results in the first peptide

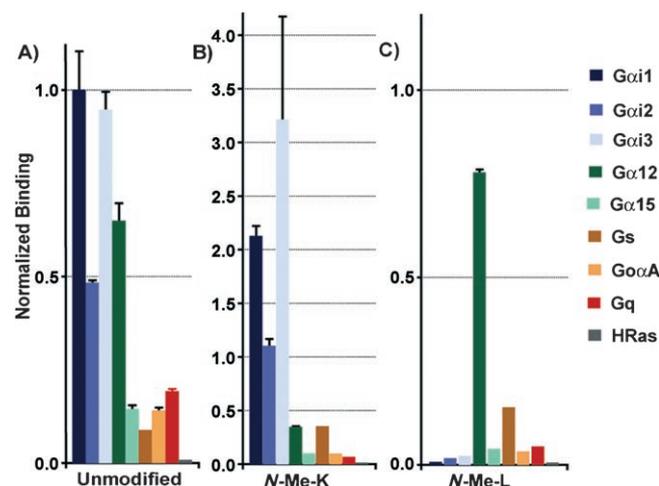


Figure 3. Binding of radiolabeled $G\alpha$ subunits to immobilized peptides. $G\alpha 1$, $G\alpha 2$, $G\alpha 3$, Gq , Gs , $G\alpha\alpha A$, $G\alpha 12$, and $G\alpha 15$ pcDNA were translated into ³⁵S-Met-labeled proteins using TnT-coupled transcription/translation system (Promega). Translation reactions were desalted and applied to A) Unmodified peptide, B) *N*-Me-K, and C) *N*-Me-L immobilized to neutravidin-agarose. Values represent the mean value from three experiments, and the error bars represent the standard deviation.

ligand able to specifically bind to $G\alpha 12$. We have shown that the incorporation of *N*-methyl amino acids can dramatically increase the function and stability of a peptide selected by mRNA display. Selective incorporation of *N*-methyl amino acids into peptides with a Phi, Psi angle similar to those stated for *N*-Me-K would likely have the same benefits from their incorporation. This relative synthetic ease and predictability makes incorporating of *N*-methyl amino acids into peptides derived from selections a practical first step in increasing bioavailability.

Experimental Section

Peptide synthesis: All solvents were purchased from Sigma. All peptides except *N*-Me-L14 were synthesized by manual solid-phase peptide synthesis with preloaded Leu-2-chlorotrityl resin (250 mg, 0.15 mmol; Anaspec). *N*-Me-L14 was synthesized after loading 2-chlorotrityl resin (Anaspec) with Fmoc-*N*-methyl leucine (Fmoc=9-fluorenylmethoxycarbonyl; Anaspec). Five *N*-terminal Asn residues were added to enhance peptide solubility. Loading was accomplished by adding Fmoc-*N*-Me-L14 to 2-chlorotrityl resin (5 equiv; 250 mg, 0.35 mmol) in DMF with *N,N*-diisopropylethylamine (DIEA; 5 equiv) for 2 h. Standard couplings were carried out with monomer (5 equiv; Novabiochem) in HATU (2 mL, 0.6 mmol; Novabiochem), HOAt (1.2 mmol; Genescript) in DMF with DIEA (1.8 mmol) at room temperature for 15 min. Coupling to an *N*-methyl amino acid followed the same procedure with a 30 min coupling time. Fmoc deprotection was carried out with 20% piperidine (Anaspec) at room temperature for 15 min. Following, deprotection, cleavage with 95% TFA, filtration and ether extraction, the crude product was purified on a Vydac C-18 reversed-phase column using gradient elution (0% B for 5 min, 10–50% B in 40 min. Solvent A: H₂O with 0.1% TFA, Solvent B: CH₃CN with 0.035% TFA). Lyophilized solid was reconstituted in DMSO and quantitated by absorbance at 280 nm ($\epsilon_{280} = 12490 \text{ L mol}^{-1} \text{ cm}^{-1}$). Yield = 20–25%.

Peptides were characterized by HPLC and MALDI-TOF. Peptides were run on a Vydac C-18 column using gradient elution (5–95% B). Buffers were the same as described above. Absorbance at 280 nm was recorded for biotin-labeled peptides excluding Bio-*N*-Me-D, Bio-R6A and Bio-unmodified peptide. Absorbance at 215 nm was recorded for the remaining peptides. The peak around 4 min for peptides at 215 nm was the solvent peak. The next major peak (or first major peak in the case of 280 nm recording) was collected and analyzed by MALDI-TOF.

Binding affinity of the *N*-methyl peptide library: TnT pulldown experiments (Promega) were carried out as described in ref. [4] with some modifications. Human cDNA clones encoding G proteins with the pcDNA3.1+ vector (Invitrogen) were obtained from the UMR cDNA Resource Center (<http://www.cdna.org>). The DNA clones for $G\alpha 1$, and HRas were translated into ³⁵S-Met-labeled proteins using a TnT-coupled transcription/translation system (Promega). Translation reactions were desalted with G-25 spin columns (GE Healthcare), and the translation yield was calculated by trichloroacetic acid (TCA) precipitation. Equal amounts of each labeled subunit were added to neutravidin-agarose (5 μL) containing prebound biotin-labeled peptide (2 nmol) in HBS-EP buffer (1 mL). Control neutravidin-agarose beads were loaded with (d)-biotin (Anaspec). Binding reactions proceeded for 2 h at 4 °C followed by filtration and washing with HBS-EP. The matrix was analyzed by scintillation counting and the percent bound was determined by the matrix counts divided by the total counts as determined by TCA precipitation. Values were normalized to the percent bound of unmodified peptide.

R6A-1 competition/equilibrium binding: R6A (MSQTKRLDDQLYWWEYL) and Bio-R6A (Bio-MSQTKRLDDQLYWWEYL) were synthesized as described above. This peptide was immobilized to NeutrAvidin beads (Promega) using the manufacturer's instructions. $G\alpha 1$ was expressed using the TnT reticulocyte lysate system (T7 promoter, Promega). (250 ng) plasmid DNA and (25 μCi) L-³⁵S-Met (MP Bio-medicals) were used in an (25 μL) expression. $G\alpha 1$ translations were desalted using MicroSpin G-25 columns (GE healthcare) into modified HBS-EP buffer: HEPES (10 mM, pH 7.4), NaCl (150 mM), EDTA (3 mM), polysorbate 20 (0.005%; Tween 20), MgCl₂ (8 mM),

GDP (30 μM), and BSA (0.05% (w/v)). Specific activity was measured by TCA precipitation.

Immobilized R6A (400 pmol) and $G\alpha 1$ (100 000 cpm) were suspended in HBS-EP buffer (1 mL). Varying quantities of free peptide (R6A, unmodified peptide, or *N*-Me-K) were added. The final DMSO concentration was adjusted to 0.5%. Samples were rotated for 3 h at 4 °C followed by brief centrifugation and supernatant removal. Samples were washed four times by resuspension in modified HBS-EP buffer at 4 °C, centrifugation, and supernatant removal. After the final wash, the immobilized sample was transferred to scintillation vials and analyzed by scintillation counting. CPM values were normalized by dividing by the average value obtained in the absence of added peptide. Binding analysis was performed by GraphPad Prism 4.0.

***N*-methyl selectivity:** $G\alpha 1$, $G\alpha 2$, $G\alpha 3$, Gq, Gs, $G\alpha A$, $G\alpha 12$, and $G\alpha 15$ pcDNA were translated into ³⁵S-Met-labeled proteins using the TnT-coupled transcription/translation system (Promega). Translation reactions were desalted using G-25 spin columns (GE Healthcare) and applied to peptide immobilized to neutravidin-agarose as previously described. The immobilized peptides were *N*-Me-D, *N*-Me-K, *N*-Me-L8, *N*-Me-Y, *N*-Me-W10, *N*-Me-W11, *N*-Me-E, *N*-Me-F, or *N*-Me-L14 as illustrated. Binding was allowed to proceed for 3 h at 4 °C after which the resin was filtered and washed. Scintillation counting of the resin yielded the cpm bound, which was normalized as detailed in the Supporting Information. Values represent the mean value from three experiments and the error bars represent the standard deviation. Binding analysis and normalization performed as outlined in R6A-1 competition/equilibrium binding.

Protease resistance experiment: Unmodified peptide, *N*-Me-D, *N*-Me-K, and *N*-Me-L8 (7.5 mM) in DMSO (2 μL) were added to buffer (98 μL) containing Tris (50 mM), CaCl₂ (20 mM), pH 7.75. This was used to reconstitute modified trypsin (20 μg ; New England Biolabs). The reaction proceeded at 37 °C for either 30 min (unmodified peptide) or 24 h (*N*-Me-D, *N*-Me-K, *N*-Me-L8, *N*-Me-Y). An aliquot (8 μL) was removed for each time point and diluted in ACN (25 μL). Samples were flash frozen until analysis was to be performed. At this time 0.1% TFA in H₂O (67 μL) was used to dilute the samples to 100 μL . The sample was injected onto a C-18 reversed-phase column and separated by gradient elution (5–90% B in 10 min. Solvent A: H₂O with 0.1% TFA, Solvent B: CH₃CN with 0.035% TFA). The area under the starting material peak was quantitated using the 32 KaratGold Software package (Beckman). The plotted values represent the mean of three experimental value and the error bars represent the standard error of the mean. The graph was generated by fitting the data to a one-phase exponential decay equation (GraphPad Prism).

Acknowledgements

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Keywords: medicinal chemistry · mRNA display · mutagenesis · peptidomimetics · proteolysis

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Supporting Information

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Supporting Information

for

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Peptide Synthesis

Analysis by MALDI-TOF MS:

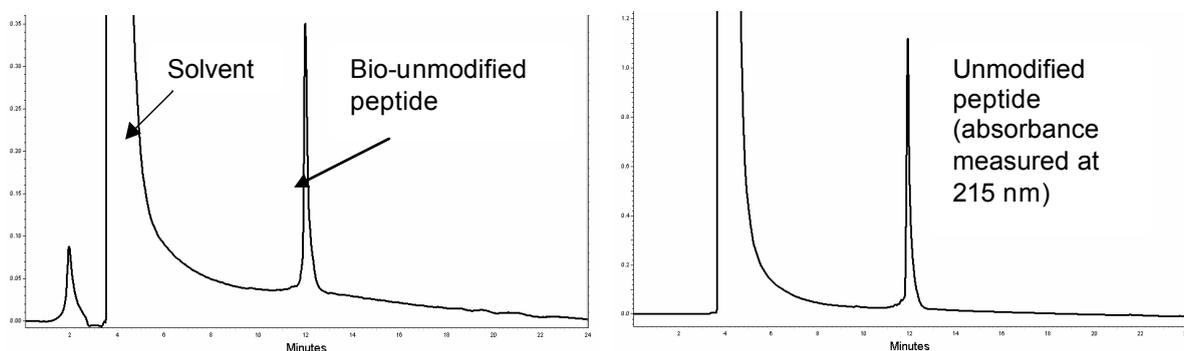
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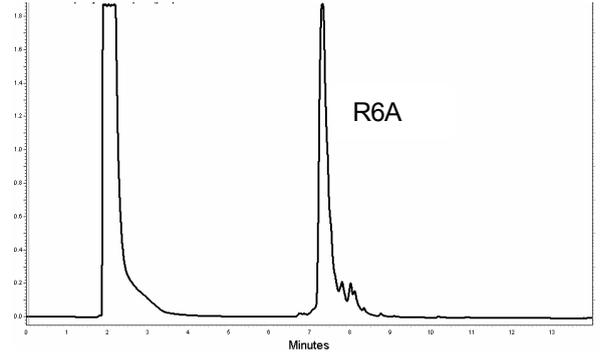
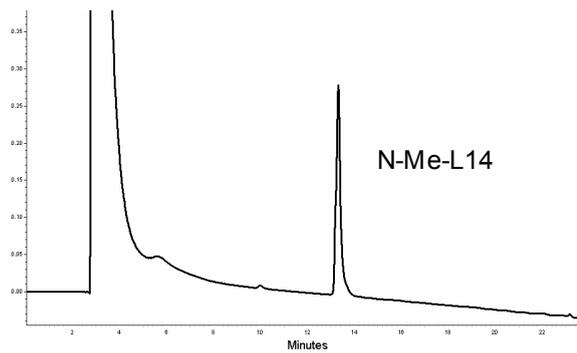
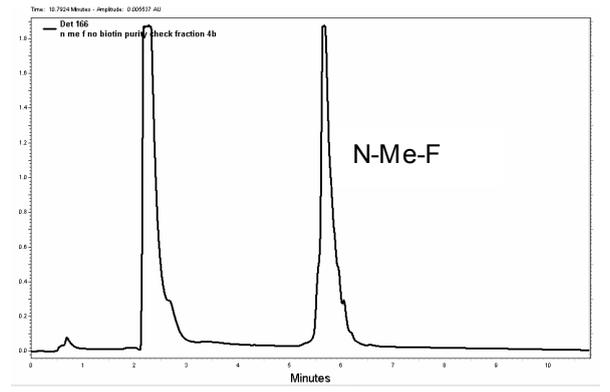
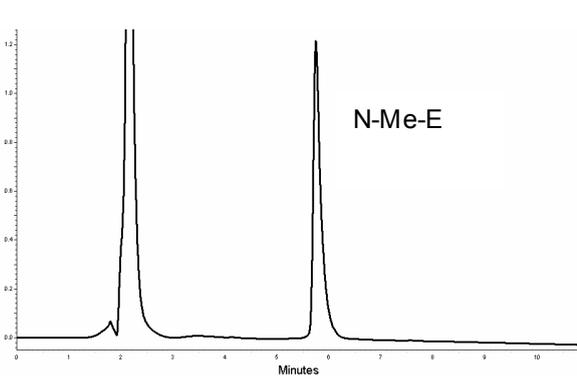
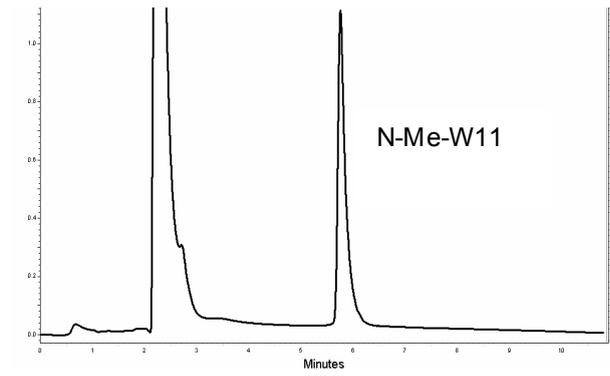
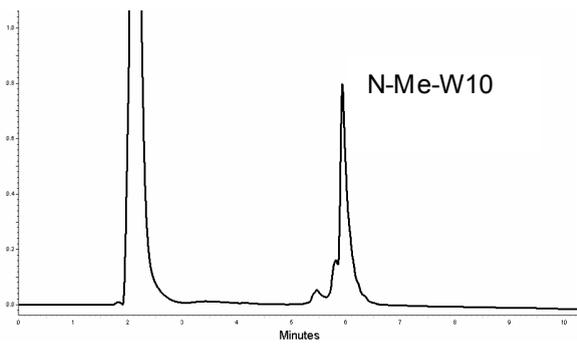
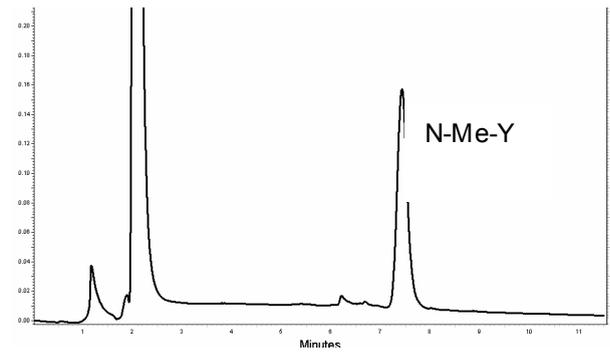
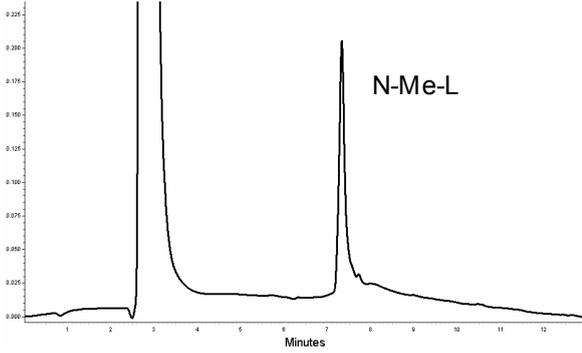
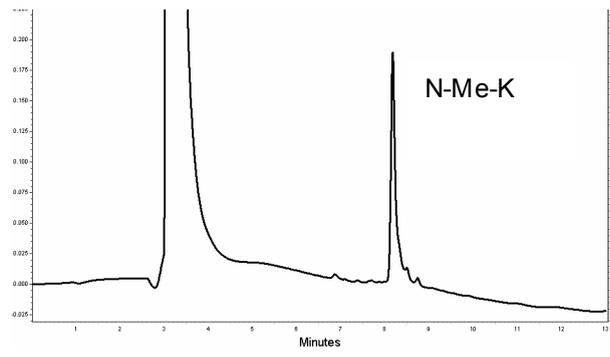
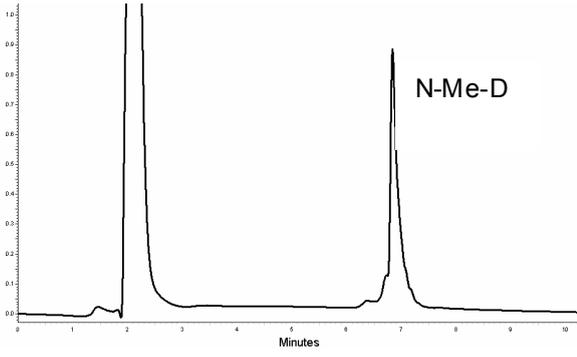
Bio-Unmodified peptide Expected $[M+H]^+ = 2095.2$, obs. $[M+H]^+ = 2096.0$ The expected mass of all N-methyl peptides $[M+H]^+ = 2110.1$, N-Me-D obs. $[M+H]^+ = 2110.9$ N-Me-K obs. $[M+H]^+ = 2110.6$ N-Me-L obs. $[M+H]^+ = 2110.2$ N-Me-Y obs. $[M+H]^+ = 2110.6$ N-Me-W10 obs. $[M+H]^+ = 2109.5$ N-Me-W11 obs. $[M+H]^+ = 2110.6$ N-Me-E obs. $[M+H]^+ = 2110.0$ N-Me-F obs. $[M+H]^+ = 2110.0$ N-Me-L obs. $[M+H]^+ = 2109.8$

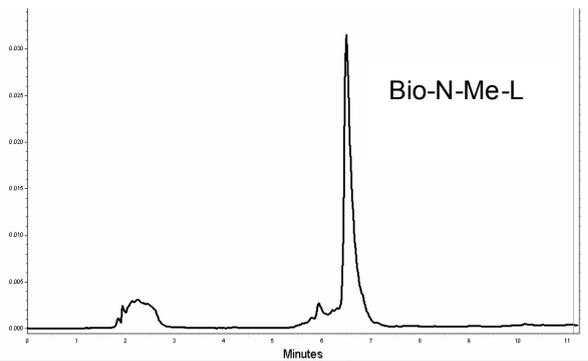
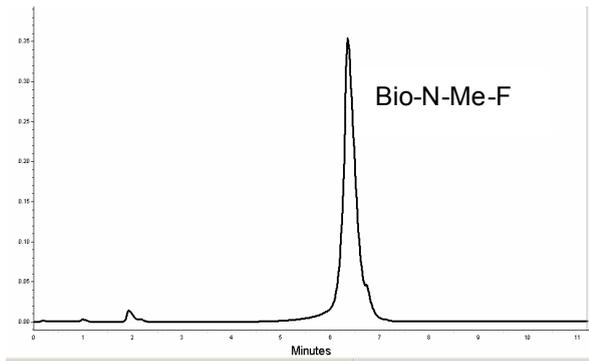
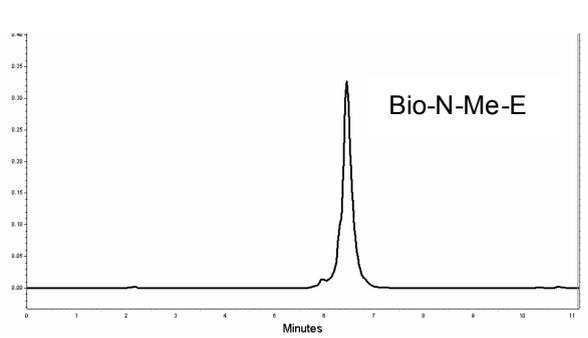
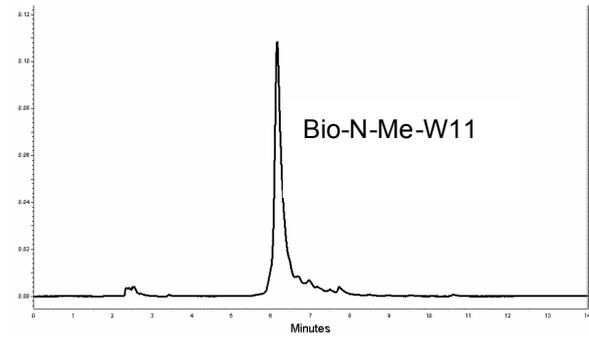
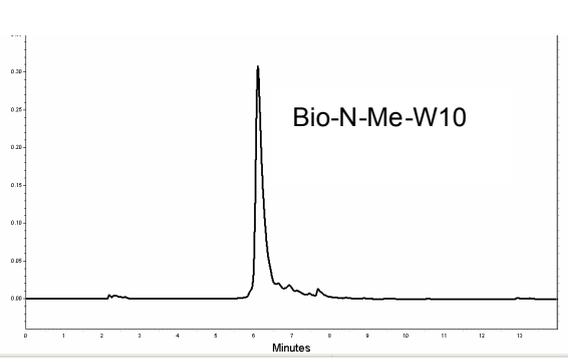
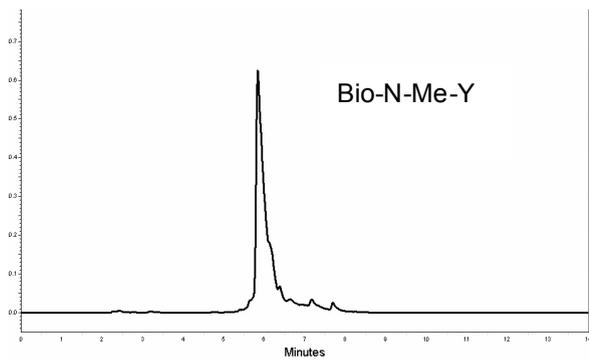
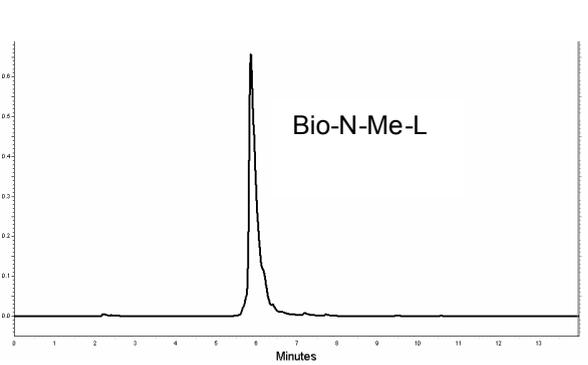
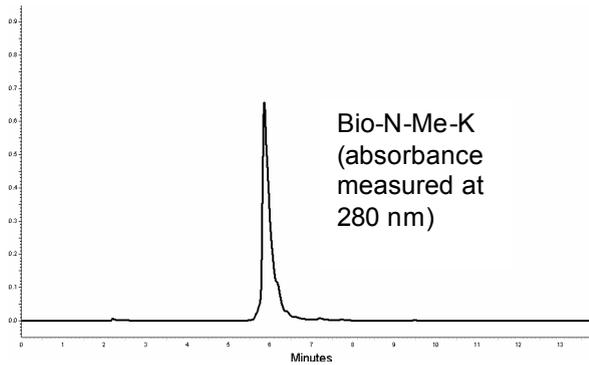
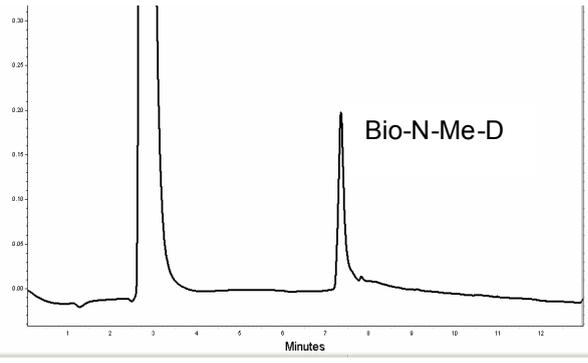
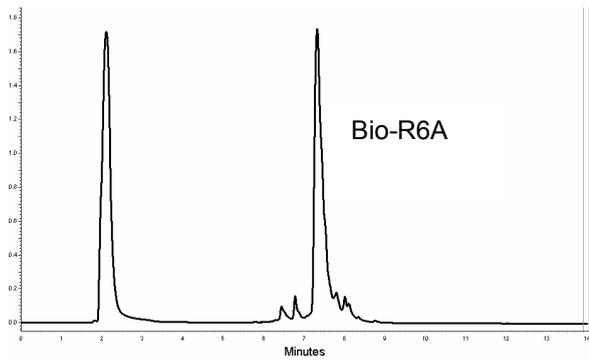
Digest product LYWWEFL Expected $[M+H] = 1056.2$, obs. $[M+H] = 1056.6$

R6A Expected $[M+H] = 2276.6$, obs. $[M+H] = 2276.9$ Bio-R6A Expected $[M+H] = 2501.7$, obs. $[M+H] = 2501.2$

HPLC traces.







N-Methyl Selectivity

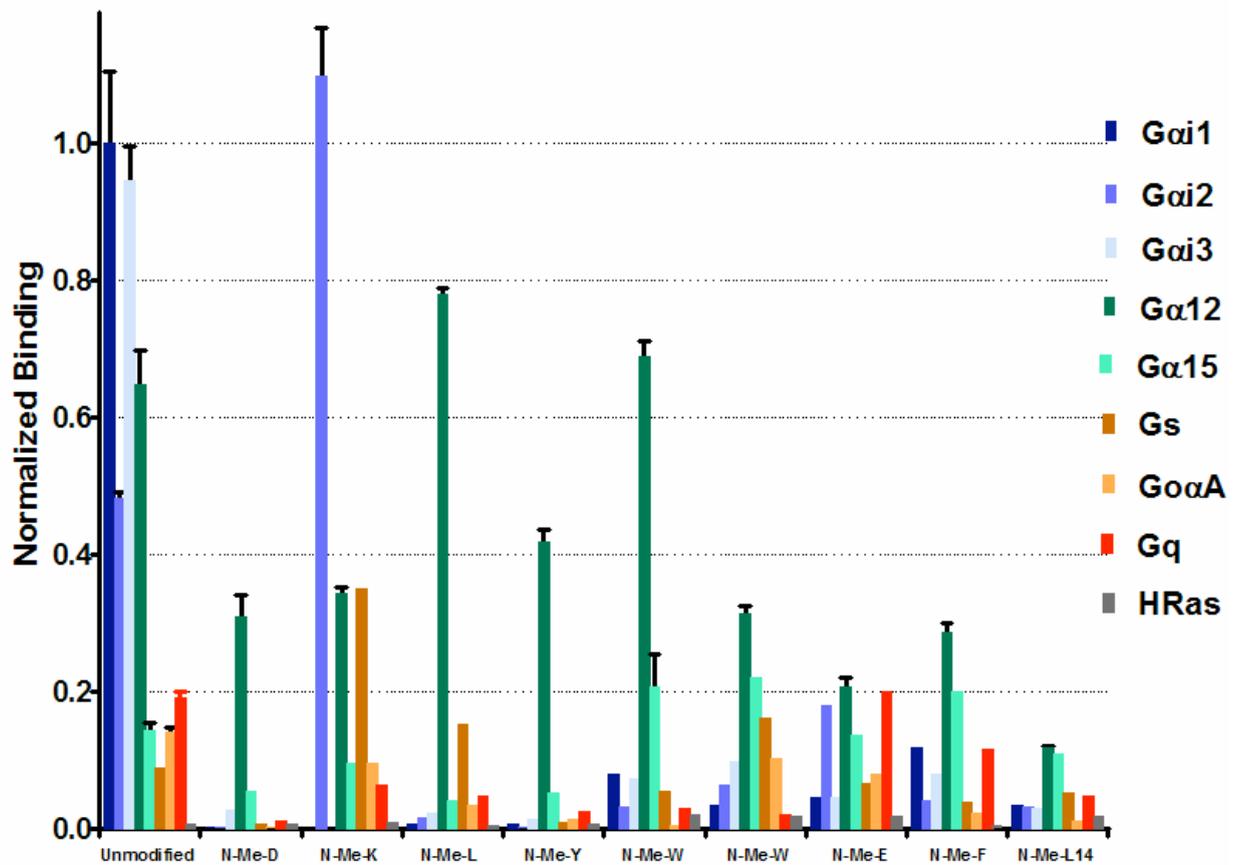


Figure S1: Selectivity for the N-methyl peptide library. G protein subunits are color coded. Binding is normalized by dividing the samples cpm by the average cpm obtained for the unmodified peptide binding to its target Gαi1. Two N-Me-K data points have been removed in order to scale the graph in a way to resolve weaker binding interactions.