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Redox-Responsive Protein Design: Design of a Small Protein Motif Dependent on Glutathionylation

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Abstract

Cysteine S-glutathionylation is a protein post-translational modification that promotes cellular responses to changes in oxidative conditions. The design of protein motifs that directly depend on defined changes to protein side chains provides new methods to probe diverse protein post-translational modifications. A canonical, 12-residue EF Hand motif was redesigned to be responsive to cysteine glutathionylation. The key design principle was the replacement of the metal-binding Glu12 carboxylate of an EF Hand with a motif capable of metal binding via a free carboxylate in the glutathione-conjugated peptide. In the optimized peptide (DKDADGWCG), metal binding and terbium luminescence were dependent on glutathionylation, with weaker metal binding in the presence of reduced cysteine, but increased metal affinity and a 3.5-fold increase in terbium luminescence at 544 nm when cysteine was glutathionylated. NMR spectroscopy indicated that the structure at all residues of the glutathionylated peptide changed in the presence of metal, with chemical shift changes consistent with the adoption of an EF-Hand-like structure in the metal-bound glutathionylated peptide. This small protein motif consists of canonical amino acids, and is thus genetically encodable, for its potential use as a localized tag to probe protein glutathionylation.

Graphical Abstract

Keywords

redox chemistry; oxidative stress; post-translational modifications; protein design; metalloproteins; lanthanides; fluorescence spectroscopy

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Supporting Information

Experimental procedures; characterization data; terbium fluorescence spectra and terbium binding isotherms of RP2-RP5 peptides, as thiols and as glutathionylated disulfides; 1-D, TOCSY, and NOESY NMR spectra of RP1-SH; 1-D, TOCSY, NOESY, ^1H - ^{13}C HSQC, and ^1H - ^{13}C HMBC NMR spectra of RP1-SSG in the absence and presence of La^{3+} ; and tabulation of NMR data. The Supporting Information is available free of charge on the ACS Publications website.

Introduction

Protein structure and function are responsive to diverse protein post-translational modifications, including phosphorylation, glycosylation, lipidation, acylation, alkylation, and oxidation. Protein post-translational modifications may be effected enzymatically (e.g. phosphorylation, O-GlcNAcylation, lysine acetylation/methylation, ubiquitination, AMPylation, sulfation) or non-enzymatically, such as in response to oxidative or nitrosative stress.^{1–3} Non-enzymatic post-translational modifications may allow for protein responsiveness to changes in redox state (e.g. cysteine disulfide, sulfenic acid formation, S-nitrosylation, or glutathionylation) or may be pathological markers of protein damage that may be associated with protein misfolding or loss of protein function (e.g. tyrosine nitration, methionine sulfoxide and sulfone formation, cysteine sulfonic acid oxidation).

Cysteine oxidation has recently emerged to be of significant importance in intracellular signal transduction, with diverse protein functions that are responsive to specific cysteine modifications, including disulfide, sulfenic acid, S-nitrosyl, and/or S-glutathionylated oxidized forms of cysteine.^{4–16} These post-translational modifications provide the capability of specific cellular responses to defined redox stresses.¹⁷ Proteomics approaches have applied a variety of techniques to identify specific oxidative modifications of cysteine in proteins, including broad observation of S-glutathionylation of proteins.^{13, 18–25}

Alternatively, a variety of small molecule and GFP-based approaches have been applied to identify general oxidative conditions and oxidants.^{17, 26–32} However, despite these significant advances that have generated new insights into the importance and dynamics of intracellular redox signaling, there is a need for additional specific, direct approaches to detect defined oxidative post-translational modifications.

Changes in glutathione oxidation state are associated with different cell types, subcellular compartments (increased oxidized glutathione in mitochondria and the ER), and oxidative stress response, with resultant S-glutathionylation at a subset of cysteine residues in proteins (Figure 1).^{11, 12, 14, 33} For example, increased protein glutathionylation is observed in ischemia, with depletion of reduced glutathione associated with oxidative damage on cardiac reperfusion.^{5, 9} Increased glutathione oxidation and protein glutathionylation are also observed in some neurodegenerative diseases, which is associated with the increased susceptibility of neuronal proteins to oxidative damage.^{34, 35} Notably, glutathionylation can result from cysteine functioning in a nucleophilic (Cys-SH or Cys-S[−] reaction with GSSG) or electrophilic (Cys sulfenic acid Cys-SOH reaction with GSH) manner, as well as via free radicals, pointing to the complexity of mechanisms possible in redox control of protein function.³⁶ Glutathionylation also may be mediated enzymatically, and appears to be of particular importance in proteins that function as sensors of cellular redox state, including the protein kinase AMPK and the E3 ubiquitin ligase adapter protein Keap1.^{37–39} In view of the importance of glutathione (GSH) as the major intracellular reductant ([GSH] = 1–10 mM) and of the ability of reduced (GSH) and oxidized (GSSG) glutathione to modulate protein oxidation state,⁴⁰ and the complex mechanisms of regulation of protein function through glutathionylation, we sought to develop a protein motif that is specifically responsive to protein glutathionylation.^{26, 41}

Experimental

Peptide synthesis.

Peptides were synthesized via standard solid-phase peptide synthesis and purified to homogeneity. Peptide synthesis, glutathionylation, and characterization details are in the Supporting Information.

Fluorescence spectroscopy.

Fluorescence experiments were conducted on a Photon Technology International fluorescence spectrometer model QM-3/2003. Samples contained 10 mM HEPES (pH 7.4), 100 mM NaCl, and 10 μ M peptide. 500 μ M DTT was present for peptides containing reduced cysteine. Tb³⁺ binding isotherms were conducted via addition of 2-fold serial dilutions of Tb³⁺ into the peptide solution. The terbium emission band at 544 nm was quantified to evaluate metal binding. Details are in the Supporting Information.

NMR spectroscopy.

1-D ¹H NMR, TOCSY, and NOESY NMR experiments were conducted at 23 °C in 90% H₂O/10% D₂O containing 10 mM NaOAc-d₃ buffer (pH 6.2 or as indicated) and 100 mM NaCl. These experiments were conducted at pH 6.2 in order to minimize broadening or exchange of amide hydrogens. ¹H-¹³C HSQC and ¹H-¹³C HMBC NMR experiments were conducted at pH 6.2 or pH 7.4, as indicated, in 100% D₂O containing 10 mM NaOAc-d₃ and 100 mM NaCl. NMR experiments were conducted using Watergate water suppression. Full experimental details and full NMR spectra are in the Supporting Information.

Terbium is a paramagnetic metal with 6 unpaired f electrons, precluding structural analysis of the terbium complex. Notably, glutathionylation-dependent binding was optimized for the lanthanide terbium, with the largest changes in terbium binding and luminescence the optimized parameters. Lanthanide-binding motifs, including both peptides and organic ligands, typically exhibit differential affinities for lanthanides as a function of ionic radius, with weaker binding affinities for lanthanides that are either smaller (to the right in the periodic table) or larger (to the left in the periodic table) than the optimized lanthanide, exhibiting a chevron-like plot of lanthanide affinity as a function of ionic radius.^{42–44} The use of La³⁺ in NMR spectroscopy experiments represents a choice necessary for any NMR characterization. The paramagnetic metal Tb³⁺ leads to severe broadening and shifting of the signals, whereas the diamagnetic La³⁺, with no unpaired electrons (0 f electrons), represents an inherent compromise compared to the optimized metal Tb³⁺ both in terms of the metal affinity and of the dynamics of the metal-peptide complex.⁴⁵ Consistent with structure being dependent on the size of the lanthanide, no evidence of metal binding was observed using the smaller diamagnetic lanthanide Lu³⁺ (14 f electrons). These compromises, combined with the minimal long-range NOEs that are inherent in the structure of a single EF Hand, preclude full NMR-based structure determination. The NMR data herein are employed to identify whether the chemical shift changes that are observed are consistent with the designed binding motif exhibiting similar metal binding to that observed in a canonical EF Hand.

Results and Discussion

We have previously described the design of protein kinase-inducible domains (Figure 2), small (12–18 amino acids) protein motifs whose structure and terbium luminescence were dependent on their phosphorylation state and which were responsive to specific protein kinase activity, both in solution and in cell extracts.^{45–55} These designs are based on a canonical EF Hand calcium-binding motif, which binds the luminescent lanthanide terbium as well as other lanthanides with greater affinity than Ca^{2+} .^{56–62} In our designs, the EF Hand was modified to replace the critical Glu12 residue, which is evolutionarily conserved and which binds the metal in a bidentate manner, with a serine, threonine, or tyrosine. When the side chain hydroxyl is non-phosphorylated, the remaining protein motif is not sufficient for metal binding. However, upon phosphorylation, a complete metal-binding motif is constituted, resulting in a large increase in terbium binding and terbium luminescence. We envisioned that a similar approach using a redesigned EF Hand could be applied to generate a motif dependent on protein glutathionylation, based on the presence of two carboxylates in glutathione. Cysteine is a poor ligand for lanthanides, which prefer hard (oxygen or nitrogen) ligands.^{44, 63–65} However, cysteine glutathionylation would introduce two new carboxylates into the peptide, either of which could potentially replace Glu12 and allow reconstitution of a complete terbium-binding motif (Figure 2b).

To test this hypothesis, a series of peptides (RP1-RP5) was synthesized, comprising the N-terminal 7 amino acids of an optimized EF Hand motif (a proto-terbium binding motif, termed the N-terminal cassette), plus a cysteine residue as a site for conjugation to glutathione (Figure 2c). The N-terminal cassette includes the following structural elements: alternating liganding residues 1, 3, 5, and 7, which bind metal via the side chains at residues 1, 3, and 5 (Asp) and via the main chain carbonyl at residue 7 (Trp); Lys at residue 2 to provide electrostatic balance to the anionic residues; and Ala and Gly at residues 4 and 6 to promote the conformational preferences of an EF Hand.^{66–70} Glutathionylation introduces additional length and flexibility for positioning of the glutathione carboxylates to the peptide backbone, compared to Glu or to a phosphorylated amino acid. In addition, metal binding may occur via either or both glutathione carboxylates. Therefore, peptides were synthesized with cysteine at each of the residues 7–11 of the EF Hand motif, in order to identify an optimal glutathione-dependent metal-binding protein motif.

An important component of this protein design is responsiveness: in contrast to the design of metal-binding motifs where the primary goal is maximum metal affinity, here maximum binding affinity (smallest K_d) is *not* the primary design goal. Optimizing the EF Hand motif for high metal affinity would likely increase terbium binding of *both* the unmodified *and* the post-translationally modified peptides under conditions of measurement, resulting in minimal or no difference in terbium luminescence between the unmodified and modified peptides. Thus, a critical aspect of the design is that there should be substantially weaker metal affinity for the unmodified peptides, such that a significant *change* in terbium luminescence is observed upon peptide modification. Indeed, this concept is central to the employment of the proto-terbium-binding N-terminal cassette, which is necessary, but not sufficient, for robust terbium binding and terbium luminescence.⁶⁹ Thus, in these designs, both the metal-binding amino acid at residue 9 (water-mediated binding, typically Asp, Ser,

or Glu) and the conserved Glu at residue 12 of an EF Hand were removed, in order to reduce the overall metal affinity in the absence of glutathionylation.

In addition to changes in binding affinity, the post-translational modification might also introduce an inherent increase in the terbium luminescence of the complex. Terbium luminescence is quenched by metal interactions with water.⁶³ These metal-water interactions are expected to be reduced in the modified peptides due to the additional ligand that is introduced by the post-translational modification. Thus, in the analysis of various designs, we considered both changes in metal affinity and changes in maximum terbium luminescence between the unmodified and modified peptides as criteria for design success.

Peptides were synthesized with free cysteines (RPx-SH) and with glutathionylated cysteines (RPx-SSG). The terbium affinities and maximum terbium luminescence of all peptides were quantified via terbium binding isotherms and quantification of terbium luminescence, using the terbium emission band at 544 nm resulting from the excitation of tryptophan at 280 nm.⁷¹ In the absence of metal binding, excitation of Trp would not lead to energy transfer and sensitized terbium emission. Moreover, terbium itself is poorly directly excited by light.⁶³ In contrast, tryptophan at residue 7 in an EF Hand is directly bound to metal via its main chain carbonyl oxygen, and effectively sensitizes terbium luminescence.^{56, 68} Thus, the terbium luminescence is significantly dependent on metal binding by the EF-Hand motif.

The peptide with Cys at residue 8 of the EF Hand (RP1) exhibited excellent glutathionylation-dependent terbium binding and terbium luminescence (Figure 3), with a 3.5-fold increase in terbium luminescence at 544 nm for the glutathionylated over the non-glutathionylated peptide at 125 μM Tb^{3+} . In contrast, all other peptides exhibited smaller increases in fluorescence and terbium binding on glutathionylation (based on maximum change in terbium luminescence between the unmodified and glutathionylated peptides, $\text{RP1} > \text{RP2} \sim \text{RP3} > \text{RP4} > \text{RP5}$; see the Supporting Information for details). These data suggest a geometric dependence for glutathione-dependent terbium-binding, as was observed for phosphorylation-dependent protein design,^{45, 46} rather than a simple electrostatic complementation, and thus are suggestive of structure being adopted in the $\text{RP1-SSG}\cdot\text{Tb}^{3+}$ complex.

In this design, a glutathionylated cysteine at residue 8, a non-metal-binding position in an EF Hand, replaced the metal-binding Glu at position 9 or 12 of the EF Hand. RP1-SSG exhibited terbium binding ($\text{RP1-SSG}\cdot\text{Tb}^{3+}$ $K_d = 182 \pm 21 \mu\text{M}$) similar to or somewhat weaker than optimized protein kinase-inducible domains or related peptides with Glu, as well as less differentiation between modified and unmodified peptides compared to the kinase-inducible domain peptides. These observations are consistent with the greater flexibility of the glutathione conjugate, with an anionic oxygen 6 or 8 atoms further from the backbone than in a phosphate or a Glu. Glutathionylation modestly increased the terbium affinity compared to the parent peptide ($\text{RP1-SH}\cdot\text{Tb}^{3+}$ $K_d = 336 \pm 62 \mu\text{M}$). Notably, glutathionylation also increased the terbium luminescence of the complex by 1.6 fold (ratio of the fluorescence of the complexes at saturation), with the overall 3.5-fold increased luminescence observed at 125 μM Tb^{3+} a factor of both the increased affinity and the

increased inherent fluorescence of the complex with the glutathionylated peptide compared to the peptide with cysteine.

To characterize the basis for this redox-responsive protein design, RP1-SSG was analyzed by NMR spectroscopy, in the absence and presence of the diamagnetic lanthanide lanthanum (La^{3+}). ^1H and ^{13}C NMR data indicated large chemical shift changes throughout the peptide upon addition of metal, consistent with the formation of an EF Hand-type structure in the RP1-SSG•metal complex (Figure 4, Table 1, and Supporting Information). Most notably, the ^1H - ^{13}C HSQC spectrum, in combination with chemical shift index (CSI) analysis (downfield $\text{H}\alpha$ and upfield $\text{C}\alpha$ chemical shifts indicate more extended structure, while upfield $\text{H}\alpha$ and downfield $\text{C}\alpha$ chemical shifts indicate more compact (α -helical) structure),^{72, 73} indicates changes in structure that are consistent with the adoption of a structure similar to that of an EF Hand. Large downfield changes in chemical shift were observed for $\text{C}\alpha$ for the metal-binding residues Asp1, Asp3, and Asp5, which adopt $\phi, \psi \sim (-90, 0)$ in an EF Hand. Substantial chemical shift changes were also observed for all Asp $\text{C}\beta$ resonances (Figure 4c), consistent with the central role of these residues in metal binding. Changes in the $\text{H}\alpha$ and $\text{C}\alpha$ chemical shifts of Lys2, which adopts an α_R conformation in EF Hand proteins, are consistent with a more α -helical conformation at this residue upon metal binding. In addition, changes in conformation were also observed for the residues Ala4 and Gly6, which adopt an α_L conformation in a canonical EF Hand. Notably, resolution of the Gly6 diastereotopic $\text{H}\alpha$ protons was observed in the metal-bound complex, suggesting a highly ordered structure in the terbium complex of the glutathionylated peptide. All of these chemical shift changes are consistent with those expected in metal binding in an EF Hand peptide. Collectively, these data indicate ordering of the structure of the entire peptide upon metal binding. Full NMR data are in the Supporting Information.

Analysis of the glutathione-derived resonances indicated relatively smaller changes of $\text{H}\alpha$ and $\text{C}\alpha$ chemical shifts upon addition of metal, with the largest change at the glutathione Glu $\text{C}\alpha$. These data suggest greater flexibility in the glutathione compared to the N-terminal cassette. Therefore, to identify the role of the glutathione conjugate in metal binding, as well as to further characterize the metal-binding motif, ^1H - ^{13}C HMBC experiments were conducted, which allow the correlation of $\text{H}\alpha$ and/or $\text{H}\beta$ resonances with carbonyl or carboxylate ^{13}C chemical shifts via their 2-bond and 3-bond couplings (Figure 5). These experiments, conducted at pH 7.4, indicated significant changes in the chemical shifts of both the glutathione Gly carboxylate and the glutathione Glu carboxylate in the presence of metal, with larger carbonyl chemical shift changes observed at the Glu carboxylate. As expected, large chemical shift changes were also observed for the side chain Asp carboxylates ($\delta = 0.74\text{--}1.92$ ppm) that directly bind to the metal in an EF Hand, consistent with their critical role in metal binding in this designed protein motif. In addition, the Trp7 carbonyl, which in an EF Hand directly binds to metal, exhibited the largest change in main chain carbonyl chemical shift in the peptide ($\delta = 0.41$ ppm). Collectively, these data strongly suggest that metal binding in residues 1–7 is similar to that of a native EF Hand, and further suggest that the glutamate carboxylate and/or the glycine carboxylate can provide an additional metal ligand in RP1-SSG, leading to increased metal affinity and terbium luminescence of the RP1 glutathione conjugate RP1-SSG over the free thiol in RP1-SH. Notably, the Glu ammonium is expected to have a $\text{p}K_a \sim 8$, potentially permitting

binding of the Glu carboxylate with minimal electrostatic repulsion between the metal and the ammonium.

In this design, as in protein kinase-inducible domains,^{45, 46} a proto-terbium binding motif (DKDADGW) is necessary but not sufficient for metal binding.⁶⁹ Upon post-translational modification of the designed peptide, a new anionic ligand is introduced (glutathione carboxylate; phosphoserine/phosphothreonine/phosphotyrosine) which mimics the native EF Hand Glu12 and recapitulates a complete metal-binding motif. Given that many protein post-translational modifications result in the introduction of negative charge (e.g. phosphorylation, sulfation, malonation, AMPylation, sulfenation, nitration), or alternatively result in the neutralization of a positive charge (e.g. lysine acetylation, arginine citrullination), these data suggest a potential generality of this design strategy for the modular design of proteins responsive to post-translational modifications.

We have described the first example of the design of a protein motif that is dependent on the specific redox-responsive post-translational modification cysteine glutathionylation. The basis of the design was the replacement of a native protein glutamic acid carboxylate with a carboxylate of a cysteine-conjugated glutathione. The structure and the terbium binding and luminescence of the designed protein motif were dependent on cysteine glutathionylation, allowing direct fluorescent detection of specific peptide glutathionylation. The designed peptide comprises a small, genetically encodable protein motif, suggesting its use as a non-obtrusive protein tag for the characterization of glutathionylation dynamics in a manner that may be localized via the protein to which it is conjugated. These results suggest general approaches both to the detection of protein glutathionylation and more generally to post-translational modification-dependent protein design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cysteine (Cys) oxidation state is regulated intracellularly by the relative concentrations of reduced glutathione (GSH) and oxidized glutathione (glutathione disulfide dimer, GSSG). In a typical intracellular environment, high concentrations of reduced glutathione maintain cysteine in a thiol (Cys-SH) oxidation state. Under oxidizing conditions, including oxidative stress, increased concentrations of GSSG lead to increased levels of cysteine disulfides, including glutathionylated cysteine via reaction of the nucleophilic cysteine with the electrophilic glutathione disulfide via disulfide exchange. Alternatively, glutathionylated cysteine may also be generated via oxidation of cysteine to the sulfenic acid followed by reaction of the electrophilic sulfenic acid with nucleophilic glutathione. In addition, cysteine glutathionylation may be effected via free radical (not shown) or enzymatic mechanisms.

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Figure 2.

(a) Structure of a calcium-bound EF Hand motif (1cll, calmodulin), with metal-binding Asp/Asn residues 1, 3, and 5 (cpk colors) and Glu12 (blue) emphasized.^{66, 67} C β and C γ 2 of Ile8, which is replaced by Cys in the peptide RP1, are shown in yellow. (b) Design principle: replace the critical metal-binding Glu12 of an EF Hand with a glutathionylated cysteine. Terbium binding could be mediated by either or both carboxylates of the glutathione conjugate. (c) Sequences of a consensus EF Hand, of a kinase-inducible domain (pKID-PKA) with a serine phosphorylated at residue 12 (blue), of a tyrosine kinase-inducible

domain peptide in which metal binding is mediated by phosphotyrosine after a proto-terbium binding motif (DKDADGW), and of redox-responsive peptides (RP) examined in this study. Residues in red and blue bind the metal via the side chains. Residue 7 (Tyr in a consensus EF Hand) binds the metal via the main chain carbonyl. Tyr or Trp (magenta) is used as a sensitizer of terbium luminescence.⁶⁸ Residue 9 (typically Asp or Ser) makes a water-mediated metal contact in a standard EF Hand. Glu12 (blue) binds the metal in a bidentate manner. The cysteine residue is indicated in green. In RP5, Cys is at residue 7, with the Trp moved to residue 8. Trp8 sensitizes terbium luminescence somewhat less effectively than Trp7.⁶⁸ Asp was used at residue 3 to generate an overall terbium-binding motif (after glutathionylation) with an optimal -4 charge of the liganding residues.⁶⁸ All peptides are acetylated on the N-terminus and contain C-terminal amides. Details of peptide synthesis and characterization are in the Supporting Information.

Figure 3.

Fluorescence data of non-glutathionylated (RP1-SH; green squares) and glutathionylated (RP1-SSG; blue circles) peptides in water with 10 mM HEPES pH 7.5 and 100 mM NaCl. Non-glutathionylated peptide also contained 500 μ M DTT to prevent disulfide formation. Error bars indicate standard error of at least three independent trials. (a) Background-corrected fluorescence spectra of 10 μ M peptide with 125 μ M Tb³⁺. (b) Binding isotherms of peptides. Fluorescence spectra and binding isotherms of non-glutathionylated and glutathionylated RP2-RP5, which exhibited smaller fluorescence changes and smaller

differences in dissociation constant between glutathionylated and non-glutathionylated forms, are in the Supporting Information.

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Figure 4.

NMR spectra of RP1-SSG in the absence of metal (red) and in the presence of 1 equivalent La^{3+} (blue). NMR data were collected using a solution of (a) 90% H_2O /10% D_2O or (b,c) 100% D_2O , with 1.5 mM peptide, 100 mM NaCl, and 10 mM sodium acetate- d_3 pH 6.2. (a) Fingerprint region of the TOCSY spectra. (b) $\text{H}\alpha\text{-C}\alpha$ region of the ${}^1\text{H}\text{-}{}^{13}\text{C}$ HSQC spectra. (c) Glycine $\text{H}\alpha\text{-C}\alpha$ and Cys/Asp $\text{H}\beta\text{-C}\beta$ regions of the ${}^1\text{H}\text{-}{}^{13}\text{C}$ HSQC spectra. Full spectral data are in the Supporting Information.

Figure 5.

^1H - ^{13}C HMBC spectra ($\text{H}\alpha$ - $\text{C}=\text{O}$ region) of RP1-SSG in the absence of metal (red) and in the presence of 1 equivalent La^{3+} (blue). NMR data were collected using a solution of 100% D_2O with 100 mM NaCl, and 10 mM sodium acetate- d_3 pH 7.4. Unlabeled peaks are due to folding over in the spectrum.

Table 1.¹H and ¹³C NMR chemical shift data for RP1-SSG in the absence and presence of La³⁺.

Residue	apo			with La ³⁺			H ^N	H α	C α
	H ^N	H α	¹³ C α	H ^N	H α	¹³ C α			
Asp1	8.29	4.57	54.65	8.30	4.58	53.88	0.01	0.01	-0.77
Lys2	8.39	4.22	56.64	8.45	4.31	56.21	0.06	0.09	-0.43
Asp3	8.34	4.60	54.87	8.19	4.66	53.14	-0.15	0.06	-1.73
Ala4	8.18	4.26	53.07	8.32	4.28	53.27	0.14	0.02	0.20
Asp5	8.31	4.60	54.87	8.24	4.65	53.88	-0.07	0.05	-0.99
Gly6	8.27	3.93	45.75	8.23	3.97, 3.91	45.58	-0.04	-0.04,+0.02	-0.17
Trp7	8.07	4.67	57.93	8.21	4.74	57.49	0.14	0.07	-0.44
Cys8	8.35	4.52	55.82	8.38	4.54	55.77	0.03	0.02	-0.05
Gly9	7.67	3.71	45.22	7.73	3.73	45.01	0.06	0.02	-0.21
Gly	8.30	3.80	46.39	8.31	3.80	46.31	0.01	0.00	-0.08
Cys	8.56	4.70	55.47	8.56	4.70	55.43	0.00	0.00	-0.04
Glu	-	3.76	57.06	-	3.77	56.88		0.01	-0.18