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Structural Studies of N-Terminal Mutants of Connexin 32 Using ^1H NMR Spectroscopy

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Abstract

The amino terminus of gap junction proteins, connexins, plays a fundamental role in voltage gating and ion permeation. We have previously shown with ^1H NMR that the structure of the N-terminus of functional connexin molecules contains a flexible turn around G12 (Arch. Biochem. Biophys. 490:9, 2009) allowing the N-terminus to form a portion of the channel pore near the cytoplasmic entrance. The mutants of nonfunctional connexin molecules G12S and G12Y were found to prevent this turn. Previous functional studies of loci at which Cx32 mutations cause a peripheral neuropathy, Charcot-Marie-Tooth disease, have shown that G12S is not plasma membrane inserted. Presently, we solve the structure of nonfunctional Connexin 32 mutants W3D and Y7D which do not appear to be membrane inserted. Using 2D ^1H NMR, we report that similar to G12S and G12Y, alterations in hydrophobic sidechain interactions disrupt (Y7D) or constrain (W3D) the flexible turn around G12. The alteration in the open turn around residue 12, observed in all nonfunctional mutants G12S, G12Y, W3D and Y7D correlates with loss of function. We propose that loss of the open turn causes the N-terminus to extend out of the channel pore and this misfolding may target mutants for destruction in the endoplasmic reticulum.

Keywords

NMR; atomic resolution structure; ion channels; voltage dependent gating; connexins; structure-function; Protein Structure and Function

INTRODUCTION

Gap junction channels are formed by the head-to-head docking of two hemichannels (connexons), each inserted into the plasma membrane of adjacent cells. A hemichannel is comprised of six protein subunits, termed connexins that span the plasma membrane four times with intracellular N- and C-termini. In humans, connexins are encoded by 21 genes, with different subsets of connexin proteins expressed in almost all tissues and organ systems forming channels with diverse physiological properties and differential sensitivity to regulation by voltage, $[\text{Ca}]_i$ and pH [1–6]. The intercellular signaling pathway provided by

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gap junctions plays a vital physiological function in excitable tissues including heart and brain, where they form electrical synapses, and in non-excitabile tissues such as liver and pancreas, where they synchronize cellular activity and secretion.

Mutations of human connexin genes are the pathophysiological basis for seven major disorders including: myelin and neuronal disorders, nonsyndromic and syndromic deafness, skin diseases, cataracts, oculodentodigital dysplasia, and idiopathic atrial fibrillation [7]. Cx32 mutations are the genetic basis of the X-linked form of Charcot-Marie-Tooth (CMTX) disease [8], a peripheral neuropathy, which afflicts 1 in 2500 individuals and is the second most common cause of CMT accounting for 10–20% of all cases [9]. Over 290 Cx32 CMT-X mutations have been identified [10]. Missense mutations leading to disease have been described at 14 of the 22 amino acids that comprise the N-terminus (NT) of Cx32. These are primarily “loss of function” mutations that either alter the functional properties of channels or prevent channel assembly and/or trafficking to the plasma membrane.

The NT plays an important role in the structure-function relation of connexin channels [11]. In channels formed by Cx26 and Cx32 the NT: 1) forms a portion of the large diameter aqueous pore by virtue of an open turn involving residues 11–15 that positions the first 10 residues into the channel pore [12–14], 2) contains at least a portion of the transjunctional (V_j) voltage sensor [15–17], 3) is an important determinant of conductance, charge selectivity, ionic rectification and permeation of both intercellular channels and undocked hemichannels formed by Cx32 [18,19]. The NT of Cx26 and Cx32 can be divided into two regions with the first 10 residues in a helical conformation (Connexin 26) or a rather structured region due to hydrophobic interactions (Connexin 32) and an unstructured second half characterized by a flexible open turn around the 12th residue. There is good agreement between inferences drawn from NMR structure of WT Cx32 and Cx26 peptides and the structure of the NT of the Cx26 channel solved by x-ray crystallography [14] and refined by molecular dynamics simulation [20]. Our past studies have shown that two loss of function Cx32 mutations G12S and G12Y disrupt the formation of the open turn, whereas the voltage-dependence of the Cx32G12P mutation, which allows the formation of the open turn, is identical to wild type [12,13]. Cx32G12S is a well characterized CMTX mutation whose loss of function is a consequence of its failure to be inserted into the plasma membrane. The mutation is characterized by low levels of expression and it appears to be trapped in the in the endoplasmic reticulum and/or Golgi network when exogenously expressed in mammalian cells [21]. G12S subunits are however stably inserted into canine microsomal membranes [22] and appear to oligomerize into hemichannels; suggesting that the trafficking defect occurs after subunit insertion into the endoplasmic reticulum membrane. Presumably, the structural defect caused by this mutation results in a channel conformation that is targeted for degradation by cellular quality control machinery.

The purpose of this paper is to investigate the solution structure of two peptides corresponding to mutations W3D and Y7D, which map to the beginning of the N-terminus using ¹H 2D NMR techniques. These mutations are at loci that are targets of loss of function CMTX mutations and also do not form functional channels in exogenous expression systems consistent with failure to be plasma membrane inserted. The structures are compared to those of the wild-type and G12P structures as well as to N-termini of G12S and G12Y to identify structural similarities in the N-termini of nonfunctional connexin molecules which are not plasma membrane inserted and which may be targeted for degradation in the endoplasmic reticulum.

MATERIALS AND METHODS

Peptide synthesis

Mutants W3D and Y7D of the Cx32 N-terminal peptide (MNWTGLYTLLSGVNRHSTAIGR) were synthesized by the New England Peptide Company. The synthesis method was standard Fmoc chemistry [23] and the sample was purified by HPLC and sequence confirmed by electron ionization spectrometry. The peptides contained free N and C termini which were not acetylated or amidated. For NMR data collection the peptides were dissolved in 100 mM KCL (ionic strength of 0.1 M) in 10% $^2\text{H}_2\text{O}$ /90% H_2O (pH 7.0) at a concentration of 1.0–1.2 mM with 100 M 3-(trimethylsilyl)propionic acid (DSS) as a chemical shift reference.

^1H 2D NMR

All NMR spectra were collected on Bruker DRX-600 MHz spectrometer or a Varian INOVA 600 MHz spectrometer with a cryoprobe. Data sets in H_2O were collected at 283, 288, 293 and 298 K to aid in proton assignment as well as for calculation of temperature coefficients. Water suppression in all 2D experiments was accomplished with either the double gradient echo method of Hwang and Shaka [24] or with Watergate [25]. The data were processed using NMRPipe [26] and extended using linear prediction and zero filling. The data were analyzed using NMRView [27].

All proton resonances were assigned by standard procedures [28]. For proton assignments both short (17ms) and long (75ms) mixing time TOCSY [29] experiments were used and proton-proton distance constraints were obtained from a 225 ms mixing time NOESY experiment at 283 K. Spin diffusion is not expected for a peptide of this size (22 amino acids) and there was no evidence of spin diffusion in comparisons of crosspeaks from NOESY spectra collected at 100 and 225 ms mixing times. NOESY crosspeak intensities were classified into very strong (2.8 Å), moderately strong (3.4 Å), and weak (5.0 Å) ranges. Atoms that were not stereospecifically assigned were treated as pseudoatoms and given correction distances according to procedures in Wüthrich [28]. Amide proton temperature coefficients were calculated from the proton chemical shift change from TOCSY spectra collected in increments of 5° in the temperature range of 278–293 K. Any amide proton which shifted less than 6.0 ppb/K was considered likely to be hydrogen bonded. Initial structures were calculated using relevant NOE constraints by simulated annealing and torsional angle dynamics as applied in the program CNS 1.1 (Crystallography and NMR Systems) [30]. A hydrogen bond analysis was performed on all structures using the program MolMol [31]. A hydrogen bond was considered to occur if it was present in greater than 70% of the structures and if the donor proton had a reduced temperature coefficient. Since no hydrogen bonds satisfied these criteria they were not used in the final structure calculations. The 20 lowest energy structures were energy minimized using CNS, evaluated with AQUA and PROCHECK-NMR [32] and displayed using MOLMOL [31].

RESULTS

The aliphatic to aromatic section of the NOESY for the N-terminal mutant W3D is shown in Figure 1. The figure shows a small number of medium to long range NOE connectivities between Y7 aromatic protons and aliphatic protons of L6, L9 and V13 in the NOESY spectrum of the W3D mutant. As expected, the number of interactions in this section of the spectrum is significantly reduced when the hydrophobic amino acid (W) is replaced with a polar amino acid (D). Similar changes were also observed in the Y7D mutant spectra.

A summary of NOEs for the W3D and Y7D mutant peptides are shown in Figure 2. Moderately strong $d_{\text{NN}}(i,i+1)$ NOE connectivities (connectivities observed between

consecutive NH protons) are observed in some areas of the W3D and in the Y7D mutant structures. There are also areas throughout the sequence with medium to strong intensity sequential $d_{\alpha N}(i,i+1)$ and $d_{\beta N}(i,i+1)$ NOES (NOE crosspeaks between the α or β proton of one residue and the NH proton of the next sequential residue) in both the mutants. Helical structure is confirmed by lower intensity $d_N(i,i+3)$, $d(i,i+3)$ and $d_N(i,i+4)$ NOE connectivities (NOE crosspeaks between the H proton and the NH proton 3 residues away, the H proton and the H proton 3 residues away and the H proton and the NH proton 4 residues away respectively). The W3D mutant contains none of these NOE patterns indicating there is no helical structure in this mutant. Examination of the mutant Y7D shows a $d_N(i,i+3)$ NOE pattern within residues D7-L10. There are also $d_N(i,i+2)$ NOE patterns located mainly within the first 12 residues of the Y7D peptide and at the end of the peptide. These patterns are indicative of turns and the lack of the signature NOE patterns for a helix suggests little helical structure in the Y7D peptide as well.

The temperature coefficients for the amide protons of W3D and Y7D mutant peptides are compared with those of the previously published wild-type and G12P mutant [12] in Figure 3a. The extent to which a temperature coefficient for an amide proton is lowered, from the solvent exposed value of 8 ppb K^{-1} , is a measure of the degree of protection from the solvent [33]. A later study reported random coil temperature coefficients for solvent exposed amide protons for each amino acid [34]. The random coil values for amide proton temperature coefficients for amino acids used in our study range from -6.43 to -9.32 ppb/K depending on the amino acid [34]. Temperature coefficients of less than 6 ppb/K are suggestive of hydrogen bonding [33]. The random coil temperature coefficients for W, Y and D are 7.98, 9.32 and 6.43 ppb/K respectively [34]. The figure shows most amino acids have lowered temperature coefficients from residues 4–6 with that of G5 being particularly reduced from random coil values (T-7.40, G-7.02 and L-8.42). In the wild-type or mutants containing W3 this may be due to the hydrophobic interactions between W3 and L6 that is expected to provide some solvent protection to these protons and/or the reported effect of an aromatic ring in close proximity to the glycine amide proton [34]. In the W3D mutant some structures show an H-bond between G5 NH and the carboxyl side chain on D3 as well as between the L6 NH and the backbone carboxyl group of T4. Other residues near the N-terminal region do not exhibit reduced temperature coefficients, consistent with hydrogen bonding. The C-terminal residues for all mutants and the wild-type show temperature coefficients that are near solvent exposed, random coil values as well. However there is a difference in the temperature coefficients in residues 11–15 between the wild-type and G12P mutants and those of the nonfunctional W3D and Y7D mutants as shown in Figure 3b. In this region, the wild-type and the G12P peptides have NH temperature coefficients that are very close to or even greater than their solvent exposed random coil values (7.02, 7.02, 8.35, 7.02 and 7.64 for SGVNR respectively, proline has no NH proton). These residues have lowered NH temperature coefficients, around 6 ppb/K, in both W3D and Y7D mutants. These values are less than the random coil values, which suggest the presence of a hydrogen bond within these residues and thus less flexibility. This data suggests less solvent exposure for residues 11–15 in the mutants W3D and Y7D and a more constrained region in these peptides as compared to the wild-type and G12P mutants.

The mutant W3D and Y7D structures were calculated in CNS using 210 (W3D mutant) and 199 (Y7D mutant) distance constraints obtained by the NOESY experiments. Structural statistics for the 20 converged structures and atomic root-mean-square differences (RMSDs) are given in Tables 1 and 2. In the Tables a medium range NOE is defined as an NOE between two protons which are 2–4 residues away. A long range NOE is defined as an NOE between two protons which are 5 or more residues away. There were no NOE violations greater than 0.5 Å and no angle violations greater than 5°. Deviations from idealized

covalent geometry are low and the Ramachandran statistics indicate that neither bad contacts nor distortions exist in the converged structures.

The 20 lowest energy structures that best fit the NMR data for the W3D and Y7D mutant peptides are shown in Figure 4. When W3 is mutated to D the structured part of the molecule was found to encompass residues 6–15. Superposition of the backbone atoms for residues 6–15 for the W3D mutant structure in Figure 4 gives an RMSD value of 1.588 Å. The 2 long range NOEs between the Y7 ring protons and the V13 methyl protons contributed to the RMSD of the W3D structure bundle since removal of these NOEs increased the RMSD by 15%. The Y7D mutant is also shown in Figure 4 and was found not to be structured for long regions of amino acids but had a structured domain encompassing residues 8–15. Superposition of backbone atoms for residues 8–15 gives a RMSD of 1.705 Å. The one long range NOE between L6 δCH_3 protons and the M1 HA proton is in the unstructured part of the molecule and had no effect on the RMSD for residues 8–15.

Figure 5 shows superposition of the 20 lowest energy structures for residues around the turn region for the nonfunctional mutants W3D and Y7D as well as for the wild-type and functional mutant G12P solved previously [12]. In the W3D mutant, superposition of backbone atoms from residues 9–15 gives an RMSD of 1.190 Å with a turn found within residues 9–13. The turn is rather narrow and constrained by hydrophobic interactions between side chains of L10 or L9 and V13 in the structures. In the mutant Y7D, superposition of residues 10–15 gives a RMSD of 1.356 Å and shows a helical-like segment rather than a turn. These bundles indicate that the structures fit the NMR data well and that each molecule can be defined by a single conformation. They also show that the structural turns around and including residue 12 are well defined by the NMR data.

The lower half of the figure shows superposition of similar residues in the wild-type and the functional mutant G12P previously solved [12]. Superposition of backbone atoms from residues 10–15 in the wild-type gives a RMSD of 0.750 Å and shows a more open, less constrained turn around residue 12. The hydrophobic sidechains of L10 and V13 are outside the turn in the wild-type rather than within it. In the functional mutant G12P, superposition of backbone atoms from residues 8–15 gives a RMSD of 1.084 Å. Similar to the wild-type a larger, unobstructed turn is observed around residue 12 where the hydrophobic interactions involve residues 9 and 10 outside of the turn with no interaction with the sidechain of residue 13.

Representative low energy structures for the W3D and Y7D mutants are shown in the top of Figure 6 with the previously published structures of the wild-type and G12P mutant peptide structures [12] shown in the lower half of the figure. The W3D structure shows the hydrophobic interactions between the sidechains of residues L6, Y7, L9, L10 and V13. The absence of W3 causes the hydrophobic associations to occur further up the sequence to include V13. The long range NOE interactions between the Y7 ring protons and the V13 methyl protons play a role in constricting the turn around G12 in this peptide. In the Y7D mutant, there are hydrophobic associations between W3 and L6 and a second group of associations which include L9, L10 and again V13. In contrast to these mutants, the functional wild-type and G12P mutant structures show that the hydrophobic associations occur mainly within residues 3–9 and sometimes include residue 10. Residue V13 is not involved in hydrophobic associations with L9 or L10 within the turn allowing for a larger, less constrained turn around residue 12 in N-terminal structures that form functional channels. The nonfunctional W3D and Y7D mutants show smaller turns encompassing residues 9–13 constrained by sidechain hydrophobic interactions. All structures show an unstructured region in the C-terminus from residues 16 to 22.

DISCUSSION

We have previously shown that the N-terminus of Connexin 32 consists of a structured region consisting of numerous hydrophobic associations within residues 3–10, an unstructured random coil in the C-terminus that is connected by an open, unconstrained turn around the 12th residue, essential for channel function. We have previously shown that the N-terminal structure of residues 1–15 in Connexin 26 consists of a helical region comprising residues 1–10 which culminated in an open, flexible turn around residue 12 [13]. The overall structural features of the Connexin 26 peptide, solved by NMR, agree well with the N-terminal structure of the Connexin 26 channel solved by x-ray crystallography [14] and further refined by molecular dynamics [20]. Agreement between the structure of peptide segments and the corresponding region of the intact protein have been reported a number of times [35,36]. This is because of the dominance of short range interactions seen in peptides [37]. Peptides from sections that are turns in the native protein display turns as peptides in solution [37–39]. For example, peptides of the loops interconnecting the helices in the membrane protein bacteriorhodopsin had the same NMR solution structure that was observed in the crystal structure of the intact protein [36]. It is quite likely the N-terminal peptide solution structures reported here resemble the conformation of the N-terminus in the native channel.

In this study we report the structure of mutations of two hydrophobic residues, W3D and Y7D. Both mutations have both local and global effects on the structure as a consequence of the reorganization of the hydrophobic interactions observed in the wild type peptide. Remarkably the mutations either constrained (W3D) or removed (Y7D) the turn formed by residues 10–15.

Peptide structures are stabilized by side chain hydrophobic interactions [40,41], salt bridge formation [42] and hydrogen bonds [43]. A large number of NOEs were observed for the wild-type and G12P functional N-terminal peptides between side chains of W3, L6, Y7, L9 and L10 indicating close contact [12]. The interactions of these hydrophobic amino acid side chains provide a stabilizing effect on the structure near the N-terminus of these peptides. The interaction of hydrophobic side chains of residues L9 and L10 with those of residues W3 and Y7 prevents interactions between the side chains of L9 and L10 with the hydrophobic side chain of V13. The lack of hydrophobic side chain associations between L9, L10 and V13 allow for an unconstrained open turn between residues 10–15 in the wild-type and the G12P mutant peptide, which we propose is required for functional channels.

In contrast, when either W3 or Y7 is mutated to a more polar residue the hydrophobic interactions change to include the sidechain of V13. The W3D structure is stabilized by hydrophobic interactions between the sidechains of residues L6, Y7, L9, L10 and V13. This results in structures where the turn is smaller around G12 (consisting of residues 9–13) and more constrained by hydrophobic side chain interactions between either residues L10 and V13 or L9 and V13 as shown in Figures 5 and 6. The Y7D mutant is stabilized by hydrophobic sidechain interactions between W3 and L6 and sidechain interactions between L9, L10 and V13. The latter group of hydrophobic sidechain interactions (L9, L10 and V13) stabilizes a helical segment around residues 7–10, allowing sidechains of L9 and L10 to interact with those of V13. This causes the loss of the open turn within residues 10–15 and a more constrained turn encompassing residues 9–13.

The temperature coefficient data supports the structural data. Both the wild-type (12G) and G12P mutant structures had temperature coefficients close to or above their random coil values in the turn regions for most of the residues 10–15 and 8–15 respectively. This suggests a lack of hydrogen bonding within the backbone protons in these turns, more

solvent exposure and thus the turns are loose and flexible. Populations of turn conformations are often observed without significant hydrogen bonding [44,45] and NOES were observed between protons from residues within these turns indicating a significant population of conformers in these conformations. In contrast, many of the backbone NH protons within residues 11–15 in the mutant peptides W3D and Y7D have temperature coefficients, around 6 ppb K^{-1} , which is less than their random coil values [34]. This indicates a less solvent exposed, more folded area of the structure and the possibility that the NH protons are involved in a hydrogen bond. Analysis of the W3D structures suggests the possible presence of a hydrogen bond between G12 NH – L9 CO, V13 NH – S11 CO, N14 NH – L10 CO, R15 NH – G12 CO and H16 NH – V13 CO in some of the models. Additional hydrogen bonds (G5 NH – D3 OD1, L6 HN – 4 CO and T8 sidechain hydroxyl hydrogen to L6 CO) are also present in some of the model structures. These serve to stabilize the N-terminal part of the W3D mutant structure. The Y7D structures suggest the presence of hydrogen bonds between L9 NH – D7 OD1, L9 NH – D7 OD2, L10 NH – D7 OD1, L10 NH – D7 OD2, S11 NH – T8 CO, G12 NH – L9 CO and V13 NH – S11 CO in several models. Additional stabilization in the N-terminal region may be provided by hydrogen bonds between T4 NH – M1 CO and T8 HN – L6 CO seen in some of the models. The presence of hydrogen bonds around G12 and the reorganization of hydrophobic side chain interactions to include the V13 sidechain in the W3D and Y7D peptides serve to constrain or eliminate the open, flexible turn correlated with the formation of functional channels seen in wild-type Connexin molecules or those with a G12P mutant N-terminus.

We have previously shown the importance of having amino acids in the residue 12 position, such as glycine or proline, which are amenable to forming turns in the N-termini of Connexin molecules. The turn at residue 12 allows the placement of the first 10 amino acids within the channel pore, as indicated by structure-function studies [12,13,15–17], the Cx26 crystal structure [14] and its refinement by Molecular dynamics [20]. We have shown that substitution of residue 12 with amino acids having longer side chains that engage in either hydrogen bonds or hydrophobic interactions such as S or Y, produce a more helical, constricted, less flexible turn in this region [12]. The substantial reduced flexibility of the open turn, which would prevent the insertion of the N-terminus into the channel pore, is so far perfectly correlated with loss of channel function. In this study we investigated the role of 2 hydrophobic amino acids at residues W3 and Y7 on the structure of the N-terminus. The current study shows that the mutations W3D and Y7D cause an alteration of hydrophobic associations which constrain the turn around residue 12. The mutations at W3 or Y7 also prevents the interaction of these hydrophobic side chains which causes the N-terminus to loop back, which in addition to the turn also aids in placing the N-terminal residues within the channel pore. This study demonstrates that the hydrophobic associations in the beginning of the N-terminus are essential to the structure of the molecule and for the formation of functional channels.

During the biogenesis of gap junctions the connexin subunits are synthesized in the endoplasmic reticulum (ER), and oligomerized into hemichannels (connexons) either in the ER or the Golgi apparatus before delivery to the plasma membrane [46,47]. Connexons in adjacent membranes then align and dock to generate gap junction intercellular channels. Misfolded or abnormally oligomerized connexins can be transported back to the ER where they are expelled for degradation [47]. We have reported that W3D, Y7D, G12Y and G12S do not form functional channels in homotypic and heterotypic pairings with wild type Cx32 and Cx26 hemichannels [13,15,16]. Thus, the loss of function is unlikely to arise as a consequence of large shifts in voltage-dependence of the mutant hemichannel, but rather strongly suggests that the mutant hemichannels are not inserted into the plasma membrane. This interpretation of electrophysiological studies has been confirmed for G12S and other mutations that fail to express currents in heterotypic and homotypic pairings with Cx26 and

Cx32 hemichannels and shown not to be membrane inserted by immunofluorescence [21,48]. It is well established that G12S is not inserted into the plasma membrane of mammalian cells but appears to be trapped in the Golgi apparatus [49]. The results reported here support a previous functional study suggesting that single point mutations at specific sites on connexin may affect the protein conformation, disturbing gap junction biogenesis, plasma membrane trafficking and channel function [49]. Certain polypeptide domains or structural features may serve as a sorting signals regulating connexin degradation [47]. All four mutants, W3D, Y7D, G12Y and G12S converge to an altered structure around residue 12; the constraint or elimination of the open turn. Our results suggest that structural defects caused by loss of function mutations at W3, Y7 and G12 may either target connexin 32 proteins for degradation or prevent oligomerization in the golgi. We propose that the loss of open turn causes the NT to extend out of the channel pore toward the cytoplasmic entrance of the channel and that connexin proteins adopting this conformation may be targeted for degradation.

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Connexin32 mutants W3D and Y7D do not form functional channels.

¹H NMR structures show alterations in the flexible turn around G12.

Altered turn around G12 was reported previously in nonfunctional G12S and G12Y mutants.

Connexin mutants G12S, G12Y, W3D and Y7D are not membrane inserted.

Loss of turn around G12 may target misfolded Connexins for degradation.

Figure 1.

The two-dimensional ^1H NMR spectrum of the W3D mutant peptide showing labeled NOE interactions between aliphatic and aromatic side chain protons.

Figure 2.
A summary of NOE restraints for the W3D and Y7D mutant N-terminal peptides. The horizontal lines indicate backbone NOES with the thickness representing intensity.

Figure 3.

a) Plots of temperature coefficients vs. residue number for the mutants W3D and Y7D as well as the previously published temperature coefficients for wild-type and mutant N-terminal peptides[12]. The proline residue (12) in the mutant G12P does not have an NH proton. Temperature coefficients were only calculated for residues whose NH proton was observable at all temperatures. **b)** Temperature coefficients of residues 11–15 for the wild-type, G12P, W3D and Y7D peptides. The error bars represent errors of 0.15 ppb/K.

Figure 4.

The 20 lowest energy conformers of the W3D and Y7D mutants are shown. Superpositions of the backbone atoms for residues 6–15 are shown for the W3D mutant. Superpositions of the backbone atoms for residues 8–15 are shown for the Y7D mutant.

Figure 5. Superposition of the backbone atoms for residues within the turn around G12 for the W3D and Y7D mutants as well as the previously published wild-type (G12) and G12P functional mutants [12].

Figure 6. Representative ribbon diagrams of the mutants W3D and Y7D and previously published functional N-termini of the wild-type and G12P mutants are shown [12].

Table I

W3D

Structural Statistics	Values for 15 converged structures
Total Constraints	210
Intraresidue	147
Sequential	49
Medium range	12
long range	2
Largest distance constraint violation (Å)	0.35
RMSD	
Backbone (Å)	1.588 (res.6–15), 1.190 (res. 9–15)
Heavy atoms (Å)	2.957 (res.6–15), 2.634 (res 9–15)
Deviations from idealized geometry	
Bonds (Å)	0.0017
Angles (deg)	0.3222
Ramachandran	69.1 % in most favorable 30.6 % additionally or generously allowed regions

Table II

Y7D

Structural Statistics	Values for 15 converged structures
Total Constraints	199
Intraresidue	105
Sequential	72
Medium range	21
long range	1
Largest distance constraint violation (Å)	0.40
RMSD	
Backbone (Å)	1.705 (res.8–15), 1.356 (res.10–15)
Heavy atoms (Å)	3.113 (res.8–15), 2.800 (res 10–15)
Deviations from idealized geometry	
Bonds (Å)	0.0023
Angles (deg)	0.3492
Ramachandran	55.4 % in most favorable 42.7 % additionally or generously allowed regions