The MuLV 4070A G541R Env mutation decreases the stability and alters the conformation of the TM ectodomain

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Abstract

Virus–cell and cell–cell fusion events are affected by various properties of the fusogenic Env protein on the cell surface. The G541R mutation within the TM ectodomain of murine leukemia virus (MuLV) 4070A arose by positive selection in viral passage and results in a reduction of cell–cell fusion events while maintaining viral titer. Size exclusion chromatography shows that the multimerization properties are similar among expressed wild-type and mutant ectodomain peptides. Circular dichroism measurements reveal decreased thermal stability of the G541R mutant as compared to wild type. The G541R mutant also renders the peptide more susceptible to Lys-C protease cleavage. The 42–114 monoclonal antibody does not bind to the G541R mutant peptides, suggesting a structural difference from wild type. These altered physical properties result in productive viral infection of G541R bearing virus with decreased syncytia.

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Introduction

Membrane fusion is a necessary event in the infectious cycle of enveloped viruses and is therefore under strict spatial and temporal control. Retrovirus membrane fusion is catalyzed by the envelope (Env) surface (SU) and transmembrane (TM) proteins. The SU protein is involved in receptor binding, therefore providing specificity, while the TM protein contains the necessary elements for membrane fusion. The Moloney murine leukemia virus (Mo-MuLV) TM is initially expressed as a precursor protein that is activated for viral fusion when the viral-encoded protease (Pr) cleaves 16 amino acids from the C-terminus. These 16 amino acids, known as the R-peptide, negatively regulate membrane fusion (Rein et al., 1994). Along with catalyzing virus–cell membrane fusion, the viral Env can also induce cell–cell fusion. This results from the cell surface expression of viral Env proteins and their interaction with receptors on neighboring cells.

Class I fusion proteins including MuLVs Env protein, human immunodeficiency virus type I (HIV-I) Env, and influenza hemagglutinin (HA) protein have evolved a similar method of controlling membrane fusion (Ekert and Kim, 2001). All contain a fusion peptide that is presumably buried in early envelope conformations and subsequently exposed either in response to a low pH endosomal compartment or conformational changes following receptor binding. Upon exposure, the fusion peptide inserts into the target cell membrane. This results in the TM protein acting as a bridge linking the virus membrane to the target cell membrane. It is at this stage that the TM protein transitions into the highly stable post-fusion hairpin conformation. This physical process brings the two membranes within close proximity thereby promoting membrane fusion and content transfer (Ekert and Kim, 2001).

Altering expression levels of the TM and SU proteins has been shown to affect membrane fusion in multiple viral systems. The density of MuLV Env present on the cell surface can be directly related to cell–cell fusion events (Chung et al., 1999). However, virus–cell fusion is less affected and Mo-MuLV
infection requires only minimal amounts of Env protein (Bachrach et al., 2000). Similarly, a low density of influenza hemagglutinin protein on the cell surface results in an increased lag time before cell–cell fusion occurs (Clague et al., 1991; Danieli et al., 1996). Consistent with this idea, cellular expression levels of the HIV-1 Env protein has been shown to alter cell–cell fusion (Lineberger et al., 2002).

Various mutations within the Env proteins also affect the fusion process. Previous work has identified a point mutation, G541R, within the ectodomain of the amphotropic 4070A TM protein that was positively selected during viral passage. The G541R mutation has been observed in chimeric (EA6 and EA7) viruses in three independent populations as well as within a wild-type (WT) 4070A virus passaged on D17 cells (O’Reilly and Roth, 2003a, b; Peredo et al., 1996). G541 and G542 comprise a Gly–Gly motif that is conserved among HTLV-1, Ebola, and MuLV TM proteins adjacent to the conserved CX_{6–7}C motif known to form a disulfide bond (Center et al., 1998; Fass et al., 1996; Maerz et al., 2001; Weissenhorn et al., 1998b). The phenotype associated with the G541R mutation is a decrease in cell–cell fusion events despite near-wild-type viral titers, a reduced cell surface density of Env protein, and a decrease in SU–TM interactions (O’Reilly and Roth, 2003a). It has been suggested that this Gly–Gly motif may act as a hinge region allowing the putative post-fusion hairpin structure to arise (Maerz et al., 2000). In the analogous region of HIV-1 gp41, a Trp–Gly sequence is highly conserved among HIV and SIV strains. In the HIV-1 gp41 ectodomain, a point mutation of the Trp residue in this conserved Trp–Gly sequence results in a decrease in cell–cell fusion and a mild decrease in gp120–gp41 interactions despite near wild-type viral fusogenicity (Cao et al., 1994). The behavior exhibited by the G541R mutation within the MuLV TM seems to parallel that observed in the mutant HIV-1 gp41 ectodomain.

In an effort to understand how the G541R mutation results in the observed phenotype, studies were performed to compare the physical properties of purified TM ectodomain peptides (TM55) encoding the WT and G541R mutation. Although multimerization is not affected, the presence of the G541R mutation within the 4070A TM ectodomain multimers results in a decrease in thermal stability and an increased susceptibility to protease digestion. The differential recognition of a monoclonal antibody provides further support that the G541R mutation alters the protein’s 3D structure.

**Results**

**Wild-type and G541R mutant protein have similar multimerization properties**

The ectodomain of the amphotropic 4070A TM was expressed and purified from *Escherichia coli*. The region expressed, from Thr503 to Asp 556 (TM55), was based upon homology with the Mo-MuLV TM ectodomain, for which biochemical analysis (Fass and Kim, 1995) and the X-ray structure (Fass et al., 1996) have been determined. Fig. 1 profiles the primary structure of the TM55 protein (region inclusive of arrows) within the TM protein, based on the structure of Mo-MuLV (Fass et al., 1996). The region encodes a putative coiled-coil region (Q503–F537) followed by a short 3_{10} helix. The G541R mutation lies between the two helical domains. Three conserved cysteines localize near the C-terminus of the peptide. The short 3_{10} helix is not similar to the alpha-helical region found to intercalate into the coiled-coil region in the crystal structures of HTLV (Kobe et al., 1999; Maerz et al., 2001) and Ebola (Weissenhorn et al., 1998b), predicted to be further towards the C-terminus.

The TM55 protein of both the wild-type 4070A (WT) and the G541R mutant were expressed in a vector encoding an N-terminal hexahistidine tag followed by a thrombin cleavage site, facilitating a one-step purification on Ni-NTA resin under native conditions. To determine if the G541R mutation would alter the predicted trimeric multimerization of the protein, both WT and G541R purified protein were size fractionated on a Superdex 75 gel filtration column (Fig. 2). The predominant protein peaks for both WT (Fig. 2A) and G541R (Fig. 2B) elute at near identical volumes. Coomassie-stained SDS–PAGE along with western blots using an anti-G541R antibody (which recognizes the G541R and WT peptides) confirm that the TM55 protein corresponds to the observed A_{280} peaks in the chromatogram at fractions 19 and 20 (Fig. 2, arrow). The co-elution of both the WT and G541R peptides in identical fractions indicates no gross structural changes or alteration in multimerization states has occurred. The majority of the protein migrated as a monomer on the SDS–PAGE. Low levels of higher molecular weight products corresponding to incompletely denatured TM55 multimers are also observed in the fractions corresponding to the chromatogram peak. Using a plot of the TM55 protein elution volume on the
Superdex 75 column versus the globular molecular weight standards, the TM55 migrated as a 36-kDa protein. Although it would be expected that a trimer would elute at 25 kDa, the coiled-coil structure likely results in a slower migration than expected when plotted against globular molecular weight standards.

The results of the Superdex 75 column chromatography indicated that the mutation at G541R did not disrupt the overall oligomerization of the TM ectodomain. This was expected given that multimerization of TM is driven primarily through the coiled-coil domain (Ou and Silver, 2005) and the G541R mutation is C-terminal to this region.

Wild-type TM peptides are more thermodynamically stable than G541R peptides

The G541R mutation lies within a Gly–Gly motif adjacent to a cysteine-bonded loop highly conserved among HTLV-1, Ebola, and MLV TM proteins (Maerz et al., 2001). In the HTLV-1 TM structure predicted to represent the post-fusion conformation, this region encompasses a turn and chain reversal, which is stabilized by the disulfide bond (Maerz et al., 2001). Experiments were therefore performed to examine whether the G541R mutation would destabilize this region and decrease the structural integrity of the peptide.

To test this hypothesis, WT and G541R peptides were thermally denatured (Figs. 3A and C), then renatured (Figs. 3B and D) and monitored for changes in the circular dichroism profile (Aviv Spectropolarimeter Model 215 CD instrument). Studies were performed using two buffer conditions: HEPES pH 7.4, 400 mM NaCl, 1 mM DTT, 5% glycerol, or phosphate buffer (200 mM K₂HPO₄–KH₂PO₄ pH 8.0, 400 mM KCl). The peak of the first derivative from the resulting curve was used as the midpoint of transition (T_m), the temperature at which 50% of the protein is folded. In both phosphate and HEPES buffer, the WT peptides displayed higher melting temperatures than the G541R peptides. In phosphate buffer the T_m of denaturation for WT is 74 °C while the T_m for G541R is only 67 °C (Fig. 3A). Protein analyzed in HEPES buffer (Fig. 3C) gives a T_m of 66 °C for WT and a T_m of 54 °C for G541R. The T_m of renaturation was also measured in both phosphate and HEPES buffers. In phosphate buffer, the T_m of renaturation for WT is 67 °C and 58 °C for G541R (Fig. 3B). Protein analyzed in HEPES buffer (Fig. 3C) gives a T_m of denaturation of 66 °C for WT and a T_m of 54 °C for G541R. The T_m of renaturation was also measured in both phosphate and HEPES buffers. In phosphate buffer, the T_m of renaturation for WT is 67 °C and 58 °C for G541R (Fig. 3B). In HEPES buffer, renaturation yields a T_m of 73 °C for WT and 62 °C for G541R (Fig. 3D).

These studies indicate that the G541R mutation decreases both the T_m of thermal denaturation and renaturation compared to the WT TM peptide. This confirms the hypothesis that G541R destabilizes the coiled-coil region. Despite these differences in thermal stability, additional wavelength scans show both proteins have an α-helix content of 65–70% and the proportion...
of secondary structural elements remains roughly the same between the two peptides (data not shown). This observation argues that the thermostability differences observed likely result from a local deformation in the structure.

Wild-type TM peptide is more resistant to protease digestion than G541R

The location of the G541R mutation along with the observed structural thermal instability it imparts suggests the possibility that the arginine substitution may reduce the flexibility in the hinge region. This limited flexibility could then prevent optimal hairpin formation resulting in weaker interactions among the N-terminal helices and the C-terminal tail in the trimer. If this is true, the G541R peptide should be significantly more susceptible to protease cleavage compared to WT TM peptide. To test this, a time course of protease digestion was carried out using Endoproteinase Lys-C (Lys-C). Lys-C enzyme cleaves peptide bonds C-terminally at lysine residues. The TM55 protein contains four lysines that could be cleaved by Lys-C (Fig. 4, top). The first two are within the triple-helical region, the third lies between the C-terminus of the coiled-coil and the G541R mutation, and the fourth lysine is within the CX6CC motif (Fig. 1). Digestion at a single lysine would result in loss of the full-length TM55 product.

Coomassie-stained SDS–PAGE analysis of WT and G541R treated with Lys-C shows the loss of the full length TM55 peptide with protease digestion and the concomitant appearance of degraded products at the bottom of the gel, denoted by an asterisk. The WT TM peptide (Fig. 4A) is highly resistant to Lys-C digestion at time points up to 24 h while G541R (Fig. 4B) displays complete digestion within the first hour after addition of protease. Given the location of the mutation it is possible the susceptibility to protease digestion results from a disruption of the predicted disulfide bond that likely adds stability to the region. However, the addition of TCEP prior to digestion had a minimal effect on digestion of the WT and G541R peptides at time points of 1 h and after (data not shown). This suggests that while the predicted disulfide that forms between two cysteines in the hairpin loop may provide some resistance to protease digestion, its presence or absence cannot explain the difference found between WT and G541R.

Wild-type and G541R protein both have an intact disulfide bond

In order to determine if the G541R mutation could interfere with the formation of the expected disulfide bond, experiments to identify the disulfide bonds were performed on both the WT and G541R TM55 peptides. The approach involved the modification of the protein samples using the alkylating agent iodoacetamide (IAM), and subsequently analyzing their mass spectra. Free cysteine residues will be alkylated by IAM, whereas cysteines that are disulfide bonded will not be modified. Following treatment with IAM, the peptide samples were treated with high concentrations of Lys-C to maximize proteolysis and analyzed by mass spectrometry to identify the presence and location of disulfide bonds. Figs. 5A and C show respectively that WT and G541R peptides treated with IAM followed by digestion with Lys-C yielded fragments with a mass of 1876.9
Control samples of the WT and G541R peptides were created by initially reducing with DTT then observing their mass spectra. Fragments of 1992.8 amu and 2091.9 amu appear for WT and G541R respectively, corresponding in size to the modification of all three cysteines (Figs. 5B and D). It should be noted that despite reduction and modification of all three cysteine residues, fragments corresponding to cleavage at the lysine residue within the CX6CC motif were never observed (including LC-MS analysis using LTQ Thermo-Fisher; data not shown). This suggests steric constraints prevent cleavage at this particular site regardless of disulfide bonding.

The analysis performed identified one disulfide bond but does not specify which two cysteines are involved in the disulfide bond. To determine whether the disulfide occurs between the first and second cysteine residues in the CX6CC motif as expected, MALDI-TOF/TOF (MS/MS) was performed. To map cysteine bonds, protein fragments from the first TOF MS were subsequently fragmented in a second TOF MS and the mass of each sequential amino acid in the peptide chain was analyzed allowing for the identification of the IAM modified cysteine. MS/MS analysis indicated that there does in fact exist a disulfide bond between the first and second cysteine residues; however, there also exists a population in both WT and G541R peptides that contains a disulfide bond between the vicinal cysteine residues (data not shown). However, because this analysis was not coupled with liquid chromatography the two species could not be separated quantitatively, and it is therefore not possible to conclude whether the G541R mutation favors one conformation over another. This unexpected finding may be a result of expression of subdomains in bacteria or disulfide shuffling during the analysis, although a possible biological function cannot be eliminated.

The G541R mutation abrogates binding of a TM-specific antibody

The observed differences in physical properties exhibited by WT and G541R suggest that disparities in physical structure were quite likely. Fig. 6 shows the results obtained from probing western blots of bacterially expressed TM55 with either a TM-specific (42–114) antibody (Fig. 6A) or an anti-polyhistidine antibody (anti-polyHis6) (Fig. 6B). Panel A shows the 42–114 antibody recognized WT (lane 3) but not G541R (lane 5) from E. coli soluble cell lysates. Total cell extract (lane 8) from E. coli expressing the G541R mutant reacts weakly. Panel B shows that both the WT and G541R proteins are present in equal amounts in the soluble fractions (lanes 3 and 5 respectively), as seen by a western blot with an anti-polyHis6 antibody. The monoclonal TM-specific 42–114 antibody differentiates between the WT and G541R peptides, either due to changes in the conformational epitope, or disruption of a sequence-specific epitope. However, weak binding of 42–114 to G541R peptides in total cell extracts (Fig. 6A, lane 8) implies that there is a population of G541R present that can bind the antibody. The differences in antibody recognition between the wild-type and
G541R mutant peptide suggest an altered conformation may result from the mutation.

Discussion

The G541R mutation within the amphotropic MuLV TM protein was previously found in multiple viral populations including WT 4070A virus as well as three independent populations of chimeric (EA6 and EA7) virus (O’Reilly and Roth, 2003b). The phenotype associated with this mutation exhibits decreased syncytia formation despite near wild-type viral titers. In addition, there is a decrease in co-immunoprecipitation equilibrium profiles (Center et al., 1998) and the phenotype associated with this mutation result from the mutation.

The G541R mutation within the TM does not alter susceptibility to protease digestion of the G541R mutant protein has shown that the G541R mutation within the TM does not alter susceptibility to protease digestion of the G541R mutant protein. The results presented in this manuscript indicate that the G541R mutation, which is outside the coiled-coil structure, affects the stability of the TM peptide. Unfortunately, the G541R mutation significantly decreased the thermal stability of the TM peptide. When analyzed in two different buffer conditions, the G541R mutant peptides denatured and renatured at consistently lower temperatures than the WT peptides. The results presented in this manuscript indicate that the G541R mutation, which is outside the coiled-coil region, affects the stability of the triple-helical regions.

G541R substitutes Arg for the first Gly within a Gly–Gly dipeptide, known to be extremely flexible in its potential rotations. Introduction of the large charged Arg within this motif restraints the peptide chain’s rotational conformations and most likely imparts unfavorable constraints on the flanking regions. G541R immediately precedes the conserved CX6CC motif that is predicted to form a disulfide bond between the first and second cysteine residues. This proposed disulfide linkage is based upon X-ray structural data of homologous fusion proteins (Fass et al., 1996; Fass and Kim, 1995; Ruigrok et al., 1988, 1986; Weissenhorn et al., 1998a, 1996). Both WT and G541R peptides also had high α-helix content (65–70% by CD); however, the G541R mutation significantly decreased the thermal stability of the TM peptide. When analyzed in two different buffer conditions, the G541R mutant peptides denatured and renatured at consistently lower temperatures than the WT peptides. The results presented in this manuscript indicate that the G541R mutation, which is outside the coiled-coil region, affects the stability of the triple-helical regions.

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Although the G541R mutation does not prevent a disulfide from forming, it did not rule out the possibility that the disulfide bond was forming between cysteine residues in the motif other than the two expected. To clarify this point, MS/MS analysis was performed and it was in fact determined that a disulfide bond occurs between the first and second cysteine residues as expected. Interestingly, MS/MS data show that there is also a population in which the disulfide bond occurs between the second and third (vicinal) cysteines. This is seen in both WT as well as the G541R mutant protein. Since the data collected is only qualitative, it is not possible to conclude whether or not the G541R mutation affects the likelihood of one bond forming over the other. Within the pre-fusion state, SU-TM interaction is through non-covalent as well as covalent interactions. For MuLV, a disulfide bond between the TM CX6CC region and the CWLC region of SU has been identified (Pinter et al., 1997), which undergoes thiol-isomerization upon receptor binding (Wallin et al., 2005a,b). While it remains possible the vicinal cysteine bond observed is an artifact of the purification procedure or mass spectrometry technique, a high energy vicinal disulfide bond in the extended pre-fusion state may have a biological role. A high energy disulfide bond stabilized by interactions with the SU protein may serve to increase the free energy release when the SU protein is shed and the transition to a post-fusion state is made. In this model the first cysteine residue would be involved in the disulfide bridge linking the SU and TM proteins. Upon receptor binding, the disulfide bridging the SU and TM proteins would be disrupted creating a redox switch in TM, similar to the redox switch believed to exist in the SU protein (Pinter et al., 1997). This would result in the breaking of the vicinal disulfide bond and the formation of the post-fusion conformation, with a lower energy disulfide bond forming between the first and second cysteine in the CX6CC motif. While a biological role cannot be ruled out, further investigation would be required to test this hypothesis.

While the G541R mutation does not appear to result in a dramatic structural change within the ectodomain, at least in the post-fusion form, the results of these studies support a detectable, but subtle local destabilization that decreases the integrity of the multimer. In addition to the physical (thermal denaturation/renaturation studies) and biochemical (Lys-C digestion) evidence, additional support is provided by the differential recognition of the wild-type and mutant proteins by the monoclonal antibody 42–114. The 42–114 anti-TM antibody binds the WT TM55 peptide; however, it shows low recognition of the TM55 bearing the G541R mutation. If the antibody is in fact recognizing a specific conformation, even subtle structural differences within this region could alter the association of TM with SU. Alteration in the positioning of the CX6CC could readily affect the ability to form the SU-TM disulfide bond as well as destabilize non-covalent interactions yielding the observed decrease in Env on the cell surface previously reported (O’Reilly and Roth, 2003a).

Fig. 7 shows a molecular model representing possible structural changes in the TM protein due to the G541R mutation. In HTLV-1 gp21, the Gly–Gly dipeptide forms a hinge fold-back region, allowing the C-terminal residues to pack tightly with the residues in the N-terminal helices (Maerz et al., 2000). Molecular modeling of G541R based on the known ecotropic MuLV TM55 protein indicates the regions flanking the Arg-Gly dipeptide are in an altered conformation. This is in agreement with the Lys-C digestion, indicating the altered conformation may render the mutant more susceptible to Lys-C digestion. The alteration in the structure near the hinge also affects the potential to maintain the disulfide bonding between the SU and TM proteins. The model shows the orientation of the two disulfide bonded cysteine residues is altered and there is an increase in the S-S bond distance. In addition, differences within the cysteine-bonded loop may negatively affect the ability of the monomers to pack tightly into a trimer. Weaker packing interactions, leading to a more solvent exposed conformation, would explain the decreased thermostability as well as the increased susceptibility to protease digestion.

A decrease in the cell surface concentration of Env protein along with the decreased structural integrity reported herein may account for the observed reduction in syncytia formation while at the same time allowing for viral titers to remain within wild-type levels. Mutations in the HIV-1 gp41 Trp–Gly motif, amino acid substitutions in the amino terminus of gp41, or proline substitutions in either of the two conserved glycines in the Gly–Gly motif result in a similar phenotype (Kowalski et al., 1991; Maerz et al., 2000, 2001). It has been suggested that a greater quantity of Env on the surface is required for cell–cell fusion than is required for virus–cell fusion (Bachrach et al., 2000; Cao et al., 1994; Kowalski et al., 1991). A decrease in fusogenic potential along with a decreased surface expression of TM harboring the G541R mutation could thus provide a means to balance cytopathogenicity with productive viral infection.
Materials and methods

Production and purification of TM peptides

The DNA sequence encoding amino acids T503–D556 was PCR amplified from wild-type and G541R proviral DNA using a forward primer introducing a 5′ NdeI site (5′-GAATTCCTATGACAGACCTCAACGAAGTC-3′) and a reverse primer encoding a 3′ BamHI site (5′-CGGGATTCCGTCAGTCTGCATAAAAAACAAACA-3′). PCR products were then cloned into a pET 11d vector (Studier et al., 1990) and a 6HisT-pET11 vector (Hoffmann and Roeder, 1991). CATAAAAACAACA-3′ TATG

cracking buffer (10 mM Na2HPO4 – NaH2PO4 pH 8.0, 4.0 M urea, 6.7 mM iodoacetamide, with and without SDS, boiled for 10 min, and stored at –20 °C prior to SDS–PAGE analysis.

A pET 11d vector (Studier et al., 1990) and a 6HisT-pET11 vector (Hoffmann and Roeder, 1991) were used to express WT and G541R TM peptides (TM55) were grown to a final concentration of 2% PAGE analysis.

Western blots

Monoclonal anti-polyhistidine (Sigma Cat. # H1029) was used at a dilution of 1/3000. Monoclonal anti-TM 42–114 (Pinter et al., 1982) was used at a dilution of 1/500 and polyclonal anti-G541R was used at a dilution of 1/15,000. Anti-G541R rabbit polyclonal antibody was generated by Cocalico Biologicals, Inc. using as antigen G541R TM 55 purified from a soluble E. coli lysate on NiNTA agarose resin via the N-terminal histidine tag under native conditions. Anti-G541R reacts with both WT and G541R TM 55 peptide. Secondary antibody used in monoclonal antibody experiments was a 1/1000 dilution of anti-mouse IgG biotin conjugate (Sigma Cat. # B7264). Secondary antibody used in polyclonal anti-G541R experiments was anti-rabbit IgG biotin conjugate (Sigma Cat. # B7389). Visualization with alkaline phosphatase was achieved using the Vectastain™ ABC kit (Vector Laboratories Cat. # AK-5000).

Circular dichroism spectroscopy

Thermal denaturation/renaturation experiments were carried out on an Aviv Spectropolarimeter Model 215 at the UMDNJ Circular Dichroism Facility. Wild-type (0.2 mg/mL) and G541R mutant (0.3 mg/mL) TM 55 proteins were analyzed in both HEPES buffer (50 mM HEPES pH 7.4, 400 mM NaCl, 1 mM DTT, 5% glycerol) and phosphate buffer (200 mM K2HPO4 – KH2PO4 pH 8.0, 400 mM KCl). Thermal denaturation experiments were performed from 10 °C to 80 °C. The CD signal was measured in steps of 0.2 °C, equilibration time 0.2 min, averaging time 5.00 s. Thermal renaturation experiments were carried out as described above starting at 80 °C and ending at 22 °C. The Tm for each protein was determined from the peak of the first derivative of the ellipticity vs. temperature curve.

FPLC gel filtration

Gel filtration was performed on an AKTA FPLC (Amersham Pharmacia Biotech) using a Superdex 75 HR10/30 column. 250 μL of a 4 mg/mL protein solution was injected into a 500-μL sample loop following the partial fill method. The flow rate was set at 0.500 ml/min and samples were collected in 500 μL fractions. Samples were run in 200 mM K2HPO4 – KH2PO4 pH 8.0, 400 mM KCl, 1 mM DTT. The molecular weight standards used for size determination consisted of a 250-μL solution containing 2 mg/mL blue dextran, 1 mg/mL bovine serum albumin, 2.4 mg/mL ovalbumin, 0.6 mg/mL carbonic anhydrase, 0.64 mg/mL cytochrome c, and 2 mg/mL aprotinin. A linear relationship between elution volume and molecular weight was generated by plotting Kav1/3 vs. Molecular weight0.555. Kav = (Vc – V0)/(Vf – V0), where Vc = elution volume of protein, V0 = void volume of column, and Vf = bed volume of column.

Mass spectrometry

Ten micrograms of wild-type and G541R mutant TM 55 proteins was prepared for mass spectrometry by first adding 8 M

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urea and 20 mM methylamine. Samples were incubated either with 10 mM DTT to reduce disulfide bonds, or without DTT as a control for 30 min at 60 °C. Twenty millimolar iodoacetamide was then added to each sample; and the samples were placed in the dark for 1 h at room temperature to alkylate the sulfhydryl groups. An equal volume of (NH₄)HCO₃ was then added to the sample prior to Lys-C digestion (1:100 protease: protein, at 37 °C overnight.) After digestion, approximately 0.5 pmol of sample was treated with ziptipC18 (Millipore, Billerica, MA) and eluted with 1 μl of 2.5 mg/ml alpha-cyano-4-hydroxynamic acid in 0.1% TFA, 50% acetonitrile directly on an opti-TOF 384 plate (Applied Biosystem/Sciex).

MALDI-TOF TOF MS analysis was performed using a 4800 TOF/TOF analyzer (Applied Biosystems/MDS Sciex). MS was collected using positive reflectron mode, mass range 800 amu to 4000 amu. Each spectrum is an average of 500 laser shots with fixed intensity. Calmix1 (des-Arg-Bradykinin, anigotensin1, Glu-Fibrinopeptide B, ACTH (Clip 1–17), ACTH (Clip 18–29), ACTH (Clip 7–38), Applied Biosystem) was used for external calibration. MS/MS was performed with collision gas on, 1000 μl of 2.5 mg/ml alpha-cyano-4-hydrocinnamic acid in 0.1% TFA, 50% acetonitrile directly on an opti-TOF 384 plate (Applied Biosystem/Sciex).

G541R modeling

The 1mof crystal structure (Fass et al., 1996) from D46 to D98 was input into the program O. Modifications comprised the addition of Q44 and T45 at the N-terminus, mutation of G83R (G541R), and substitution of the 4070A sequence at residue A94C. The φ/ψ angles of G541R were adjusted to reside in the allowed loop region of a Ramachandran plot. K81 and K90 were placed with idealized side chains, in their statistically most probable conformations. This model was then modestly minimized, using only torsion angle dynamics. A trimer model was then created via superpositioning onto the known crystallographic model of 1mof (using symmetry elements 1, 4, and 7). The trimer model was subjected to full gradient minimization in vacuo using the Amber 8 Suite of programs (Case et al., 2005). The final model remained as a symmetric trimer, with one monomer rendered in the figure.

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