Long Range Communication in the Envelope Protein Domain III and Its Effect on the Resistance of West Nile Virus to Antibody-mediated Neutralization

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The envelope protein domain III (ED3) of West Nile virus is the major virus-specific neutralization domain and harbors most of the critical mutations that induce resistance against antibody-mediated neutralization. We investigated the molecular mechanisms of neutralization resistance by studying the biophysical perturbations of monoclonal antibody (mAb)-resistant mutations on ED3 wild type. Our results showed that although the solution structure between ED3 wild type and mutants was preserved, the mutations that confer the highest degree of resistance to mAbs showed low protein stability and high local dynamic motions. Interestingly, the latter was observed in regions outside the mutation sites, indicating long range communications within ED3. Thus, we hypothesized that the mechanisms involved in resistance to mAb neutralization may include, in addition to mutations in the epitope, long range effects among distant structural elements. This hypothesis is consistent with reported mutations in other flaviviruses whose surfaces are not exposed for the interaction with other macromolecules, yet they confer mAb neutralization resistance.

The genus Flavivirus of the family Flaviviridae consists of a number of pathogens of major public health importance that cause diseases, including yellow fever, Japanese encephalitis, tick-borne encephalitis, dengue, and, most recently, in the Americas, West Nile virus (WNV) (1). Since it was first isolated in 1999, the number of human cases reporting West Nile viral encephalitis has increased dramatically. Currently there is no human vaccine to prevent WNV infections. All studies to date indicate that neutralizing antibodies are the major mechanism of protective immunity against flaviviruses (2), as demonstrated by the effectiveness of vaccines to prevent other flavivirus diseases (yellow fever, Japanese encephalitis, and tick-borne encephalitis). The primary target of virus-neutralizing antibodies is the envelope (E) protein (3). The E protein is the major structural protein on the surface of flaviviruses and consists of three distinct structural domains (ED1–3). ED1 and ED2 are the central and dimerization domains, respectively, and ED3 (amino acids 295–395 in the E protein) is the putative receptor-binding domain (4, 5). Consistent with the functional role of ED3 is its orientation in the context of the intact virion. The ED3 of WNV clearly projects above the rest of the E protein facilitating the potential interaction with other macromolecules, such as antibodies or cell receptors (6).

Although all three domains in the E protein contain immunogenic sites, suggesting that there is not a single defined antigenic site, most of the experimental evidence strongly indicates that ED3 is the major immunogenic domain for virus type-specific neutralizing monoclonal antibodies (mAbs) (3). Many studies have shown that mAb binding to ED3 is most efficient at blocking virus attachment to cells (7–10), and recently it has been suggested that some anti-ED3 mAbs may also impair virus membrane fusion (11, 12). We have previously determined using virological techniques that residue Thr-332 in ED3 is a major neutralization site of WNV (13, 14) (Fig. 1). Subsequent to our studies, Nybakken et al. (11) also identified the same critical neutralization site in ED3 using a different panel of mAbs. In addition, they demonstrated the binding of a Fab fragment of a neutralizing anti-WNV mAb to ED3 on and around residue Thr-332. Thus, the direct interaction of a mAb with the critical neutralization site on WNV ED3 has been determined.

Although epitopes have been viewed as structural elements that exhibit higher dynamic motions compared with the rest of the protein antigen (15–19), there is much not known about the biophysical properties of epitopes. One issue that has yet to be addressed is the mechanism on the perturbation of these dynamic properties of the epitope by distant sites to generate mAb neutralization resistance in the virion. For instance, a possible mechanism is by mutations that generate a differential perturbation of the dynamic motions of the epitope, without altering the stability of the rest of the protein antigen (i.e. the mutation and its perturbation are localized only to the epitope). Conversely, but not excluding the previous mechanism, a mutation outside the epitope can exert a long range effect that results in a change in the dynamic properties of distant posi-
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FIGURE 1. NMR structure of rED3-WT (22) (Protein Data Bank code 156N) showing the distance (in Å) between residues Thr-332 (red), Lys-310 (magenta), and Trp-397 (green). Residue numbering corresponds to amino acids 296–406 of the entire E protein of WNV. Residues in yellow correspond to tyrosine residues Tyr-302, Tyr-329, and Tyr-383. The ribbon diagram was rendered using PyMOL version 0.97 (DeLano Scientific LLC, San Carlos, CA).

The identification of structural elements of the protein antigen that are important for mAb neutralization. Furthermore, is there a correlation between the changes in the dynamics of the protein induced by mAb neutralization-resistant mutations and changes in protein stability? If so, to what extent can the protein antigen be “destabilized” to evade mAb neutralization without adversely affecting the virus viability? At present there is no direct experimental information to answer these questions involved in the mechanism of mAb neutralization resistance.

To resolve these issues, we studied the effect of naturally occurring single mutations on the biophysical properties of ED3 from the North American WNV prototype strain, NY99. The single mutations K310T, T332A, and T332K were chosen because they cover a broad spectrum of degree of resistance to neutralization by a panel of WNV subtype-specific mAbs (13, 14). The substitution T332K confers full resistance of the virus to antibody-mediated neutralization, whereas K310T and T332A exhibit partial resistance. Furthermore, residue Lys-310 is located at a distant site, ~14 Å away from Thr-332 (Fig. 1). First, we demonstrated that the recombinant protein ED3 preserves the antigenic properties of the wild type protein as in the context of the intact virus. Thus, the findings about ED3 in this investigation are relevant to the in vivo behavior of WNV. From our biophysical studies we showed significant perturbations in protein local dynamics and structural stability for the mutations that generate the highest degree of resistance (i.e. T332K). Furthermore, we identified that mutations affecting the dynamic motions, and surface electrostatics are at regions localized approximately ~20Å away from the mutation sites, which provide direct experimental evidence of physical perturbations in distant structural elements or long-range communications within ED3. This observation is in agreement with other studies in flaviviruses that reported that mAb binding (or neutralization) can be perturbed by mutations in regions other than the epitope or not accessible to the solvent (14). Thus, based on our experimental evidence of long range perturbations, together with these previous observations, we hypothesize that in WNV, and potentially in other flaviviruses as well, the mechanisms of mAb neutralization resistance may not only depend on mutations of residues in direct interaction of ED3 with the mAb (epitope) but also on distant structural elements via long range effects.

MATERIALS AND METHODS

Chemicals and Buffers—2,2,2-Trichloroethanol (TCE), potassium iodide, thallium acetate, N-acetyl-l-tryptophanamide (N-AcTrpNH2), N*-acetyl-l-tyrosinamide (N-AcTyrNH2), and phenylmethanesulfonyl fluoride were from Sigma. Acrylamide was from Roche Applied Science. Quinine bisulfate was from Eastman Kodak Co. Ultrapure guanidine hydrochloride (GdnHCl) was from ICN Biochemicals. TN200 buffer was 20 mM Tris, pH 8.0 (at 20 °C), 200 mM NaCl, 1 mM EDTA and 5% glycerol (v/v). Phosphate buffer was 100 mM NaHPO4, pH 7.4, at 20 °C, 200 mM NaCl, 1 mM EDTA, and 5% glycerol (v/v). GdnHCl stock solution (7.5 M) was prepared in TN200 buffer or phosphate buffer, filtered, degassed, and corrected for pH. The GdnHCl concentration, [GdnHCl], was determined by density measurements at 20 °C, as described previously (20).

Protein Preparation—The recombinant E protein structural domain III (rED3) incorporates amino acids 296–406 of the E protein from WNV strain 385-99. The rED3 gene was harbored in a pMAL-c2x vector (New England Biolabs) and expressed as a maltose-binding protein (MBP) fusion protein (21). rED3 wild-type and single mutants were prepared identically by sequential chromatography on Bio-Rex 70 (Bio-Rad), amylose affinity resin (New England Biolabs), and Bio-Gel P-30 (Bio-Rad). All chromatographic steps were carried out at 4 °C unless stated otherwise. The purification scheme was as follows. Escherichia coli (DH5α) cells were grown at 37 °C in rich media with glucose (2 g/liter) and induced with isopropyl 1-thio-β-D-galactopyranoside (0.2 mM) at A600 nm of 0.7. The cells were resuspended in MES buffer (20 mM MES, pH 5.5, 20 mM NaCl, 1 mM EDTA, and 5% glycerol (v/v)) with 1 mM phenylmethylsulfonyl fluoride and lysed at 10,000 p.s.i. After centrifugation, the resulting supernatant was passed over Bio-Rex 70 pre-equilibrated with MES buffer, and the fusion protein was eluted out at 20 °C and stored at -70 °C.

4) The fusion protein was eluted out at room temperature with a salt gradient (0.2–1.0 M NaHPO4, pH 8.3, 5% glycerol (v/v)), followed by extensive dialysis against TN200 buffer. The dialysate was passed over amionexy resin, and the fusion protein was eluted out with a gradient of 0–0.01 M maltose in TN200 buffer, concentrated to ~10 mg/ml, and cleaved (0.1% w/w) with factor Xa (Novagen-EMD Biosciences) at room temperature for 14 h. The rED3 was separated from the MBP by size exclusion chromatography using Bio Gel P-30 pre-equilibrated with degassed TN700 buffer (same as TN200 buffer but with 700 mM NaCl). All rED3s were >95% homogeneous as judged by Coomassie Blue-stained SDS-polyacrylamide gels with a loading of ~30 μg of protein per lane. The rED3s
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correction was determined using an extinction coefficient of $e_{280 \text{ nm}} = 10,875 \text{ M}^{-1} \text{ cm}^{-1}$ based on the protein aromatic amino acid composition (22). Before using, the rED3s were dialyzed against fresh buffer and centrifuged for 20 min at 14,000 rpm, 4 °C.

Fluorescent Measurements—All steady-state fluorescent measurements were carried out at 20 °C in TN200 buffer. The excitation and emission polarizers were set at 90 and 55° (magic angle) to avoid potential artifacts because of fluorescence anisotropy of the sample. All measurements were corrected for buffer contribution to the fluorescence intensity and inner filter effects (23).

Tryptophan Fluorescence Quenching—Quenching experiments were monitored at the emission wavelength ($\lambda_{\text{em}}$) 340 nm with $\lambda_{\text{ex}} = 295$ nm. Four molecules (quenchers) were used as follows: acrylamide (neutral), iodide (negatively charged), thallium (positively charged), and TCE (hydrophobic). Because of the low solubility of thallium in the presence of chloride, we used Tris acetate buffer (20 mM Tris acetate, pH 8.0, 200 mM sodium acetate, 1 mM EDTA, and 5% glycerol (v/v)). The Job angle) to avoid potential artifacts because of fluorescence anisotropy of the sample. All measurements were corrected for buffer contribution to the fluorescence intensity and inner filter effects (23).


described by Stern and Volmer (25) as shown in Equation 1,

$$F_Q = (1 + K_{SV}[Q]) \cdot e^{V[Q]}$$

(Eq. 1)

where $F_{Q=0}$ and $F_Q$ are the corrected steady-state fluorescent intensities in the absence or presence of quencher at concentration [Q], respectively. $K_{SV}$ and $V$ are the Stern-Volmer and static quenching constants (respectively) in $\text{M}^{-1}$.

Tryptophan Fluorescence Quenching Efficiency by Iodide, Thallium, and TCE—The efficiency of a hydrophobic or ionic quencher (Q.) relative to the accessibility of the tryptophan residue, determined acrylamide quenching ($Q_a$), is defined by Equation 2.

$$\frac{\gamma_1}{\gamma_2} = \frac{K_{SV}^1}{K_{SV}^2} \cdot \frac{K_{SV}^f}{K_{SV}^g}$$

(Eq. 2)

The subscripts 1 and 2 refer to the quencher $Q_1$ (TCE, iodide, or thallium) and the reference quencher $Q_2$ (acrylamide), respectively. The superscripts b and f refer to fluorophore in the bound (i.e. in the native state of the rED3s) and free (free N-AcTmNh2 in solution) states, respectively. The quantity $\gamma_1/\gamma_2$ reflects the accessibility of the fluorophore to $Q_1$ as compared with $Q_2$ considering steric hindrances and physicochemical properties of the fluorophore surroundings (26).

Tyrosine Exposure Determination—The fractional exposure of tyrosine residues, $\alpha$, in the native state of the rED3s was calculated from the second derivative absorbance spectra as described previously (28) using the following Equation 3,

$$\alpha = \frac{(r_N - r_a)}{(r_U - r_a)}$$

(Eq. 3)

where $r_{i=\text{N,L,U}}$ is the ratio between two peak-to-peak distances in the second derivative spectra, corresponding to 287 to 283 nm and 295 to 290.5 nm. The ratios $r_{i=\text{N,L,U}}$ depend mainly on the polarity of the tyrosine environment; $r_{i=\text{N}}$ and $r_{i=\text{L}}$ correspond to the ratio in the folded and unfolded states of the protein; and $r_{i=\text{a}}$ corresponds to the ratio of the molar mixture of N-AcTmNh2 and N-AcTyrNh2, as in the studied protein, dissolved in ethylene glycol, which represents an environment similar to the complete burial of the aromatic amino acids in the interior of the protein. The absorbance spectra were recorded using a Hitachi U-3010 spectrophotometer equipped at 20 °C. Spectra were recorded in triplicate every 0.2 nm with 2 nm bandwidth over the range of 340–240 nm at 30 nm/min. Savitzky and Golay (27) smoothing was performed on the spectral data using a 30-point moving window size and fourth degree polynomial approximations to acquire the second derivative spectra.

Chemical Unfolding—Unfolding of rED3s was evaluated by measuring changes in the fluorescence intensity at $\lambda_{\text{em}} = 340$ nm with $\lambda_{\text{ex}} = 280$ or 295 nm as a function of [GdnHCl]. Fluorescence recordings were monitored with an SLM 8000C spectrophotometer at 20 °C. All samples were incubated at 4 °C for 14 h. The data were analyzed by fitting Equation 4, which describes a two-state unfolding process,

$$\Delta F_D = \frac{(a_N + m_N[D]) + (a_U + m_U[D]) \cdot e^{\frac{-m_D[\lambda - C_N]}{RT}}}{1 + e^{\frac{-m_D[\lambda - C_N]}{RT}} \cdot e^{\frac{-m_U[\lambda - C_U]}{RT}}}$$

(Eq. 4)

where $\Delta F_D$ is the change in maximum emission intensity at a given denaturant concentration, [D]; $a_N$, $m_{N,SV}$, $a_U$, and $m_{U,SV}$ are the intercepts ($a$) and slopes (m) of the base lines corresponding to the native (N) and unfolded (U) states, respectively. $C_N$ is the [GdnHCl] in the unfolding transition midpoint. $m_D$ is the slope of the linear dependence of $\Delta G$ on [GdnHCl] as described by the linear extrapolation method (29).

Thermal Unfolding—Thermal denaturation measurements were evaluated by changes in the fluorescence intensity ($\lambda_{\text{em}} = 340$ nm with $\lambda_{\text{ex}} = 280$ or 295 nm) using an LSS50B spectrophotometer (PerkinElmer Life Sciences). The temperature in the cuvette was controlled with circulating water with a precision of ±0.1 °C at 1 °C/min. To avoid temperature differences between the circulating water and the cuvette, the temperature of the protein solution was measured directly using a thermocouple. The temperature scans were performed as a function of increasing [GdnHCl] from 0.0 to 1.0 M. Samples were prepared by mixing first stock denaturant solution and phosphate buffer followed by degassing and pH correction. Then the protein was added, and the samples were incubated at 4 °C overnight for 14 h. The data were simultaneously fitted for a two-state unfolding process described by Equation 5,

$$\Delta F_{D,0} = \frac{(a_N + m_N[T + m_{N,SV}[D]) + (a_U + m_U[T + m_{U,SV}[D]) \cdot e^{\frac{-m_D[\lambda - C_N]}{RT}}}{1 + e^{\frac{-m_D[\lambda - C_N]}{RT}} \cdot e^{\frac{-m_U[\lambda - C_U]}{RT}}}$$

(Eq. 5)
where $\Delta F_{T,D}$ is the change in maximum emission intensity at a given temperature ($T$) and denaturant concentration, $[D]$; $a_{N}, m_{N,T}, m_{U,T}, a_{D}, m_{D,T}$, and $m_{U,D}$ are the intercepts ($a$) and slopes ($m$) for the temperature ($T$) and denaturant ($D$) dependences of the base lines corresponding to the native ($N$) and unfolded ($U$) states, respectively. $\Delta G_{N\leftrightarrow U}(T,D)$ is defined by Equation 6,

$$\Delta G_{N\leftrightarrow U}(T,D) = [\Delta H^0 + \Delta C_p^0(T - T_m)] - T \cdot \left[ \frac{\Delta H^0}{T_m} + \Delta C_p^0 \cdot \ln \left( \frac{T}{T_m} \right) \right] - m_D[D] \quad (\text{Eq. 6})$$

where $\Delta H^0$ and $\Delta C_p^0$ are the enthalpy and heat capacity changes for conversion of native to unfolded protein, respectively, and $T_m$ is the unfolding transition midpoint.

**CD Spectroscopy**—CD measurements were performed on an AVIV model 60 DS spectropolarimeter using published procedures (21).

**Analytical Ultracentrifugation**—Sedimentation velocity and equilibrium experiments were carried out at 60,000 and 40,000 rpm (respectively), at $20 \, ^\circ\text{C}$ in TN200 buffer. Velocity and equilibrium data were collected and analyzed as described previously (21).

**Quantum Yield Determination**—The quantum yield of the rED3s was determined by the comparative method of Parker and Rees (30) using as reference fluorophore quinine bisulfate in $0.1 \, \text{N H}_2\text{SO}_4$ (31).

**Monoclonal Antibody Binding**—Binding of WNV type-specific mAb 3A3 and 5H10 (Bioreliance Corp., Rockville, MD) to purified MBP-rED3 fusion proteins was evaluated by indirect enzyme-linked immunosorbent assay at $20 \, ^\circ\text{C}$, as described previously (14, 32). Briefly, enzyme-linked immunosorbent assay plates were coated overnight at $4 \, ^\circ\text{C}$ with $30 \, \text{ng}$ of pure MBP-rED3 in borate saline buffer, pH 9.0. The coated plates were blocked with a solution of PBS containing 3% bovine serum albumin for 1 h. The mAbs were diluted in PBS with 0.5% Tween 20 to a concentration of $10 \, \text{nm}$, which corresponds to $\sim 100$-fold greater than the previously reported $K_{d,\text{app}}$ value for rED3-WT (32). Varying amounts of this stock solution of mAb were titrated to each well. As control, an anti-MBP serum was included. All binding reactions were performed in triplicate. After a 45-min incubation time between the mAb and the fusion protein, the plates were washed three times with PBS containing Tween. Then a peroxidase-labeled anti-mouse IgG antibody was added to each well for 45 min. The plates were again washed three times, and the colorimetric reaction was started by adding 3,3',5,5'-tetramethylbenzidine substrate (Sigma) for 10 min. The reaction was stopped with 3.0 M HCl, and the absorbance values were read on a model 3550-UV plate reader (Bio-Rad) at 450 nm with reference at 595 nm. Triplicate experiments were performed and averaged for each MBP-rED3. The apparent dissociation constant ($K_{d,\text{app}}$) was obtained by fitting the data to a single-site binding isotherm using a standard package from Sigma Plot version 8.0. The reported standard deviation was derived from the data fitting (Table 1).

**RESULTS**

**Correlation between in Vitro mAb Neutralization Resistance and mAb Binding Affinity**

We first assessed the biological relevance of using the recombinant ED3s (rED3s) from wild type (WNV prototype strain USA99b) and the mutants rED3-K310T, rED3-T332A, and rED3-T332K to investigate the phenomenon of virus mAb neutralization resistance in cell assays. The effects of mutations on mAb binding affinity were assessed in the interaction between naturally occurring mutants of ED3 and two mAbs, namely mAb-3A3 and mAb-5H10. The apparent dissociation constant ($K_{d,\text{app}}$) was correlated with neutralization index, which is a measurement of reduction of the number of virus plaques (or virus infectivity titer) in plates of virus-infected cells in the presence of mAb, compared with a medium-only control (14, 32). The neutralization index quantitatively characterizes the degree of resistance conferred to the virus because of a mutation relative to wild type virus.

The binding isotherms of the rED3s are shown in Fig. 2, in which the upper and lower panels showed the results for mAbs 3A3 and 5H10, respectively. The data were fitted to a single-site
binding isotherm to obtain the apparent dissociation constant \( K_{\text{dapp}} \). The rank order in decreasing affinity for mAb 3A3 is WT > K310T > T332A > T332K. The same trend was observed for mAb-5H10, although the magnitude of the effect was lower compared with mAb-3A3, as summarized in Table 1. The binding affinity to mAbs for rED3-T332A and rED3-T332K was reduced by ~5 to >100-fold relative to rED3-WT, respectively (Table 1).

By directly comparing the \( K_{\text{dapp}} \) values from the binding isotherm and neutralization index for mAb-5H10, for rED3-WT and the single mutants, we found an inverse correlation of systematic increase in \( K_{\text{dapp}} \) for decreasing neutralization index values as shown in Table 1. For example, the mutation T332K has the lowest neutralization index of 0.2, indicating a high degree of resistance in vitro, and the highest \( K_{\text{dapp}} \) (>10 nm), indicating the weakest binding affinity. The opposite holds for rED3-WT (\( K_{\text{dapp}} \) of 0.13 ± 0.03 nm and neutralization index of 2.3). Therefore, the rED3s preserve their immunological properties as in its native environment in the intact viral particle.

These results validate the functional and biological significance of using the purified rED3s to assess the effects of single mutations on the solution biophysical properties of the rED3-WT to understand the basic mechanism(s) of mAb neutralization resistance induced by mutations. In addition, the results for rED3-K310T mutant demonstrated the impact of mutation in a distant site on mAb-ED3 interactions because residue Lys-310 is ~14 Å away from residue Thr-332 (Fig. 1), which is in contact with mAb. Furthermore, the magnitude of the effect is mAb specific e.g. the K310T mutation does not significantly affect the interaction with mAb-5H10 but exhibits a significant 2-fold decrease in \( K_{\text{dapp}} \) for mAb-3A3.

### Dynamic Motion and Communications between Distant Sites

**Tryptophan Fluorescence Quenching with Acrylamide, a Neutral Quencher**—The single tryptophan residue in WNV rED3 (Trp-397) is at least 19 Å away from sites of mutation (see Fig. 1). Thus, we used the spectroscopic response of Trp-397 to monitor long range perturbations as a consequence of mutation. We investigated the dynamic motions around Trp-397 by monitoring its collisional (or dynamical) quenching process. The dynamic fraction of the quenching process shown in Fig. 3A (open symbols) revealed a 3-fold lower Stern-Volmer quenching constant, \( K_{\text{SV}} \), for rED3-WT (5.6 ± 0.2 m\(^{-1}\)) compared with N-AcTrpNH\(_2\) (17.0 ± 0.1 m\(^{-1}\)), indicating that Trp-397 is located in a hydrophobic environment and has 30% of surface area (ASA) accessible to the solvent. The experimental ASA (ASA\(_{\text{exp}} = 32 ± 2\%\)) is in agreement with the calculated ASA (ASA\(_{\text{cal}} = 27 ± 3\%\)) of Trp-397 based on N-AcTrpNH\(_2\) as reference molecule. The error bars of \( K_{\text{SV}} \), and \( V \) were obtained from the data fitting using Equation 1.

![Table 2](image)

| WNV strain | mAb-3A3 \( (K_{\text{dapp}}) \) nM | mAb-5H10 \( (K_{\text{dapp}}) \) nM | N.I. * |
|------------|-----------------|-----------------|-------|
| rED3-WT    | USA99b          | 0.17 ± 0.05     | 0.13 ± 0.03 | 2.3  |
| rED3-K310T | AUS60           | 0.39 ± 0.01     | 1.6 ± 0.02  | 1.1  |
| rED3-T332A | ISR53           | 1.00 ± 0.16     | 0.30 ± 0.03 | 0.9  |
| rED3-T332K | MAD88/SAS8a     | >10.0           | >10.0       | 0.2  |

* Neutralization index (N.I.) is the log\(_{10}\) reduction in virus titer in the presence of mAb-5H10 compared with a culture medium only as control. Values were obtained from Ref. 14.

![Figure 3](image)

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**Tryptophan Fluorescence Quenching with Acrylamide, a Neutral Quencher**—The dynamic fraction of the quenching process shown in Fig. 3A (open symbols) revealed a 3-fold lower Stern-Volmer quenching constant, \( K_{\text{SV}} \), for rED3-WT (5.6 ± 0.2 m\(^{-1}\)) compared with N-AcTrpNH\(_2\) (17.0 ± 0.1 m\(^{-1}\)), indicating that Trp-397 is located in a hydrophobic environment and has 30% of surface area (ASA) accessible to the solvent. The experimental ASA (ASA\(_{\text{exp}} = 32 ± 2\%\)) is in agreement with the calculated ASA (ASA\(_{\text{cal}} = 27 ± 3\%\)) of Trp-397 based on N-AcTrpNH\(_2\) as reference molecule. The error bars of \( K_{\text{SV}} \), and \( V \) were obtained from the data fitting using Equation 1.

![Table 3](image)

| K\(_{\text{SV}}\) | V | ASA\(_{\text{exp}}\) | Maximum |
|----------------|---|-----------------|---------|
| N-AcTrpNH\(_2\) | 17.0 ± 0.1 | 2.2 ± 0.1 | 100 | 358 |
| rED3-WT | 5.6 ± 0.2 | 1.5 ± 0.1 | 32 ± 2 | 341 |
| rED3-K310T | 5.6 ± 0.2 | 1.2 ± 0.1 | 32 ± 2 | 341 |
| rED3-T332A | 6.2 ± 0.3 | 1.2 ± 0.1 | 36 ± 2 | 344 |
| rED3-T332K | 6.8 ± 0.4 | 1.1 ± 0.2 | 40 ± 2 | 344 |

* Expressed in (%), the ASA\(_{\text{exp}}\) was determined using N-AcTrpNH\(_2\) as reference molecule.

**TABLE 3**

| K\(_{\text{SV}}\) | V | ASA\(_{\text{exp}}\) | Maximum |
|----------------|---|-----------------|---------|
| N-AcTrpNH\(_2\) | 10.6 ± 0.1 | 20.7 ± 0.7 | 15.6 ± 0.3 | 1.0 | 1.0 | 1.0 |
| rED3-WT | 3.1 ± 0.3 | 20.9 ± 0.7 | 6.3 ± 0.2 | 0.9 | 3.0 | 1.2 |
| rED3-K310T | 1.8 ± 0.3 | 24.5 ± 0.3 | 6.0 ± 0.1 | 0.5 | 3.6 | 1.2 |
| rED3-T332A | 5.0 ± 0.2 | 20.9 ± 0.5 | 7.1 ± 0.3 | 1.3 | 2.8 | 1.2 |
| rED3-T332K | 1.5 ± 0.1 | 23.3 ± 0.3 | 5.7 ± 0.1 | 0.5 | 2.8 | 0.9 |

ASA (33) (ASA\(_{\text{cal}} = 27 ± 3\%\)) of Trp-397 based on the rED3-WT NMR structure (32) (Table 2).

The Stern-Volmer plots for rED3-WT, rED3-K310T, rED3-T332A, and rED3-T332K are shown in Fig. 3B. The values of \( K_{\text{SV}} \) for rED3-K310T (5.6 ± 0.2 m\(^{-1}\)) and rED3-WT are the same within experimental error; however, the \( K_{\text{SV}} \) for rED3-T332A and rED3-T332K were 6.2 ± 0.3 and 6.8 ± 0.4 m\(^{-1}\), respectively. These results indicate that mutations at position Thr-332 (rED3-T332A/K) lead to greater tryptophan ASA\(_{\text{exp}}\), whereas rED3-K310T does not (Table 2).

**Tryptophan Fluorescence Quenching with Iodide, Thallium, and TCE**—Ionic and Hydrophobic Quenchers—Having revealed that mutations of different residues can differentially perturb the dynamic environment of Trp-397, we probed the physicochemical nature of these perturbations. Tryptophan fluorescence quenching with TCE was used to investigate the hydrophobicity of the Trp-397 environment (34) (Table 3). The data are expressed as \( Q_{\text{TCE}}/Q_{\text{acrylamide}}\). A value of >1 or <1 indicates...
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A more hydrophobic or hydrophilic environment, respectively. The quenching efficiency of TCE relative to acrylamide was identical for rED3-K310T, rED3-T332A, and rED3-WT (\(\gamma_{\text{TCE}}/\gamma_{\text{acrylamide}} = 1.2\)) whereas it was 0.9 for rED3-T332K. The lower value of \(\gamma_{\text{TCE}}/\gamma_{\text{acrylamide}}\) is indicative of the environment of Trp-397 in rED3-T332K is more hydrophilic than an rED3-T332A mutation (Table 3).

The results of the series of studies using the negatively and positively charged quenchers (iodide and thallium, respectively) further showed a distinct pattern of perturbation of the environment of Trp-397 (Table 3). The data for rED3-K310T showed \(\gamma_{\text{iodide}}/\gamma_{\text{acrylamide}} = 0.5\) and \(\gamma_{\text{thallium}}/\gamma_{\text{acrylamide}} = 3.6\). These results indicate that Trp-397 in rED3-K310T has a higher negatively charged environment relative to rED3-WT. A similar conclusion is derived for rED3-T332K (\(\gamma_{\text{iodide}}/\gamma_{\text{acrylamide}} = 0.5\) and \(\gamma_{\text{thallium}}/\gamma_{\text{acrylamide}} = 2.8\)). However, the opposite conclusion is evident for rED3-T332A (\(\gamma_{\text{iodide}}/\gamma_{\text{acrylamide}} = 1.3\) and \(\gamma_{\text{thallium}}/\gamma_{\text{acrylamide}} = 2.8\)). Given that residues 332 and 310 are located 31 and 19 Å, respectively, away from Trp-397 (Fig. 1), the ionic nature of perturbation is transmitted through a long distance.

Tyrosine-accessible Surface Area to the Solvent—To complement the studies on the tryptophan at residue 397, the effect of mutations on the degree of exposure to solvent of the three tyrosine residues in rED3 at positions 302, 329, and 383 (Fig. 1) was investigated by UV absorption spectroscopy (35, 36). The UV absorbance spectra displayed a blue shift in the maximum absorption band from the native to the unfolded state (Fig. 4A). The peak shift for rED3-WT, rED3-K310T, and rED3-T332K between the native and unfolded states was 2.80 ± 0.65, 2.10 ± 0.37, and 2.60 ± 0.16 nm, respectively. In the case of rED3-T332A, the peak distance was shorter with a value of 1.30 ± 0.61 nm (Table 4). Because the change in the maximum absorption band is proportional to the degree of change of solvent exposure of the tyrosine residues, then the shortest peak shift observed for rED3-T332A suggests that the three tyrosine residues are more exposed to the solvent in its native state as compared with the other rED3s. This conclusion is consistent with results determined by second derivative UV absorbance spectroscopy (Fig. 4B). This method allows the selective investigation of the fractional solvent exposure of tyrosine residues (defined as \(\alpha\)) in a protein in the native conformation (27). In the case of rED3-T332A, \(\alpha\) was the highest with a value of 0.31 ± 0.04, corroborating that these tyrosine residues were more exposed to the solvent in the native state. There was no significant difference in \(\alpha\) between rED3-WT, rED3-K310T, and rED3-T332K (Table 4).

Protein Structural Stability

Chemical and Thermal Denaturation—We probed the effects of mutation on the structural stability of ED3 by performing unfolding experiments. Unfolding of the rED3s was monitored by changes in the emission intensity of Trp-397 with excitation wavelength (\(\lambda_{\text{ex}}\)) 295 nm, and in coordination with the tyrosine residues (\(\lambda_{\text{ex}} = 280\) nm). Within experimental error, the thermodynamic parameters obtained for all rED3s were independent of the excitation wavelength.

Mutations in rED3s lowered the stability, as indicated by a shift toward lower values of \(C_{1/2}\), the [GdnHCl] at the midpoint of the unfolding transition (Fig. 5A). The \(C_{1/2}\) values for rED3-WT, rED3-K310T, rED3-T332A, and rED3-T332K were 1.45, 1.23, 1.34, and 1.16 M, respectively. The lowest \(\Delta G^{\text{H}_{2}O} - \Delta G\) of unfolding, observed for rED3-T332A, was 3.9 kcal mol\(^{-1}\) compared with rED3-WT (4.6 kcal mol\(^{-1}\)). Higher \(m_{f}\) values were observed for rED3-T332A (23 kcal mol\(^{-1}\) K\(^{-1}\)), rED3-K310T (18 kcal mol\(^{-1}\) K\(^{-1}\)), and rED3-T332K (16 kcal mol\(^{-1}\) K\(^{-1}\)) as a function of [GdnHCl] (\(\lambda_{\text{ex}} = 340\) nm with \(\lambda_{\text{em}} = 280\) nm). The protein concentration was 18 μM. The solid lines are the best fit to Equation 4.8, temperature-induced unfolding of ED3-WT (1 M) as a function of [GdnHCl] (\(\lambda_{\text{ex}} = 340\) nm, \(\lambda_{\text{em}} = 280\) nm). The [GdnHCl] values were 0.0 M (○), 0.3 M (□), 0.5 M (△), 0.8 M (▲), and 1.0 M (▲). The solid lines are the best fit to Equation 5. Both chemical and thermal unfolding experiments were performed twice and averaged. The error bars in this figure and Table V correspond to the standard deviation of the fitting procedure.

### Table 4

| Residue     | Fractional Exposure (\(\alpha\)) | ASA<sub>\text{solvent}</sub> (%) | \(d_{\text{ex}-\text{eq}}\) nm |
|-------------|----------------------------------|----------------------------------|-------------------------------|
| rED3-WT     | 1.05                             | 0.22 ± 0.04 (0.7)               | 23 ± 7 (0.69)                 |
| rED3-K310T  | 1.00                             | 0.17 ± 0.03 (0.5)               | 2.10 ± 0.37                   |
| rED3-T332A  | 1.25                             | 0.31 ± 0.04 (1.0)               | 1.30 ± 0.61                   |
| rED3-T332K  | 0.98                             | 0.17 ± 0.04 (0.5)               | 2.60 ± 0.16                   |

- Data were determined by using Equation 3.
- \(d_{\text{ex}-\text{eq}}\) is the peak distance between the native and unfolded states of the rED3s in the UV absorption spectra.
- Based on \(\alpha\), the number in parentheses corresponds to the average number of tyrosine residues exposed to the solvent in the rED3.
- The number in parentheses is the summation of the three tyrosine residues fractional ASA<sub>\text{solvent}</sub> (see Ref. 33) in the rED3-WT: Tyr-302 (0.48), Tyr-329 (0.0), and Tyr-383 (0.21).
observed for rED3-K310T and rED3-T332K, implying that the cooperativity of unfolding is higher than that of rED3-T332A and rED3-WT (Table 5).

The thermal unfolding of all rED3s was reversible as judged by the complete recovery of the spectral properties upon refolding (supplemental Fig. 1). Global analysis of temperature scans as a function of [GdnHCl] allowed us to extract all the thermodynamic parameters that characterize the thermally induced unfolding process (Fig. 5B). Despite the small, but significant, differences in the stability among these rED3s, all single mutants affected the different thermodynamic components that are embedded in \( \Delta G_{N-U} \) (Table 5). The rED3-T332A exhibited a lower \( \Delta H^0 \) value by ~5 kcal mol\(^{-1}\), whereas \( \Delta C_P^0 \) and \( m_P \) values remain essentially identical to rED3-WT. However, the rED3-K310T and rED3-T332K mutations led to an increase in both \( \Delta C_P^0 \) and \( m_P \) values, whereas \( \Delta H^0 \) value remains similar to that of rED3-WT. No significant differences in \( \Delta S^0 \) value were observed between the rED3s. These results indicate that these single-site mutations exert a change in protein stability; however, the source of change is dependent on the specific residue and the nature of mutation.

**rED3 Solution Structure**

**CD Spectroscopy**—The secondary structure of rED3 was monitored by CD spectroscopy. All rED3s have similar negative ellipticity at 216–217 nm, which is characteristic of \( \beta \)-strand structure (37) and is in agreement with the NMR structure of WNV rED3-WT (22). However, each rED3 exhibited a different spectrum at 205–207 nm that corresponds to \( \beta \)-turn structures (37, 38) (Fig. 6). Quantitative analysis of the CD spectra by curve fitting confirms that mutations at residue Thr-332 perturb the local environment of \( \beta \)-turn structures (Table 6). These differences and the ascribed cause of these differences are consistent with the fact that residue 332 is spatially located in a \( \beta \)-turn between strands 2 and 3 in ED3. Residue Lys-310 is located on \( \beta \)-strand 1, and mutation of this residue does not perturb the \( \beta \)-turn environment as shown in the CD spectra (at 205 nm) and fractional content (Table 6).

**Hydrodynamic Properties**—None of the single mutants affected the hydrodynamic size and shape of the rED3s. The weight average sedimentation velocity coefficients, \((s_{w,av})\), were 1.15, 1.09, 1.00, and 1.02 for rED3-WT, rED3-K310T, rED3-T332A, and rED3-T332K, respectively. Thus, the proteins remained as a monomer with a very similar, if not identical, hydrodynamic structure.
of Trp-397 (higher $K_{SV}$), i.e. a consistent result showing long range communication within the protein antigen.

Having characterized the environment of Trp-397, the local environments of the three tyrosine residues 302, 329, and 383 were assessed by estimating the contribution of these residues to the total protein fluorescence. We observed that the emission intensity of the tyrosine residues in all rED3s in the unfolded state was greater than in the native state (supplemental Fig. 2A), indicating that the spatial locations of at least some of the tyrosine residues are close enough to Trp-397 for fluorescence energy transfer, and this process is disrupted upon denaturation. We corroborated this interpretation with the observed higher quantum yield of the tyrosine residues (39) in the unfolded state ($\phi_{U,Tyr}$) as compared with the native conformation ($\phi_{N,Tyr}$) for all rED3s. The higher $\phi_{U,Tyr}$ implies the elimination of the quenching process upon destruction of the native architecture around the tyrosine residues. Only rED3-T332A showed a significant decrease (~30%) in $\phi_{N,Tyr}$ as compared with rED3-WT (supplemental Table 1). Results from this study imply that a T332A mutation leads to perturbation of the average local environments surrounding the aromatic residues. This phenomenon can happen only if there is communication between distant sites.

DISCUSSION

Like all flaviviruses studied to date, WNV has a critical neutralization site on the surface of ED3 where interaction of antibodies results in neutralization of the virus. Previous virologic and structural studies have identified the residues in WNV ED3 that constitute the points of contact of antibody with the antigenic site (11, 13). In this study we have compared the biophysical properties of wild-type WNV rED3 to three mutant rED3s that each has a single amino acid mutation at a residue involved in the neutralization site (K310T, T332K, and T332A).

Escape Mutations Preserve the Solution Properties of rED3-WT—None of the single mutations significantly affected the protein fold relative to rED3-WT, as determined by CD spectroscopy. The tertiary solution structure around the Trp-397, assessed by fluorescence spectroscopy, showed that the local environment of Trp-397 in all rED3s is highly structured (supplemental Fig. 2B). In addition, the hydrodynamic size and shape of these proteins were essentially identical. Altogether, the measurements of the solution structure of these proteins suggest that the rED3 harboring the single mutations are not escaping from mAb by generating large structural or conformational changes. This observation is consistent with the putative role of ED3 as the primary receptor binding domain, which presumably places significant constraints on the range of mutations that can be tolerated in this domain without adversely affecting virus viability.

Escape Mutations Affect Structural Stability of rED3s—Although the solution properties of rED3s studied here were very similar, there were differences in their structural stability (Table 5) consistent among chemical and thermal denaturation, indicating that the thermodynamic parameters obtained in this study represent intrinsic properties of the rED3s.

There were no significant differences in $\Delta H^\circ$ between rED3-WT, rED3-K310T, and rED3-T332K. The average $\Delta H^\circ$ for these proteins was 73.7 ± 1.5 kcal-mol$^{-1}$. However, the single mutant rED3-T332A had a lower value of ~67.1 ± 1.1 kcal-mol$^{-1}$, which can possibly be attributed to changes in the protein hydration (i.e. changes in van der Waals interactions or hydrogen bonding) (40), because of the hydrophobic nature of the substitution on a solvent-exposed residue (98% ASA) on the surface of rED3 (Fig. 1). Additional evidence showing that rED3-T332A subtly affects the solution structure in the local area of the mutation site was observed in the higher calculated $\beta$-turn content (30% for rED3-T332A and ~18% for the other rED3s) and the 30% reduction of the tryptophan and tyrosine quantum yields ($\phi_{N,Trp}$ and $\phi_{N,Tyr}$, respectively) in the native state (Table 6 and supplemental Table 1).

The most significant variation among the thermodynamic parameters was the heat capacity change of unfolding ($\Delta C_p^\circ$). The rED3-K310T and rED3-T332K had higher $\Delta C_p^\circ$ values than rED3-WT and rED3-T332A. Differences in $\Delta C_p^\circ$ are associated either with changes in the solvent-accessible hydrophobic surface area that is exposed upon unfolding (41) ( ÄASA$_{N\rightarrow U}$) or changes in the surface electrostatics (40), or both. Changes in ÄASA$_{N\rightarrow U}$ are directly correlated to changes in the $m_p$ value (42). The $m_p$ values for rED3-K310T and rED3-T332K are higher than those of rED3-WT and rED3-T332A. These observations indicate that mutations K310T and T332K generate subtle changes in the native conformation of the protein. Moreover, because the mutation sites for rED3-K310T and rED3-T332K are located on the surface of rED3 (Fig. 1), it is expected that these substitutions will perturb the surface electrostatics of rED3-WT. Direct evidence for changes of the surface electrostatics was observed in the reduced iodide quenching efficiency of ~50% for these two mutants in comparison with the rED3-WT (Table 3).

Long Range Communication between Distant Sites of rED3s—We observed a redistribution of charge density on the surface of rED3 as indicated by differences in $K_{SV}$ and quenching efficiency for iodide and thallium induced by single-site mutations. These results are more remarkable considering that the changes are sensed by Trp-397, which is 19 and 31 Å away from the mutation sites (Fig. 1). Thus, these is strong evidence of long range communication between distant sites in rED3.

The results from the tryptophan fluorescence quenching with acrylamide provided additional evidence of long range communication within rED3. Significant differences in $K_{SV}$ between rED3-WT and rED3 mutants were observed indicating that the Trp-397 is more accessible to the solvent for rED3-T332A and rED3-T332K compared with rED3-K310T (Table 2). Thus, the mutations are affecting not only the surface electrostatics around Trp-397 but also the structural dynamics of that region in the folded state, with rED3-T332K being the most dynamic.

Linear Correlation between mAb Neutralization Resistance, Stability, and Dynamics of rED3s—In this study, we obtained a linear correlation between the stability and dynamics of rED3 (the latter was monitored by changes in the dynamics of the tryptophan residue and surrounding areas; see Fig. 1) and binding affinity to mAb expressed as $-\log(K_{d,app})$ shown in Fig. 7. There is an inverse relationship between protein stability and dynamics, namely the less stable the protein, the more dynamic
Mechanism of Resistance to Antibody-mediated Neutralization

Concluding Remarks—The results derived from these studies most probably reflect the intrinsic solution properties of the native ED3. Although we have investigated rED3 as an isolated domain and not in the context of the entire E protein or in the context of the intact virion, it has been observed that the Ig-like fold of the rED3 from WNV (32) is preserved as in the whole E protein ectodomain (4, 5). For example, the root mean square deviation between backbone atoms of the WNV rED3 NMR structure and WNV E protein x-ray structure (5) is 1.1 Å, despite the fact that the latter structure was determined as part of the complete ectodomain of the E protein and by x-ray crystallography. Furthermore, the agreement between the degree of virus neutralization (using the intact viral particle harboring the mutations studied in this report) (13, 14) and our results on mAb binding using the rED3s (Table 1) indicates that the rED3, as an isolated domain, has very similar antigenic properties as compared with the ED3 in the context of the intact virion.

The molecular mechanism of mAb neutralization resistance of flaviviruses, including WNV, has been elusive despite the intense investigation. However, for the first time, as a result of this study, a quantitative correlation between the biological phenomenon of antibody-mediated neutralization resistance and the physical properties of the domain ED3 is established as follows. 1) The substitution that conferred the highest degree of mAb neutralization resistance to the virus possesses the least stable and most dynamic ED3 (Fig. 7). 2) The viability of the virus is not affected even though there is a decrease of 1 kcal/mol in stability of ED3, which corresponds to the maximum difference in stability between rED3-T332K (the most resistant mutation) and rED3-WT. 3) These mutations elicit physical perturbations at distant sites that are spatially located many angstroms away, i.e. long range communications between distant residues in ED3 (Fig. 1).

These experimental results are evidence in support of our hypothesis that the mechanism of resistance to mAb neutralization in WNV involves not only mutations in regions of direct interaction with the antibody (epitope) but also mutations in distant residues of the protein via long range communication. In previous studies in other flaviviruses, several investigators observed that escape from mAb neutralization occurred (in cell assays) because of a mutation, but without loss of the specific mAb binding (14, 43–45). This observation could indicate that mutations (or perturbations) in other structural elements besides the epitope are able to generate mAb neutralization resistance in vivo. Thus, our hypothesis provides a rationale for the role of long range interactions in mAb neutralization resistance.

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