Single point mutation induced alterations in the equilibrium structural transitions on the folding landscape of HIV-1 protease

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Equilibrium folding–unfolding transitions are hard to study in HIV-1 protease (PR) because of its autolytic properties. Further, the protease exhibits many tolerant point mutations some of which also impart drug resistance to the protein. It is conceivable that the mutations affect protein’s function by altering its folding characteristics; these would clearly depend on the nature of the mutations themselves. In this background, we report here NMR studies on the effects of D25N mutation, which removes one negative charge from the protein at the active site, on the equilibrium folding behaviour of PR starting from its acetic acid denatured state. It is observed that in PR\textsubscript{D25N} two slowly exchanging conformations are present at the N-terminal. One of them is similar to that of PR. Though the conformational and dynamics preferences of PR and PR\textsubscript{D25N} are fairly similar in 9 M acetic acid, they seem to undergo different folding transitions when acetic acid concentration is reduced. The differences are seen in the active site, in the flap, and in the hinge of the flap regions. The present study suggests that such differences, though different in detail, would occur for other mutations as well, and also for different initial denatured states. These would have significant regulatory implications for the efficacy of protease function.

Keywords: HIV-1 protease; acid denatured state; equilibrium folding transitions; nuclear magnetic resonance; active site mutation; secondary structural changes

Introduction

HIV-1 protease is one of the proteins inside the HIV virus, and in the virus life cycle it catalyzes the cleavage of the polyprotein, Gag-Pol, to produce its own copy and various other functional proteins required for the virus (Kay & Dunn, 1990; Skalka, 1989); Gag-Pol itself is coded by the viral genome. This process is very crucial for the virus maturation and propagation (Kaplan et al., 1993; Kaplan, Manchester, & Swanstrom, 1994; Karacostas, Wolfe, Nagashima, Gonda, & Moss, 1993; Krausslich, 1991). Due to this vital role it has always been a prominent target for protease inhibitors as a anti-AIDS drugs (Erickson & Burt, 1996). Several of these inhibitors are in clinical use. However, it is observed that during repeated life cycles of the virus, mutations occur naturally in PR due to the infidelity of the reverse transcriptase (Bennett et al., 2009). These mutations occur throughout the protein sequence. Functionally tolerant mutations survive in the cell and the other mutant proteins will get degraded. Some of these tolerant mutations affect the binding efficacy of the protein to the drugs and this leads to drug resistance.

Mutations can also affect the stability and folding characteristics of the protease. For example, mutations Q7 K, L33I and L63I do not alter the overall structure, or the activity of the protein but render the protease fairly resistant to autolysis (Louis, Clore, & Gronenborn, 1999). Functionally active native protein folds in such a way as to form a homodimer, with the active site lying in a pocket at the dimer interface; the active site contains Asp-Thr-Gly (Asp25, Thr26 and Gly27) sequence which is common to aspartic proteases (Oroszlan & Luftig, 1990; Pearl & Taylor, 1987). It is known that mutation, D25 N, makes the protein inactive (Sayer, Liu, Ishima, Weber, & Louis, 2008). It was observed that when PR and PR\textsubscript{D25N} were co-expressed \textit{in vivo}, there was a decrease in protease activity (Babe, Rose, & Craik, 1995). This suggested that the inactive protease domain PR\textsubscript{D25N} is indeed capable of adopting a native-like fold and competes with PR to form the dimer; and the heterodimer would also be inactive. But the dissociation constant of the heterodimer was found to be more

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than 100-fold higher compared to that of the wild type (Sayer et al. 2008). In other words, the stability of the heterodimer or of the mutant dimer is much less than that of the wild type.

The structure of the folded monomer PR\textsubscript{D25N} containing the autolysis resistance mutations has been reported earlier (Ishima, Torchia, Lynch, Gronenborn, & Louis, 2003). The structure of PR containing the same autolysis resistance mutations, but without the active site mutation has also been reported. In either case, the secondary structural elements are the same; β-sheets: β1,12–15; β2,18–21; β3,23–24; β4,32–34; β5,43–47; β6,57–66; β7,69–78; β8,83–85 and α-helix, 87–91 (Sayer et al. 2008).

Thus in the context of structure–folding–function relationships, it would be crucial to investigate the effects of mutations on the folding transitions, and such studies will have to start from a denatured state. Even inside a cell, a newly synthesized protein is in some kind of a denatured state, and folding starts from there; however, the nature of this state is generally not known. If the folding is thermodynamically driven, then \textit{in vitro} investigations starting from a denatured state of the equilibrium structural transitions, as folding conditions are provided by dilution of the denaturant, would provide valuable insights into the folding mechanism of the protein. In this context, we address both the issues here in a limited manner using PR containing D25N mutation in addition to the standard mutations conferring some resistance to autolysis (Q7K, L33I and L63I) and to oxidation (C67A and C95A). We observe that the single point mutation (D25N) in the active site region of the protein sequence results in substantial differences in the structural preferences and dynamics characteristics of PR in the acetic acid denatured state; incidentally, choice of acetic acid as denaturant here has no special significance, except for the fact that purification protocols of PR use acetic acid denaturation as the first step. The studies could very well be done starting from other denatured states, such as those created by urea, guanidine hydrochloride, etc. All of these would sample different structural transitions on the folding landscape of the protein.

In the present investigation, we find interesting differences in the folding transitions in PR and PR\textsubscript{D25N} which are seen even at regions very remote from the site of the mutation. The initial states in the two proteins have some differences and this may have a bearing on the folding transitions.

Materials and methods

Protein preparation

The protease constructs PR (11 kD, 99 amino acids) and PR\textsubscript{D25N} in the vector pET11a were expressed in \textit{Escherichia coli} BL21 (DE3) host cells. Protein expression and purification from inclusion bodies have been described previously (Panchal, Pillai, Hosur, & Hosur, 2000). The protein solutions were exchanged by dialyzing the diluted proteins in desired concentration of acetic acid solution. Then the diluted protein solutions were concentrated up to 1 mM using centrifugal filter units for various NMR experiments.

NMR spectroscopy

All NMR experiments were performed on Bruker Avance 800 MHz NMR spectrometer at 25 °C.

The HSQC spectrum recorded at the end of all the 3D experiments was identical to the one recorded at the beginning indicating that the protein was stable over the duration of the experiments. The 3D triple resonance experiments included: HNN, HN(C)N, HNCA, HNCO, HNCACB and CBCA(CO)NH. A 3D \textsuperscript{15}N resolved TOCSY was also recorded. Standard experimental parameters were used. The N–C\textsuperscript{α} and N–CO transfer times for HNCA, HN(CO)CA, HNN and HN(CN) were in the range 24–30 ms. The mixing time for TOCSY was 80 ms. The time domain data along the indirect \textsuperscript{15}N and CO dimensions consisted of 40 and 48 complex points, respectively. Complex (60–80) increments were used along the aliphatic carbon dimension. Along the indirect \textsuperscript{1}H dimension 80 complex increments were used for TOCSY. In all the experiments 4–16 scans per FID were collected. For the two-dimensional (2D) relaxation experiments, 2048 and 256 complex points were used along the \textit{t}_2 and \textit{t}_1 dimensions, respectively. \textsuperscript{15}N transverse relaxation rates (\textit{R}_{2}) and longitudinal relaxation rates (\textit{R}_{1}) were measured with CPMG and inversion recovery experiments, respectively.

All the spectra were processed using Felix, apodized with a sine-squared weighting function, shifted by 60° in all the dimensions for both 2D and 3D experiments. The data were Fourier transformed after zero filling to 1024, 256 and 256 points along the F3, F2 and F1 dimensions, respectively. The 2D HSQC experiments were processed with 4096, 1024 points, respectively, along the F2 and F1 dimensions. All the spectra were analyzed using CARA (Keller, 2004) and Felix. The \textit{R}_{1} and \textit{R}_{2} values were calculated by fitting the intensity values of the peaks to the equation, \textit{I}(\textit{t})= \textit{B} \text{ exp}(–\textit{R}_{1,2} \textit{t}).

Results and discussion

Backbone resonance assignment

NMR spectra of PR and PR\textsubscript{D25N} were recorded under three denaturing conditions, namely, 9, 7 and 5 M acetic acid concentrations. Figure 1 shows the \textsuperscript{1}H–\textsuperscript{15}N HSQC spectrum of PR\textsubscript{D25N} in 9 M acetic acid at temperature 25 ° C. The chemical shift dispersion in the \textsuperscript{1}H dimension is ~1 ppm, which is very small compared to that in normal...
folded proteins (~4 ppm). The same observation was made at 7 and 5 M acetic acid concentrations in both PR and PRD25N. Nearly complete backbone assignments were obtained for all the denatured conditions using the experiments HNCA, HN(CO)CA, CBCANH, CBCA(CO)NH, HNCO (Ferentz & Wagner, 2000) and HNN and HN(C)N (Panchal, Bhavesh, & Hosur, 2001). Table 1 gives a summary of the assignments and the assignments for PRD25N in 9 M acetic acid are annotated in Figure 1. During the assignment of PRD25N we observed that the stretch of residues 2–7 displayed two sets of peaks (Figure 2). Both these sets showed contiguous sequential connections. It is worthwhile to mention that residue eight is connected to residue seven of each of the above mentioned two different stretches representative of two different slowly exchanging conformations. Among these, chemical shifts of one conformer are identical to those of PR in all the denatured states.

Chemical shift perturbations due to D25N mutation

Figure 3 shows a comparison of the chemical shifts of PRD25N and PR in 9 M acetic acid. Evidently, chemical shift perturbations due to the mutation are primarily observed in two regions, one at the mutation site and the other at the N-terminal. The perturbations throughout the rest of the protein are small. The change in chemical shifts of aa-25 is larger compared to those of aa-24 and aa-26, which is a direct consequence of the D25N mutation. A closer look at Figure 3 reveals that the chemical shift difference of the alternate conformation (at residues 2–7) with respect to PR increases progressively towards the N-terminal end. This could be construed to indicate a population with a stable structure at the N-terminal, but more interestingly, this is seen far from the mutation site. Further, it becomes even more interesting considering that residues 2–5 are involved in the dimer formation in the native structure. That means, even in the denatured state, there could a tendency to form transient dimers. We noted that these spectral features were present at 7 M acetic acid as well (Figure 4).

Table 1. Number of residues in PR and PRD25N assigned at each of the acetic acid concentrations.

| Acetic acid concentration (M) | PR  | PRD25N |
|------------------------------|-----|--------|
| 9                            | 84  | 85     |
| 7                            | 81  | 84     |
| 5                            | 79  | 83     |
Earlier studies from literature show that the activity of HIV-1 protease is almost zero below pH 3.0 (Todd, Semo, & Freire, 1998). Hence any possibility of native-like dimerization or autolysis can be ruled out in all these acid denatured states where the pH is nearly 2.3. In the native dimer, several segments of the chain occur at the interface and this includes the N-terminal at the bottom β-sheet. In the denatured state, it may not be that the native type dimer is transiently formed, but portion of the contacts can be occurring in a transient manner. Such transient structures would be kinetically driven rather than by thermodynamics within the time frame of the experiments. They may or may not produce peaks depending upon the exchange rates. Thus such structures cannot be ruled out in WT protease, but it may just be that either their populations are below detection level or the exchange rates are such that additional peaks are not seen in the spectra.

Structural transitions caused by acetic acid dilution in PR and PRD25N

We investigated the structural transitions as the acetic acid concentration was progressively reduced from 9 to 5 M. At lower acid concentrations the NMR spectra of...
wild type PR displayed many extra peaks. These could be either due to multiple conformations in slow exchange or because the protein is getting slowly degraded perhaps due to some low level of autolytic activity from partially folded forms (the autolysis resistance mutations may not be providing 100% protection under these conditions). The former may be a more plausible reason as the protein is known to be inactive below pH 3. In any case, the extra peaks rendered analysis of spectra difficult and ambiguous. Thus, the present investigation reflects on early equilibrium transitions in the folding process driven by acetic acid dilution.

Figure 4 displays $^1$H–$^{15}$N HSQC spectra of the two proteins at 9, 7 and 5 M acetic acid concentrations. It can be seen that up to 5 M acetic acid both the proteins have high tendency to be unfolded. However, we also observe a few weaker intensity peaks in 7 and 5 M conditions of acetic acid, which suggest presence of some minor conformations in the ensemble.

Figure 5 shows the relative changes in peak positions at 7 and 5 M condition for both PR and PR$_{D25N}$ compared to 9 M acetic acid concentration. The chemical shift perturbations of most of the peaks in the protein are increasing with decrease in denaturant concentration,
which reflect on the overall changes in the population distribution of partially structured and unstructured species in the ensemble. In other words, the folding transitions have created some structured species in the ensemble. Amino acids in the core domain aa: 67–77 (β-7) show significant change in the chemical shifts in 7 M acetic acid concentration, which increases and becomes more prominent when the denaturant concentration is decreased to 5 M in both the proteins. This domain is mainly responsible for dimer stabilization and catalytic site formation. The hinge of the flap region (aa: 34–38) also shows major chemical shift perturbation in PR\textsubscript{D25N}. In case of PR, this region does not exhibit any distinct change when acetic acid concentration decreases from 9 to 7 M, but, on further decrease to 5 M the perturbations are quite distinct. This part of the protein plays a major role in the flap opening and closing on substrate binding to the folded protein. The terminal residues are showing enhanced chemical shift perturbations with decrease in acetic acid concentration. This indicates that these are not truly random coils even in the strong denaturing conditions. The earlier discussion about slow exchange between two conformers in the N-terminal also corroborates this result.

Secondary structural changes

Secondary chemical shifts (Wishart, Sykes, & Richards, 1992; Schwarzinger et al., 2001) (deviation of the chemical shifts from corresponding sequence corrected random coil values) provide information about the residual structural elements present in the denatured states of the protein. Typically, such perturbations in the secondary structural preferences along the length of the protein are probed using differences in the \( C_\alpha \), \( H_\alpha \) and CO chemical shifts.

Figure 5. Backbone amide chemical shift changes (in Hertz) on lowering the acetic acid concentration; data for 9–7 M and 9–5 M are shown for both PR and PR\textsubscript{D25N} separately. The square root of the sum of the squares of individual difference in \( ^1H \) (\( \Delta_\rho \)) and \( ^15N \) (\( \Delta_\nu \)) chemical shifts are plotted for each residue. Residues belonging to secondary structural elements of the folded protein are shown on the top of the panel. Average values of deviations are shown by horizontal lines in each of the panels. Arrows and cylinders represent β-strands and α-helices, respectively.
Firstly, considering the two proteins, PR and PRD25N, we calculated $\Delta H_\alpha$ as below:

$$
\Delta H_\alpha = (H_2^\alpha_{\text{obs}} - H_\alpha^{\text{ran}}) - (H_1^\alpha_{\text{obs}} - H_\alpha^{\text{ran}}) = H_2^\alpha_{\text{obs}} - H_1^\alpha_{\text{obs}}
$$

where $H_1^\alpha_{\text{obs}}$ and $H_2^\alpha_{\text{obs}}$ are the observed chemical shifts for PR and PRD25N, respectively. $H_\alpha^{\text{ran}}$ is the corresponding sequence corrected random coil shift for the particular residue. Figure 6(A) shows the $\Delta H_\alpha$ values for HIV-1 protease at various acetic acid concentrations. A cut-off of 0.1 ppm was taken to identify the residues having significant structural perturbation due to the mutation under different denaturing conditions. Typically, if a contiguous stretch of 3–4 residues shows a magnitude of $H_\alpha$ higher than the cut-off, that would be taken to indicate a structural difference.

From Figure 6(A) it is quite clear that $\Delta H_\alpha$ values are zero or nearly zero in 9 M acetic acid denatured state. Here the protein is maximally denatured, secondary structural propensities are small and thus the changes in $\Delta H_\alpha$ values are also small. As the denaturant concentration decreases to 7 M the protein backbone tends to form secondary structures, but differentially in the two proteins. This is clear from the fact that the $H_\alpha$ values increase throughout the length of the protein. There are both positive and negative changes indicating differential changes in both $\beta$ and $\alpha$ propensities, and this varies along the sequence implying that the structural perturbations due to the mutation might be different in different parts of the protein. For example, a positive deviation in any region can result from either greater $\beta$ propensity in PRD25N than in PR or from a larger $\alpha$ propensity in PR than in PRD25N or from combinations thereof (e.g. $\beta$ propensity in PRD25N and $\alpha$ propensity in PR). In 5 M acetic acid almost all the residues show negative deviations and this is uniform along the length of the polypeptide chain. Here the $\Delta H_\alpha$ values for most of the residues are larger than or close to 0.1 ppm (nearly 80 Hz in 800 MHz NMR spectrometer) while the error in measurement in $\Delta H_\alpha$ values

![Figure 6](image_url)

Figure 6. (A) $\Delta H_\alpha$ values for HIV-1 protease at various acetic acid concentrations are shown. A cut-off of 0.1 ppm was taken to show the residues having significant structural perturbation due to the mutation. Perturbation of transient secondary structure can be considered in a region if a contiguous stretch of 3–4 residues shows $\Delta H_\alpha$ values more than the cut-off. (B) Residues having $\Delta H_\alpha$ values higher than 0.1 ppm are shown (blue) on the native dimer (PDB ID 1G61). The increasing number of residues having higher $\Delta H_\alpha$ with decreasing acetic acid concentration reflects on the differential structural perturbations in PR and PRD25N. These figures are made by the software Pymol.
are closer to 0.03 ppm (24 Hz). This most likely indicates that at 5 M the patterns of residual structures in both the proteins, PR and PRD25N, are qualitatively similar but the propensities are uniformly different along the backbone in the mutant protein. This progression of differences in the structural changes in the two proteins is displayed on the native structure of the protease dimer in Figure 6(B). From the above analysis it is clear that the two proteins follow different folding transitions as we go from 9 to 5 M acetic acid, i.e. the equilibrium structures on the folding landscape are altered due to the single point mutation D25N.

A similar analysis was carried out using C′ chemical shifts and the data are included in Figure S1. We observe that, this data overall, corroborate the general conclusions derived from Hα chemical shift analysis. There is very little difference in the structural preferences in 9 M acetic acid between the two proteins. When the acid concentration is reduced to 7 M, the types of preferences change differently in the two proteins resulting in positive and negative differences at similar locations along the length of the protein, as seen with Hα. With further decrease in acetic acid concentration to 5 M, both the proteins attain similar types of secondary structural preferences, though the degrees are different which is why we see small but uniform differences across the length of the protein. We could not carry out such an analysis with Cα because of poor chemical shift dispersion.

Dynamics perturbations due to D25N mutation in the equilibrium folding transitions

Dynamics preferences in the folding transitions from 9 M acetic acid to 5 M acetic acid were characterized using amide 15N relaxation rates R1 and R2. Longitudinal relaxation rates (R1) can give information about high frequency motion (picoseconds–nanoseconds), whereas transverse relaxation rates (R2) give information on both (picoseconds–nanoseconds) timescale as well as slow conformational transitions on microsecond to millisecond timescale (Kay, Torchia, & Bax, 1989). Here we use R2 to monitor the dynamics perturbation in the intermediate denatured states.

The observed transverse relaxation rates R2 values have two contributions:

\[ R_2 = R_{2,\text{int}} + R_{\text{ex}} \]

where, \( R_{2,\text{int}} \) is the intrinsic transverse relaxation rate which reflects ps-ns timescale motions, and \( R_{\text{ex}} \) reflects exchange which occurs on μs-ms timescale. The overall pattern of R2’s for PRD25N was found to be similar to that of PR. The residue wise R2 values of both the proteins are shown in Figure 7; clearly, the magnitudes seem very similar and thus the differences, if any, must be very subtle. In general, as the denaturant concentration decreases protein starts to fold; more and more structure forming and breaking events occur. Structure forming leads to restriction of dynamic motions or

Figure 7. Residue wise deviation of 15N transverse relaxation rates (R2) of PR from those of PRD25N in the different denatured states. The individual R2 values of PR and PRD25N along with ΔR2 values for different acetic acid concentration are shown in the respective panels.
increase in slow motions. Hence the $R_2$ values should increase with decrease in acetic acid concentration from 9 to 5 M. In contrast, we observe here that the $R_2$ values are decreasing progressively as acetic acid concentration is lowered. This implies that acetic acid dilution is associated with changes in solution conditions which affect the relaxation rates. For example, the viscosity of acetic acid–water mixture increases with increase in acetic acid concentration (Mazurkiewicz & Tomasik, 1990) and this definitely alters the rotational motions which in turn affects the $R_2$ values. The current patterns of $R_2$ values at different acetic acid concentrations seem to reflect the dominance of the solvent viscosity effect rather than the protein folding effect.

In order to extract the dynamics changes in the individual proteins (PR and PR$_{D25N}$) along the folding process we obtained difference plots (where viscosity effects should cancel out) at 9, 7 and 5 M acetic acid conditions and these are shown in Figure 7. Cut-off values of 1.38, 0.85 and 0.94 s$^{-1}$ (three times of the average error in the $\Delta R_2$) were taken for 9, 7 and 5 M, respectively to identify significant perturbations. The error values for the differences can be roughly approximated to $\sqrt{((\delta R_{2_{D25N}})^2 + (\delta R_{2_{mut}})^2)}$ where $\delta R_2$ is the error in the measured $R_2$ value. If the maximum value of the two errors was considered for both the values, then the error would be $\sqrt{2} \times \delta R_2$. Here the average error in $\Delta R_2$ over all the residues was considered instead of calculating errors for the individual difference. Hence a very conservative cut-off of three times of average error in $\Delta R_2$ was taken which is well above the likely errors for the individual residues. Interestingly, the change in $R_2$ ($\Delta R_2$) shows sequence-wise variation. The stretches 26–29 (P) and 66–67 (Q) show enhanced conformational exchange at 9 M acetic acid in the mutant. The perturbation at P is perhaps not too surprising considering that it is in the neighbourhood of the D25N mutation. In 7 M the $\Delta R_2$ values are more prominent than in 9 M acetic acid condition, which are suggestive of greater differential in slowing down of motions due to the folding transitions. The stretches showing conformational exchange extend to some neighbouring residues as well. Enhanced $R_2$ values are seen in the areas 2–4, 25–36, 51–53, 67–68 and 82–84. In contrast, in 5 M acetic acid no such stretches of enhanced $\Delta R_2$ values are visible. Lower $\Delta R_2$ values in this state indicate similar dynamics in the two proteins, suggesting that the two proteins are poised to progress towards the native structure when the acetic acid concentration gets further reduced.

**Folding implications**

The fact that the D25 N mutation causes structural perturbations across the length of the polypeptide chain as seen from the propensity changes at different denaturant concentrations has an important implication in the context of characteristics of denatured states and the mechanisms of protein folding, in general. This suggests that there could be preferred topologies in the denatured ensemble through which the perturbations are transferred from the site of mutations to other parts of the protein. Some of these topologies may be native like which drive the protein towards the native structure as the acetic acid concentration is reduced. Further, the nature of the preferred topologies and the relay of perturbations from the mutation site would be influenced by the nature of the mutation itself. In the present case, the D25 N mutation removes one negative charge from the protein. This could cause local environmental changes, which will be seen differently by different solution conditions, which, in turn, can affect the folding transitions differently.

**Concluding remarks**

We have obtained here useful insights into the equilibrium folding transitions in HIV-1 protease starting from the acetic acid denatured state. With the help of NMR parameters such as chemical shift perturbation, secondary shifts and amide $1^5$N relaxation rates we characterized different denatured states of the protein in acetic acid. Residues having native-like structural preferences have been identified and these are seen to be distributed over the active site, the flap and the hinge regions. Very interestingly, though the single point mutation D25 N does not alter the protein structure, it does alter the equilibrium folding transitions in the protein. The loss of a negative charge as a result of the mutation affects the local environment and since the protein is not completely a random coil even under the strong denaturing conditions used, such a change in the local environment is seen to have a global effect on the equilibrium folding transitions driven by acetic acid dilution in the protein. It is conceivable that such effects, but different in detail, could occur due to other mutations as well. This has an important implication for the efficacy of the protease, since it is known that the protease is prone to undergo various mutations at different places along the sequence due to the infidelity of the reverse transcriptase. The perturbation of N–H chemical shifts and relaxation dynamics at remote sites from the site of the mutation suggest influence of mutations on topological preferences in the denatured states which, in turn, could dictate the folding pathways when appropriate conditions are provided. In this context, the denatured state considered here is only one particular instance and such observations could be envisaged for other environmental situations that could be occurring inside a cell. All of these would have significant implications for the efficacy of PR function.
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