Chapter 3

Analysis of the structures of N14K and N14D

As discussed before, the mutations at the 14^{th} position are necessary to aquire knowledge about the contribution of the scaffolding residue Asn14 towards the stability of the reactive-site loop. To start with, overall three dimensional structures of the mutants, N14K and N14D were compared with rWCI. The main-chain atoms of 176 amino-acid residues of N14K and N14D were superposed on rWCI with r.m.s.d values of 0.2 Å and 0.4 Å (LSQKAB, CCP4, 1994), which revealed that the mutations at this conserved position by Lys and Asp almost have no effect on the overall topology of the protein. Hence in the next step of analysis, the individual refined models of the mutants were analyzed, with a special emphasis on the mutation site and the reactive site loop, to check the local structural changes caused by the mutations.

The reactive site loops of serine protease inhibitors, belonging to Kunitz (STI) family, have a natural in-built stability. Low B factors and well defined electron densities for the reactive site loop regions of ETI (Onesti *et al.*, 1991), STI (Meester *et al.*, 1998) and WCI (Dattagupta *et al.*, 1996) support the fact. An inspection of the temperature factors (B factors) of the mutants showed that, like WCI, the B values for the reactive site loop residues are much lower compared to the residues belonging to other surface exposed loops, which is reflected in their well defined electron density maps (Fig. 3.1).

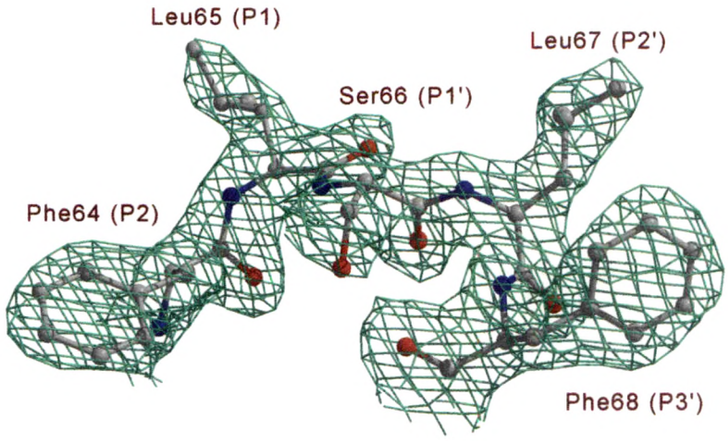


Figure 3.1.a. $2F_o$-F_c electron density map (contoured at 1.3σ), around the first reactive site loop region of N14K.

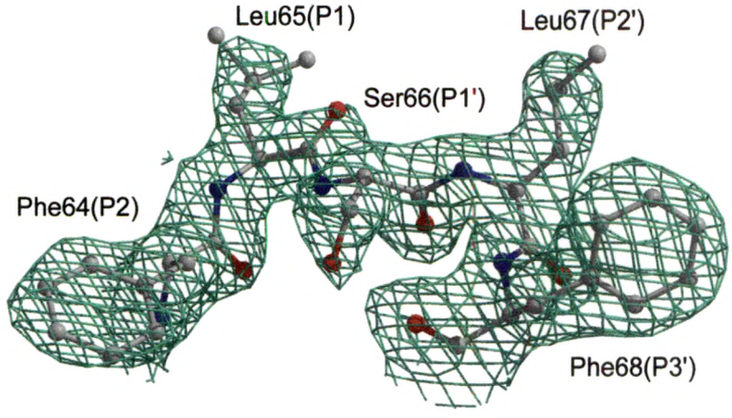


Figure 3.1.b. $2F_o$-F_c electron density map (contoured at 1.3σ), around the first reactive site loop region of N14D.

3.1. Superpositioning study and comparison of dihedral angles

The backbone atoms of the first reactive-site loop (P3-P3') of N14K and N14D were superposed (Fig. 3.2) on that of rWCI with r.m.s. deviations of 0.09 Å and 0.27 Å respectively. These low rmsd values point to the fact that, in each case, the reactive-site loop maintains more or less an identical canonical conformation. In N14K, the long side-chain of Lys14, instead of destabilizing the canonical conformation of the reactive-site loop, has itself folded back with an unusual rotamer (Fig. 3.2), whereas, in N14D, the side-chain carboxylate group of Asp14 orients in a direction perpendicular to the corresponding amide group of Asn14 in rWCI.

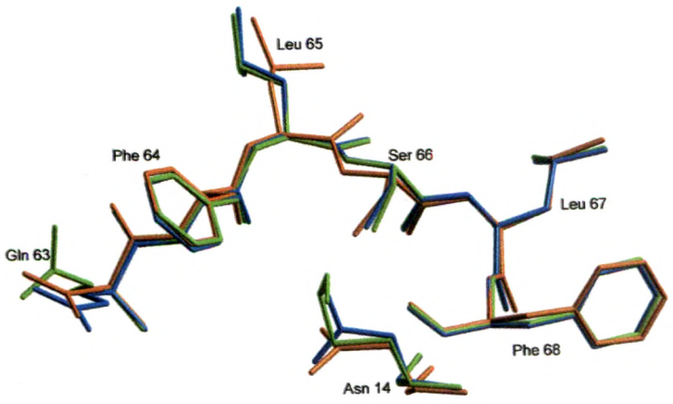


Figure 3.2 Superposition of the loop regions of N14K (green) and N14D (gold) on that of rWCI (blue)

The dihedral angles around the scissile bond of WCI and its mutants were calculated (Table 3.1) and compared with the dihedral angle values for the canonical conformations of serine protease inhibitors (Bode and Huber, 1992). According to Bode and Huber, the reactive site loops of the inhibitors exhibit a characteristic conformation, mainly from P3 to P3′. In case of Kunitz (STI) family, however, the curvature of the reactive site loop is different from other families of serine protease inhibitors. This can be shown, by measuring the distance between the C_α atoms of P4 and P3′. It is reported that the extension of the reactive site loop of STI family (P4-P3′) is among the smallest; 9 Å for STI, 9.2 Å for ETI and 8.8 Å for WCI compared to 11 3 Å for BPTI, 15 Å for MCTI of Squash family, 15 6 Å for PSTI of Kazal family and 15.9 Å for eglin-C of PI-1 family (Song & Suh, 1998). On that basis, we restricted our comparison of the dihedral angles with the canonical conformation of serine protease inhibitors, defined by Bode and Huber, mainly around the region P3-P2′. The comparison of the dihedral angles indicates that the values for N14K and N14D belong to the range of canonical conformations and the loop conformation of the mutants are almost identical to that of rWCI. Only in case of N14D, a slight flipping (of about 30°) around the scissile bond is noticed.

Table 3.1. The dihedral angles of the loop region (P3-P2′) of rWCI and the mutants, N14K and N14D

	P3 (ϕ/φ)	P2 (ϕ/φ)	P1 (ϕ/φ)	P1′ (ϕ/φ)	P2′ (ϕ/φ)
rWCI	-71.3/-17.5	-97 8/168 1	-82.4/-9.1	-55.4/158.4	-73 7/-29.2
N14K	-74.6/13.5	-90.3/172.2	-92.1/-8.9	-50.7/160.4	-76.0/-32.0
N14D	-67.4/-17.4	-99.8/163.0	-88.3/33.8	-90.2/158.7	-74.3/-22.8

3.2. Hydrogen bonding interactions in the loop region of N14K and N14D

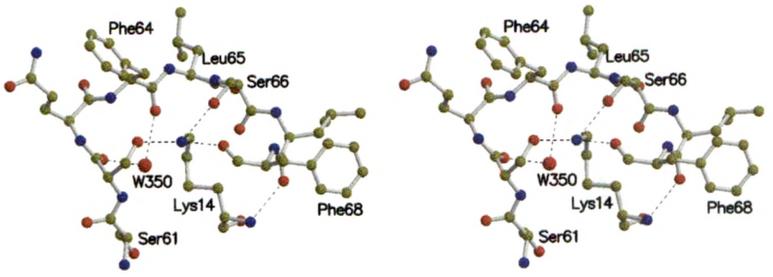
In general, the canonical conformation of the reactive site loop is maintained by an extensive system of hydrogen bonds and hydrophobic interactions, which involve residues both from the loop and the inhibitor scaffolding (Otlewski *et al.*, 1999; Iwanaga *et al.*, 1999). It has been observed that occasionally water molecules also mediate the interactions between the scaffolding and the loop residues and provide additional stability to the loop and the nature and type of such interactions vary among different classes of proteins.

The criteria for hydrogen bonds used in these structure analyses were as follows: for N-H···O bonds the maximum H···O distance was taken as 2.60Å, and minimum values for the angles N-H···O and C-O···H were 90°. For O-H···O and N-H···O bonds, for which the hydrogen positions were not assigned, the maximum distance of O···O (N) was 3 6 Å, and the minimum angle of C-O···O (N) was 90°.

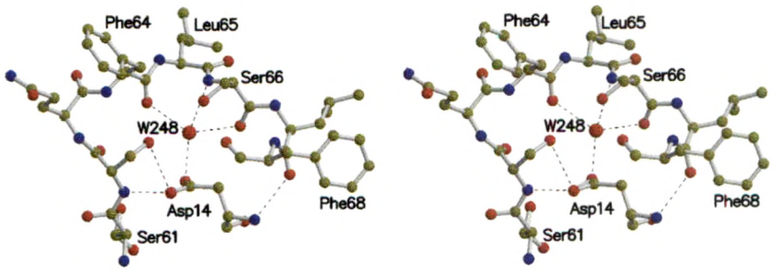
In N14K, the main-chain N atom of Lys14 forms a hydrogen bond with carbonyl O atom of Leu67 (P2′) whereas the side chain NZ atom of Lys14 is found to form hydrogen bonds with OG atom of Ser62 (P4; 2 33Å), OG atom of Ser66 (P1′; 2.54Å) and carbonyl O of Phe68 (P3′; 2.61Å). In addition to this, the water molecule Wat350 connects the carbonyl O atoms of Ser62 (P4; 2.8Å) and Phe64 (P2; 3.13Å) through hydrogen bonding interactions (Fig. 3.3.a).

In N14D, Asp14 forms hydrogen bonds with loop residues, P4, P2 and P1′, through the side-chain atoms of 14th residue and through a bridging water molecule Wat148. The OD1 atom of Asp14 forms hydrogen bonds with main-chain N atom (2.73Å) and OG (2 93Å) atom

of Ser62 (P4) whereas OD2 atom of Asp14 is linked with Wat248 through a strong hydrogen bond of distance 2.46Å. Wat248 forms other three hydrogen bonds with carbonyl O of Phe64 (P2; 2.74Å), main-chain N atom of Ser66 (P1′; 3.18Å) and carbonyl O atom of Ser66 (P1′; 2.96Å). Like rWCI and N14K, the main-chain N atom of Asp14 (of N14D) forms strong hydrogen bond with the carbonyl O atom of Leu67 (P2′; 2.93Å) The hydrogen bonding interactions in the loop region of N14D are shown in Figure 3.3.b.



(a)



(b)

Figure 3. 3. Stereoscopic representation of the hydrogen bonding interactions in the (a) loop region of N14K (b) loop region of N14D

3.3. Discussion

Owing to its crucial position, the role of the conserved scaffolding residue, Asn14 (WCI numbering), was emphasized in the case of Kunitz (STI) family of serine protease inhibitors (Onesti *et al.*, 1991; Dattagupta *et al.*, 1996; Meester *et al.*, 1998). It is observed that the interactions made by the side-chain atoms of Asn14 are more pronounced than its main-chain atoms in stabilizing the conformation of the reactive-site loop. The hydrogen-bonding pattern around the first reactive-site loop of rWCI indicates that the side-chain amide group of Asn14 makes three major hydrogen bonds with the reactive-site loop residues: ND1 with carbonyl O atoms of Phe64 (P2; 2.91Å) and Ser66 (P1'; 2.86Å) and OD2 with the main-chain N atom of Ser62 (P4; 2.80Å); whereas the main-chain N atom of Asn14 forms hydrogen bond with the carbonyl O atom of Leu67 (P2'; 2.81Å). Similar stabilization of the reactive site loop through hydrogen bonding interactions are also observed in ETI and STI, although the details of hydrogen bonding pattern are not identical (Song and Suh, 1998).

The first reactive-site loop region of N14K and N14D are analyzed and compared with that of rWCI. In case of rWCI, apart from the interactions made by Asn14 side-chain, additional stability comes from two water molecules (Wat260 and Wat241) located near the reactive-site loop. Wat260 forms hydrogen bonds with the carbonyl O atoms of P4 (Ser62) and P2 (Phe64) residues while Wat241 is hydrogen bonded with the side-chain OG atom of Ser62 and the carbonyl O atom of P3' residue (Phe68).

In case of mutants, the hydrogen bond between the 14^{th} residue side-chain and P2 residue is no more present. However, like rWCI the main-chain N atom of 14^{th} residue of both the mutants, maintain hydrogen bond with the P2' residue (Leu67). In N14K, the side chain NZ atom of Lys14 was found to form hydrogen bonds with loop residues P4, P1′ and P3′. Beside

this, a water molecule, Wat350 in N14K occupies a position corresponding to Wat260 of rWCI and form similar pattern of hydrogen bonding. But no water molecule corresponding to Wat241 is observed in N14K because of the side-chain orientation of Lys14. Rather the side-chain NZ atom of Lys14 occupies the position corresponding to Wat241 and makes hydrogen bonds with the side-chain OG atoms of two serine residues (P4 and P1') and the carbonyl O atom of P3' (Phe68). It is observed that the accessible surface area for the Lys14 side-chain is 2.44 $Å^2$ and this value is the lowest among the values calculated for the remaining Lys side-chains in the model. Moreover, it is reported that Lys side-chains can display environment-dependent conformational changes and their flexibility becomes higher during ligand binding to proteins (Najmanovich *et al.*, 2000). Hence it can be comprehended that, the inherent conformational flexibility of the Lys side-chain and its limited accessible surface area are responsible for the unusual fold of the Lys14 side-chain.

In N14D, Asp14 formed hydrogen bonds with P4, P2 and P1′ residues, either through the side-chain atoms or through a bridging water molecule. In this case, Asp14 can no more act as a hydrogen bond donor like Asn, rather, the two carbonyl oxygens of P2 and P1′ exert a repulsive force on Asp14 side chain causing a 90° rotation of the same compared to that of Asn14. Here a water molecule Wat248 occupied the position, corresponding to ND1 atom of Asn14, and maintained the hydrogen bonding interactions with the loop residues. Wat248 forms hydrogen bonds with the main-chain N and carbonyl O atoms of Ser66 (P1'), carbonyl O atom of Phe64 (P2) and the carboxylate O atoms of Asp14. Such water-mediated interactions between the reactive-site loop and the protein scaffold was observed in few other protease inhibitors too, like in BPTI, where the similar interaction is mediated through a water molecule to Gly12 (Otlewski *et al.*, 1999). Apart from this, one of the conserved water molecules

(Wat260) of rWCI is absent in N14D, as this region is occupied by one of the alternating positions of the side-chain OG atom of Ser61 (P5).

From the consideration of the retention volumes of the complexes, it is evident that rWCI, N14K and N14D form both types of complexes (1:1 and 1:2) with α-chymotrypsin, depending on the stoichiometry of the incubation mixture. This means that the replacement of Asn14 with Lys or Asp has minimum effect on the binding property of the inhibitor molecules. However, minor change in the hydrogen-bonding pattern could be a plausible reason behind the subtle alterations in the dissociation constant values (rWCI 1.14×10^{-9} M, N14K 2.4×10^{-9} M, N14D 2.58×10^{-9} M). This result also agrees with the observation of a scissile bond hydrolysis experiment performed for a point mutant, N33S of ovomucoid third domain (a Kazal family inhibitor), where the change in hydrolysis constant K_{hyd} was noted as relatively small, not exceeding a factor of 3 to 5 (Ardelt and Laskowski, 1991).

Hence it is clear that the reactive site loop (Gln63-Phe68) of WCI demands a hydrogen bonding network for its stability and in these two mutants, this need is satisfied either by the residues of the scaffold or by the solvent molecules, keeping the loop conformation intact. However, the mutations of Asn14 by two polar residues only (Lys, Asp) influence the activity of the inhibitor very marginally and these replacements are not considered enough to understand the role of Asn14 in the loop stability. Therefore, we felt the necessity of replacing Asn14, with some other residues of different sizes and charges, which will be discussed in next section of this thesis.

SECTION II