Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structures of porcine β-trypsin–detergent complexes: the stabilization of proteins through hydrophilic binding of polydocanol

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Received 10 January 2001
Accepted 3 July 2001

PDB References: PD1, 1fni; PD2, 1fmg; PD3, 1fn6.

Polydocanol has a wide range of medical applications, especially in sclerotherapy of many diseases such as gastrointestinal antiplastia, oesophageal haemangioma *etc*. It is of interest to study the mode of binding of this medically important detergent and its subsequent action on proteins. Here, three crystal structures of serine protease trypsin are reported in the presence of varying concentrations of polydocanol in order to elucidate its mode of binding and interactions with proteins. Polydocanol binds to the protein with its hydrophilic head rather than the hydrophobic tail as is the case with other detergents such as SDS and MEGA-8. This hydrophilic binding mode results in the binding sites of polydocanol being distributed on the surface of the enzyme. There are at least 11 binding sites for polydocanol in trypsin. Polydocanol forms part of the large-scale water networks which connect distant regions of the enzyme, thereby stabilizing it. The hydrophilic binding of polydocanol also results in cross-linked pairs of trypsin molecules.

1. Introduction

A great deal of information is now available through X-ray crystallography about the mode of interaction between proteins and smaller molecules such as inhibitors and haptens (Bode & Huber, 1992), resulting in a better understanding of the function of proteins. Detergents are known to unfold proteins if used in the required concentration. They are also used as an additive in crystallization to produce and improve the quality of crystals (McPherson *et al.*, 1986*a*,*b*; Reiss-Husson, 1992). Detergents are surface-active agents (surfactants) containing a hydrophobic portion soluble in oil-like solutions and a hydrophilic portion soluble in water; hence, they also play a major role in solubilization and crystallization of membrane proteins (Kuehlbrandt, 1988; Reiss-Husson, 1992). Based on their physicochemical properties, detergents are classified as non-ionic, ionic and zwitterionic (Helenius *et al.*, 1979). They migrate to the interface of a solvent, reducing the surface tension. At low concentrations they form monolayers, whereas they tend to aggregate to form micelles or clusters at high concentrations (Helenius *et al.*, 1979). Spectroscopy has been used to study the effect of various detergents on protein folding and unfolding (Ogorzalek Loo *et al.*, 1994), but it does not provide any comprehensive knowledge of molecular conformation and mode of binding of detergents. In the present work, we have studied protein–detergent interactions and the effect of detergents on the protein conformation. In particular, we have cocrystallized native porcine β-trypsin (BPT) in its orthorhombic form with varying concentrations of a non-ionic detergent,

polyoxyethylene (9)-lauryl ether or polydocanol [C_{12}–O–(C–C–O)$_9$]. Polydocanol has a wide range of medical applications, especially in sclerosis (Di Liberato *et al.*, 1999; Cisneros *et al.*, 1998; Aoki *et al.*, 1997; Dell'Abate *et al.*, 1991).

Crystal structures of only a few proteins in complex with detergents are available. The crystal structure of cross-linked triclinic lysozyme exposed to SDS (ionic detergent; Yonath *et al.*, 1977) and bromoethanol (non-ionic; Yonath *et al.*, 1978) showed that these detergents bind to proteins with their hydrophobic tail. Similarly, the crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MEGA-8 (octanoyl *N*-methylglucamide, a non-ionic detergent; Knighton *et al.*, 1993) also shows the hydrophobic binding of the detergent to the protein. Despite differences in the chemical nature and size of the above-mentioned detergents, their mode of interaction with the protein is quite similar, suggesting the existence of a general mechanism for binding of these detergents to proteins (Yonath *et al.*, 1978). However, our study shows that polydocanol completely differs from these detergents in its mode of binding.

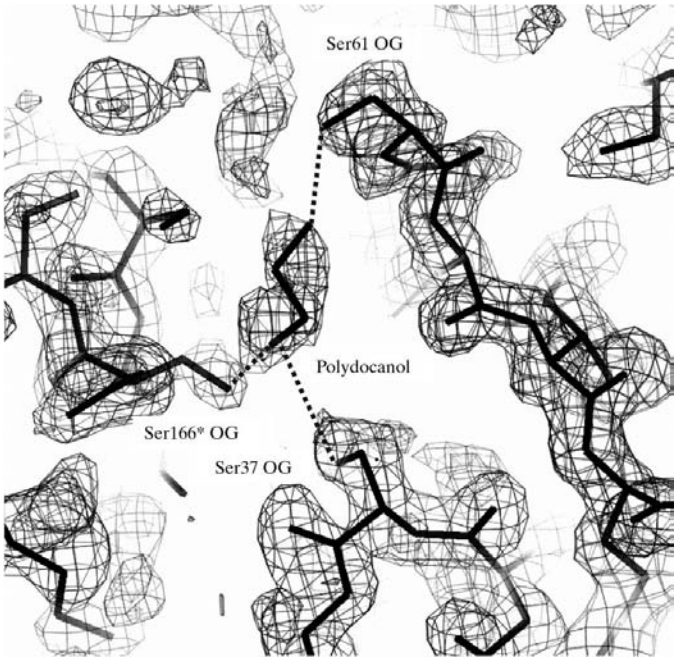

Figure 1
$2F_o - F_c$ (darker lines) with 1σ and $F_o - F_c$ (lighter lines) with 2σ level omit maps (residues 29–56 are omitted) showing GYC density and GYC-mediated intermolecular interactions of the protein.

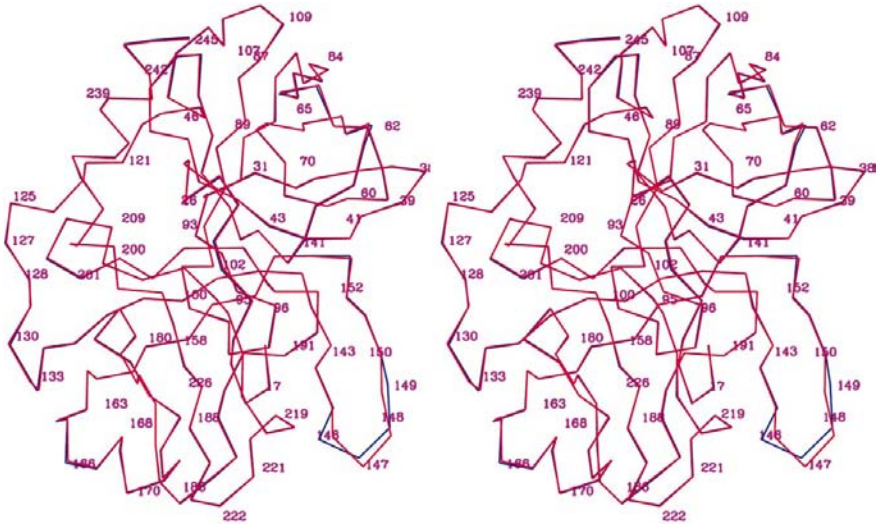

Figure 2
Stereoview of superposition of C^{α} trace of BPT (blue) and PD1 (red).

2. Experimental

2.1. Crystallization and data collection

Porcine β-trypsin (BPT) was purchased from Sigma Chemical Company (St Louis, USA) and was used without any further purification. Crystals of BPT were grown in the presence of 0.01% (PD1), 0.04% (PD2) and 0.1% (PD3) polydocanol (purchased from Sigma) using the vapour-diffusion hanging-drop technique. Concentrations of polydocanol higher than 0.1% did not yield crystals. The drop contained 15 mg ml^{-1} of protein in 0.05 *M* sodium acetate buffer at pH 6.7 in the presence of polydocanol and 20 m*M* $CaCl_2$. The precipitant and reservoir solutions were 0.7 and 1.5 *M* ammonium sulfate, respectively. The precipitant and protein were mixed in a ratio of 1:1 in the hanging drop. Good-quality crystals of BPT were obtained within few days at 293 K. The harvested crystals were washed with the well solution and found to be 100% active against BAPNA (benzoyl arginine *p*-nitroanilide), the standard substrate. The enzymatic assay was performed using the standard method after incubating at an elevated temperature for a designated period of time. The dimensions of the crystals used for data collection were 0.4 × 0.4 × 0.3 mm, 0.4 × 0.4 × 0.4 mm and 0.3 × 0.3 × 0.2 mm for 0.01, 0.04 and 0.1% polydocanol concentrations, respectively. The crystals belong to the space group $P2_12_12_1$ with $Z = 4$. The diffraction data were collected on a MAR Research imaging-plate system and were scaled using *MAR-XDS* and *MARSCALE*. Table 1 summarizes the results of data processing and scaling.

2.2. Structure solution and refinement

The coordinates of the previously refined orthorhombic structure of native BPT (1qqu; Johnson *et al.*, 1999) were used as the basis for refinement, as the crystals PD1, PD2 and PD3 are all isomorphous with BPT. The initial *R* factors for data with $F_o > 2\sigma(F_o)$ were 0.45, 0.44 and 0.45, respectively. Rigid-body refinement and conjugate-gradient refinement of positional parameters using *X-PLOR* resulted in models with *R* factors of 0.30, 0.31 and 0.30 and $R_{\rm free}$

Table 1
Data statistics.

Values in parentheses refer to the last resolution shell.

Data set	PD1	PD2	PD3
Resolution (Å)	1.64	1.9	1.8
Last resolution shell (Å)	1.8–1.64	2.0–1.9	2.0–1.8
Unit-cell parameters (Å)			
a	77.80	77.95	77.90
b	53.95	54.16	54.04
c	47.20	47.27	47.24
No. of reflections measured	88932	55748	67986
No. of unique reflections	22228 (4475)	15084 (2021)	17686 (4514)
Completeness (%)	82.68	92.39	92.63
Multiplicity	4.0	3.7	3.8
$R_{\rm merge}$ (%)	6.5 (41.5)	7.0 (36.8)	6.8 (36.1)

Table 2
Refinement statistics of the three detergent structures.

	PD1	PD2	PD3
Resolution range (Å)	8–1.64	8–1.9	8–1.8
No. of unique reflections used for refinement	21993	14881	17473
No. of protein atoms	2032	2032	2032
Calcium	1	1	1
Water	158	174	156
Sulfate	1	1	1
Acetate	—	—	—
Glycol	4	5	2
Methanol	—	—	1
R value	0.183	0.156	0.157
$R_{\rm free}$	0.234	0.231	0.238
R.m.s. deviations from ideal geometry			
Bond length (Å)	0.01	0.012	0.015
Bond angle (°)	2.4	2.3	2.5
Torsion angle (°)	28	28	28
Improper angle (°)	2.0	2.4	2.6
Estimate mean coordinate error (Luzzati, 1952)	0.22	0.23	0.21

values of 0.38, 0.36 and 0.37 for the three data sets, respectively. A cycle of simulated-annealing refinement (Brünger & Kurkawski, 1990) lowered the *R* factors to 0.26, 0.29 and 0.24, respectively. The model was improved using $2F_o - F_c$ and $F_o - F_c$ maps employing *O* (Jones & Kjeldgaard, 1991). At this point, the models were refined with *REFMAC* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994) using the maximum-likelihood principle. The solvent molecules were added to the model at stereochemically reasonable positions using peaks in the $2F_o - F_c$ and $F_o - F_c$ maps at the 1σ and 3σ levels, respectively. A methanol molecule and a few detergent molecules were identified along with the solvent atoms. The electron densities fitting the glycol-like monomer (GYC) of polydocanol and methanol were initially considered as water molecules. Refinement of the structure showed the presence of two more water molecules in the case of GYC and the distances between them were rather too short (<2 Å). Eventually, we fitted a monomer (O–C–C–O) of the polydocanol head and methanol which were stable during the refinement process. The positions of the GYC were rechecked in omit maps (Fig. 1). The dictionary files used for the refinement of the detergent and methanol were made using

Table 3
R.m.s. deviations (Å) in atomic coordinates.

M, main chain; MS, main chain and side chain.

Structure		PD1	PD2	PD3
BPT	M	0.24	0.14	0.16
	MS	0.79	0.52	0.75
PD1	M		0.24	0.21
	MS		0.69	0.50
PD2	M			0.16
	MS			0.66

energy-minimized models. All the reflections, with 0σ cutoff, were used during the final stages of refinement. At the end of refinement, the $F_o - F_c$ maps were essentially featureless. Table 2 shows the refinement statistics of the three structures. Structure solution, fitting and refinement were performed on a Silicon Graphics Octane workstation. The packages *Insight*II (Biosym Technologies, 1993) and *PROCHECK* (Laskowski *et al.*, 1993) were used to compare the secondary-structural elements and Ramachandran plots of the three detergent structures.

3. Results and discussion

3.1. The structure

The overall features of the independently refined structures of native BPT in the PD1, PD2 and PD3 structures show very high agreement (Fig. 2). The peptide torsion angles fall within the most favoured or additional allowed regions of the Ramachandran plot (Ramachandran *et al.*, 1963). Compared with BPT, even the residues in the loop regions have clearly defined electron density, which is probably a consequence of the addition of the detergent. This is similar to the observation that addition of MEGA-8 during the crystallization of cAMP-dependent protein kinase improves the diffraction quality of the crystals and also the stability of the protein (Knighton *et al.*, 1993). The overall r.m.s. deviation varies between 0.14 and 0.24 Å for main-chain atoms when the three structures (Table 3) are superposed. A detailed comparison, however, shows large variations in the loop regions of the polypeptide chain (Fig. 3). The maximum deviation of 1.46 Å in the main chain occurs in Ser146, which is a part of the most unstable loop in trypsin *viz*. the autolysis loop (Gly142–Ser153; Johnson *et al.*, 1997). Similarly, there are large variations in φ and ψ angles in the autolysis loop of PD1 compared with BPT. The maximum variations of 139.2 and 167.3° occur at Ser149 and Ser150, respectively. The deviations in the main chain coupled with variation in the dihedral angle enable the PD1 structure to form a main-chain hydrogen bond (3.14 Å) between the carbonyl group of Ser146 and the N atom of Gly148, thereby increasing the stability of the autolysis loop.

Although the agreement in main-chain atoms is high between the structures containing the detergent and BPT, a number of side chains exhibit alternative conformations in the presence of the detergent. For example in PD1, Ser37, Ser49,

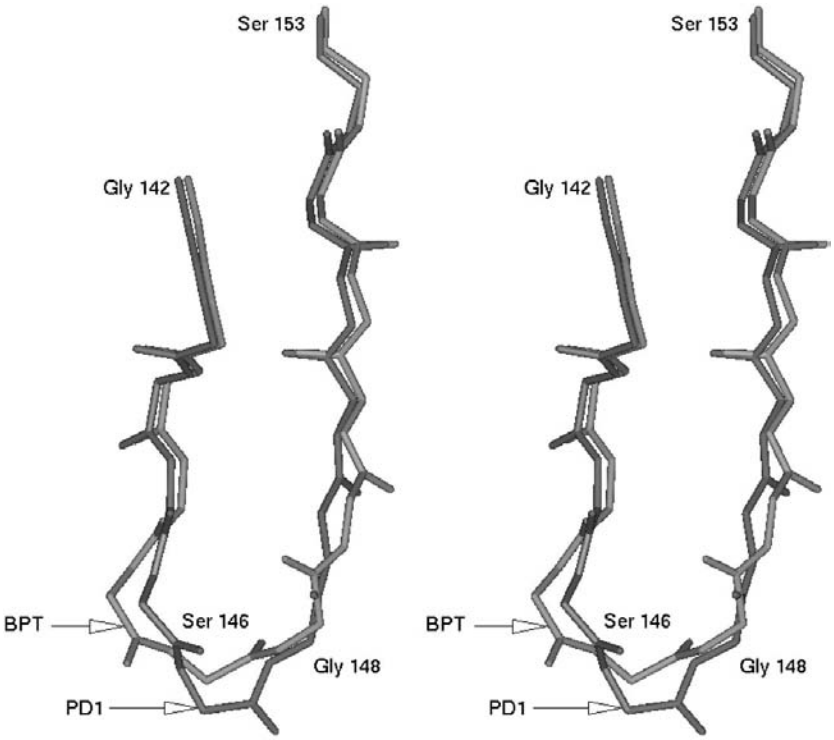

Figure 3
Stereoview of superposition of backbone atoms of autolysis loop (Gly142–Ser153): PD1, darker lines; BPT, lighter lines

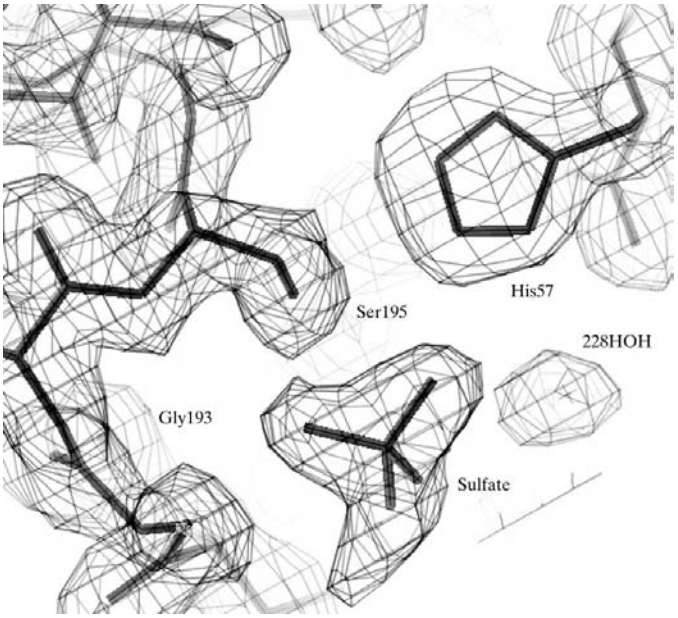

Figure 4
$2F_o - F_c$ map showing electron density for a sulfate molecule at the active site. Hydrogen bonds with His57, Ser195 and Gly193 are also shown.

Leu99, Ser122, Leu158 and Ser166 show alternative conformations. It is interesting that Leu99 shows alternate conformations in all three structures, indicating increased flexibility for this residue. The flexibility of Leu99 has been implicated in the retention of trypsin activity (Huang *et al.*, 1994; Johnson *et al.*, 1997).

The geometry of the active site in the structures containing the detergent is the same as that in the native structure. A sulfate molecule is present with well defined electron density at the active site of all three detergent structures (Fig. 4). Two of the sulfate O atoms, O2 and O4, form hydrogen bonds with Ser195 OG; O4 also forms hydrogen bonds with His57 NE2 and a water molecule. Apart from Ser195, O2 of the sulfate forms bifurcated interactions with the N atom of Gly193 and a water molecule. O1 and O3 interact with one water molecule each. The sulfate ion replaces a water molecule at a similar position in some of the other trypsin structures solved at different pH values, namely BAE (pH 7.0; Bode & Schwager, 1975), DEBB (pH 5.0; Bode & Schwager, 1975) and DEBA (pH 8.0; Walter *et al.*, 1982). An acetate ion found at a similar position in BPT (Johnson *et al.*, 1999) showed that the acetate ion and the sulfate ion binding sites and their interactions are the same in trypsin. The observability of the sulfate ion strongly depends on the contrast in the electron-density map (Bartunik *et al.*, 1989) and in the present study it is able to be identified owing to the high resolution of the data obtained in the presence of detergent.

Several serine proteases have calcium-binding sites that stabilize the enzyme against thermal or chemical denaturation and proteolytic degradation (Kretsinger, 1976; Martin, 1984). The octahedral environment of the calcium ion in the detergent structures is similar to that of BPT (Johnson *et al.*, 1999). The distance between Ca^{2+} and Glu70 OE1 is 2.50 Å, Asn72 O is 2.56 Å, Val75 O is 2.54 Å, Glu80 OE2 is 2.48 Å and 232OH is 2.68 Å in PD1.

3.2. Binding of polydocanol

The hydrophilic heads (GYC) of polydocanol were observed in 11 regions around the trypsin molecule in the three structures PD1, PD2 and PD3 (Fig. 5), of which two pairs are closer than the others to each other (<4.5 Å). The rest of the hydrophilic and hydrophobic portion of polydocanol could not be traced in the electron density, probably owing to disorder caused by the solvent environment. Table 4 shows the interactions of polydocanol in all three structures. All the polydocanol-binding sites are on the surface of the trypsin molecule, as the mode of binding is hydrophilic rather than hydrophobic (Fig. 5). Six of the 26 residues interacting with the hydrophilic head of the detergent are serine.

The addition of polydocanol is known to enhance the stability and preserve the activity of the enzyme molecule (Pikula *et al.*, 1991). The binding of the detergent molecule to trypsin is non-specific and seems to stabilize the various

Table 4
Glycol interactions.

O3 and O4 are O atoms in the O–C–C–O group.

Atoms	Structure	Nitrogen	Carbonyl	Side chain	No. of interacting water molecules
424O3	PD1			Ser37 OG, Ser166 OG†, Lys60 CD	
424O4	PD1			Ser61 OG, Ser164 OG	
425O3	PD1		Ser116		
425O4	PD1				
426O3	PD1		Gly178	Lys230 NZ	1
426O4	PD1			Asn233 ND2, Tyr151 OH	2
427O3	PD1			Gln239 NE2	1
427O4	PD1				2
435O3	PD2	Ser164†		Ser164 OG†	
435O4	PD2			Ser61 OG	
436O3	PD2		Tyr151		
436O4	PD2			Asp74 OD1, Asp74 OD2	
437O3	PD2			Ser116 OG	
437O4	PD2				
438O3	PD2			Asn95 ND2, Thr98 OG1, Thr98 CG2	1
438O4	PD2				1
439O3	PD2			Ile242 CG2	1
439O4	PD2		Ile242, Ala243		
436O3	PD3	Asn25	Glu70	His71 ND1	
436O4	PD3	Ser26, Ile27	Ile27		1
438O3	PD3		Gly216†, Gly219†, Arg125	Gln192 OE1†	2
438O4	PD3				2

† Symmetry-related molecule.

autolytic sites by making direct interactions with the residues in the autolytic site or with the residues that are present in the immediate vicinity of the autolytic site. For example, the GYC molecule has interactions with Ser61, which is an ε-trypsin autolytic site (Lys60–Ser61) (Huang *et al.*, 1994), with Tyr151, which is present in the autolytic loop of α-trypsin (142–153) (Johnson *et al.*, 1997), and with Ser116, which is close to the δ-trypsin autolytic site (Arg117–Val118) (Maroux & Desnalle, 1969; Smith & Shaw, 1969; Guo *et al.*, 1985). The modes of binding of detergents in crystal structures solved so far are listed in Table 5.

Table 5
Modes of binding of detergents.

Detergent	Binding environment	Residues involved
MEGA-8	Hydrophobic	Val
SDS	Hydrophobic	Ala, Ile, Thr, His
Polydocanol	Hydrophilic	Mainly hydrophilic

In the crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with a peptide inhibitor (Knighton *et al.*, 1993) and cocrystallized with MEGA-8, the binding environment is almost exclusively hydrophobic. The hydrophilic region of the MEGA-8 detergent could not be identified because of disorder. Similarly, Yonath *et al.* (1977) identified the hydrophobic region of the SDS molecule bound to cross-linked triclinic lysozyme. In contrast to these detergents, polydocanol binds in hydrophilic regions. The detergent binding on the surface of the trypsin molecule offers additional stability to the enzyme. This conclusion is supported by the melting curve of the PD1 crystals (Fig. 6).

In PD1, PD2 and PD3, more than a third of the GYC molecules have intermolecular interactions (Fig. 1) and this may bring the protein molecules close to each other, promoting the formation of dimers or aggregates as in the case of BAX proteins (Hsu & Richard, 1998). Thus, the mode of binding of polydocanol as seen in the present study explains the enhancement of stability and aggregation of proteins in the presence of low concentrations of polydocanol.

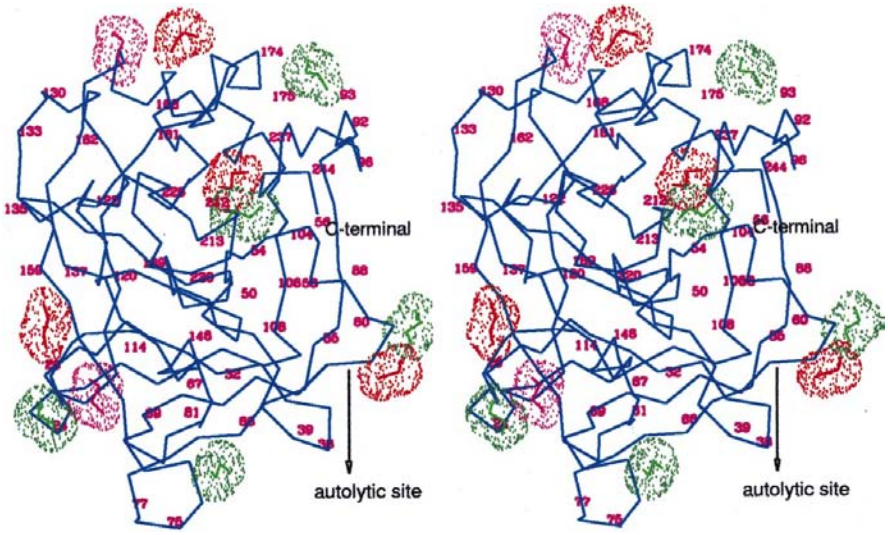

Figure 5
The backbone of the trypsin with bound glycol monomers (Connolly surface) of the hydrophilic head of polydocanol. PD1, red; PD2, green; PD3, pink.

3.3. Hydration

Comparison of the oligomeric arrangement of water molecules present in BPT and the three detergent structures shows that addition of polydocanol has not prevented or altered the formation of oligomers observed in the native structure (Johnson *et al.*, 1999). Almost all the oligomers present in the detergent structures are located near the hydrophobic residues, except the tetramer of water molecules which covers Lys60. Three of these water molecules have interactions with Lys60 NZ. There is no correlation between the number or size of the oligomers and the concentration of detergent. The tendency of polydocanol to bind the hydrophilic regions brought out by the present study could probably

be the reason why the oligomers, which are mostly present in the hydrophobic regions, are not altered in the detergent structures. 53 of the water sites in the native structure are conserved in all the three structures containing the detergent. All these water molecules belong to first hydration shell. A total of 48 of the 53 conserved sites have interactions with main-chain carbonyl O and/or amide N atoms.

The identified hydrophilic portions of the polydocanol also forms hydrogen bonds with the networks of water molecules in the hydration shell (Fig. 7). These networks appear to be more extensive in PD1, PD2 and PD3 than in the native structure. They cause indirect interactions between several β-strands, α-helices and loops which are distant from each other. For example, a set of six water molecules mediate an interaction between Thr98 and Lys169, which are almost 13 Å apart. The network around helix *B* involving water molecules and detergent molecules is an example of the identified hydrophilic head of polydocanol stabilizing the structure. Some of the networks are conserved in all the three detergent structures and these might play an important role in stabilizing the tertiary structure of trypsin.

Theoretical studies (Hayward *et al.*, 1993) have suggested that water of hydration damps the internal vibrations of proteins in the low-energy collective motion. Rupley & Careri (1991) showed that heat capacities and denaturation temperatures of soluble proteins are strongly correlated with the hydration level of the protein. Thus, the increased stability of the structures containing the detergent could well be a consequence of the extensive water networks and polydocanol binding on the surface of the protein.

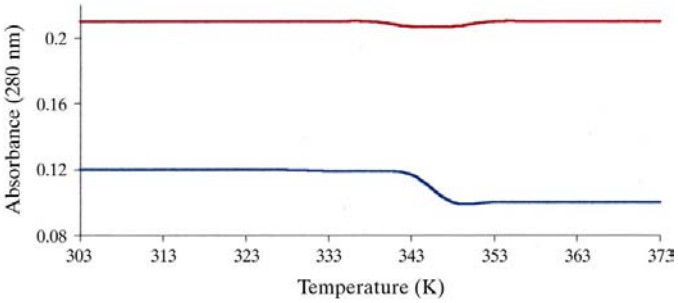

Figure 6
Denaturation curve obtained for PD1 and native BPT using a CARY-UV spectrophotometer. PD1 remains active up to 363 K, although a slight decrease in absorption around 343 K is seen. BPT, blue; PD1, red.

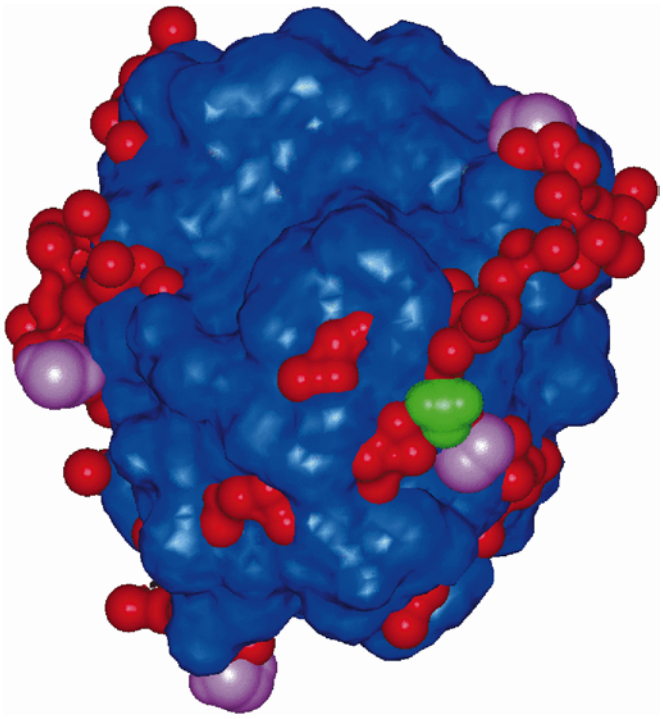

Figure 7
The distribution of bound water molecules (small red spheres) in PD1 that connect the polydocanol heads (violet spheres) and cover a large portion of the protein (blue surface), thereby enhancing the stability of the protein. The sulfate molecule is shown as a green sphere. The figure was prepared using *Insight*II.

SD was supported by Council of Scientific and Industrial Research (CSIR, India) in the form of a Senior Research Fellowship. Diffraction data were collected at the X-ray Facility for Structural Biology at the Indian Institute of Science, Bangalore.

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