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Tryptophan as a three-way switch in regulating the function of the secretory signalling glycoprotein (SPS-40) from mammary glands: structure of SPS-40 complexed with 2-methylpentane-2,4-diol at 1.6 Å resolution

Pradeep Sharma,[a] Nagendra Singh,[a] Mau Sinha,[a] Sujata Sharma,[a] M. Perbandt,[b] Christian Betzel,[b] Punit Kaur,[a] A. Srinivasan[a] and Tej P. Singh[a]*

[a]Department of Biophysics, All India Institute of Medical Sciences, New Delhi, India, and [b]Department of Biochemistry and Molecular Biology, Hamburg University, c/o DESY, Notkestrasse 85, Hamburg, Germany

Correspondence e-mail: tpsingh.aiims@gmail.com

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The 40 kDa secretory signalling glycoprotein (SPS-40) is the first example with Trp78 in three functional orientations: (i) a resting state with a pinched conformation, (ii) a stacked conformation when bound to hexasaccharide and (iii) an obstructive conformation when inhibited by 2-methylpentane-2,4-diol (MPD). Trp78 is present in the core of the sugar-binding groove. The hexasaccharide *N*-acetylglucosamine ($GlcNAc_6$) has been shown to bind to SPS-40. As a result of this, the conformation of Trp78 alters from the native pinched conformation ($\chi^1 = -65.5^\circ$, $\chi^{2,1} = -78.8^\circ$, $\chi^{2,2} = 97.5^\circ$) to the stacked conformation ($\chi^1 = -170.0^\circ$, $\chi^{2,1} = -114.3^\circ$, $\chi^{2,2} = 61.6^\circ$). Further binding experiments showed that saccharide binding does not occur in the presence of 20% MPD. The crystal structure determination of the complex of SPS-40 with MPD revealed the presence of two MPD molecules in the sugar-binding groove. The very tightly bound MPD molecules at subsites −2 and −1 induced an unexpected and a rarely observed conformation of Trp78 ($\chi^1 = 55.9^\circ$, $\chi^{2,1} = 90.2^\circ$, $\chi^{2,2} = -88.9^\circ$) which is termed an obstructive conformation. The binding of MPD molecules also twisted the side chains of Glu269 and Ile272 considerably. These residues are also part of the sugar-binding groove. The observed obstructive conformation of the side chain of Trp78 in the present structure is the exact opposite of the stacked conformation. This rarely observed conformation is stabilized by a number of hydrogen bonds between Trp78 and Asn79 through water molecules W49, W229, W269, W547 and W557.

1. Introduction

The growth and development of the mammary gland is regulated by a complex set of factors. The lactational function of the mammary gland occurs in cycles that begin with mammary-gland development and lactogenesis and conclude with mammary-gland involution. During the period between successive lactations, the mammary gland undergoes several functional transitions including active involution after cessation of milk removal. During involution, drastic changes take place that result in tissue remodelling and a massive loss of epithelial cells. This cell loss is a consequence of programmed cell death or apoptosis (Strange *et al.*, 1992; Walker *et al.*, 1989). It is a highly regulated process and a number of proteins may contribute to protect the cells against apoptosis in a variety of ways. The increased production of a 40 kDa inactive chitinase-related glycoprotein (SPS-40) during the process of mammary-gland involution suggests an important role for this protein. A similar protein called breast-regression protein (BRP-39) with a sequence identity of 69% to SPS-40 has been discovered in certain types of breast-cancer cells (Morrison & Leder, 1994). SPS-40 was isolated from sheep mammary-gland secretions which were collected during the early period of involution. Several isoforms of this protein have been studied from other species such as goat (SPG-40) and cow (SPC-40) in native forms (Mohanty *et al.*, 2003; Kumar *et al.*, 2006). SPS-40 shows sequence identities of 53% with chitinase (Renkema *et al.*, 1995) and 46% with mouse macrophage novel lectin (YM1; Sun *et al.*, 2001; Tsai *et al.*, 2004). Despite considerable sequence and structural similarities to chitinases, it lacks chitinase-like activity as a conse-

Table 1
Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell

PDB code	2pi6
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 62.9, b = 66.4, c = 105.7
No. of molecules in the unit cell	4
Resolution range (Å)	50.0–1.6
Total No. of measured reflections	1432448
No. of unique reflections	52135
Redundancy	27.4
Mosaicity	0.53
R_{merge} (%)	3.5 (46.5)
$I/\sigma(I)$	13.3 (4.0)
Completeness (%)	99.9 (99.7)
R_{cryst} (%)	16.7
R_{free} (%)	21.7
Protein atoms	2905
Water O atoms	590
NAG residue (N-linked) atoms	28 [2 molecules]
MAN residue (linked to NAG) atoms	42 [3 molecules]
Ethanol atoms	9 [3 molecules]
Ligand MPD atoms	16 [2 molecules]
R.m.s.d., bond lengths (Å)	0.01
R.m.s.d., bond angles (°)	1.5
R.m.s.d., torsion angles (°)	16.6
Mean B factor for main-chain atoms (Å^2)	19.0
Mean B factor for side-chain and water atoms (Å^2)	27.7
Mean B factor for all atoms (Å^2)	24.2
Ramachandran plot	
Residues in most favoured regions (%)	91.8
Residues in additionally allowed regions (%)	8.2

quence of the replacement of an essential Glu residue by Leu in the active site of SPS-40 (Mohanty *et al.*, 2003). Binding studies have shown that oligomers of *N*-acetylglucosamine (GlcNAc) bind to SPS-40 (Srivastava *et al.*, 2007). The lack of hydrolytic activity with a residual capability to bind saccharides at the carbohydrate-binding groove suggests a binding role for SPS-40. In this paper, we demonstrate that 2-methylpentane-2,4-diol (MPD) inhibits the binding of saccharides to SPS-40. In order to understand the mode of binding and the inhibitory effects of MPD, we have crystallized the complex of SPS-40 with MPD. Structure determination of the complex at 1.6 Å resolution shows that two molecules of MPD bind to SPS-40. It may be mentioned here that Trp78 plays the most critical role in the binding of carbohydrates to SPS-40. Upon binding to MPD, the side chain of Trp78 undergoes an unexpected but dramatic conformational change which prevents the binding of carbohydrates to SPS-40. This conformation is named the obstructive conformation. Previous results from the structures of the native protein and its complex with a hexasaccharide ($GlcNAc_6$) have shown that Trp78 adopts two other more commonly occurring conformations called the pinched and stacked conformations, respectively. With these three distinct conformations of Trp78, the protein SPS-40 exists in three states: (i) a resting state with pinched conformation, (ii) a saccharide-bound state with a stacked conformation and (iii) an inhibited state as observed in the complex with MPD with obstructive conformation.

2. Experimental procedure

2.1. Purification and crystallization

The protein was purified using the procedure described previously (Kumar *et al.*, 2006; Srivastava *et al.*, 2007). Purified samples of SPS-40 were incubated in buffer containing 50 m*M* NaCl, 25 m*M* Tris–HCl pH 7.8 and 20% MPD. These samples were ultrafiltered using a membrane with a 1 kDa cutoff and lyophilized. They were dissolved in buffer containing 50 m*M* NaCl, 25 m*M* Tris–HCl pH 7.8 to a final protein concentration of 30 mg ml^{-1}. Crystallization setups were prepared for the hanging-drop vapour-diffusion method. 10 µl drops of protein solution were equilibrated against reservoir solution containing 50 m*M* NaCl, 25 m*M* Tris–HCl pH 7.8 and 15% ethanol. Plate-like crystals were obtained at 298 K after a month.

2.2. Data collection, structure determination and refinement

The crystals were transferred to 50 m*M* NaCl, 25 m*M* Tris–HCl pH 7.8 and 25% ethanol for data collection at 100 K. Data were collected on the consortium beamline X13 at DESY, Hamburg, Germany with a wavelength of 0.98 Å using a 165 mm MAR CCD detector. The data were processed with *DENZO* and *SCALEPACK* from the *HKL* package (Otwinowski & Minor, 1997). The final data set was 99.9% complete to 1.6 Å resolution. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 62.9, b = 66.4, c = 105.7 Å. The presence of four molecules in the unit cell gave a crystal volume per protein mass (V_M) of 2.8 Å^3 Da^{-1}, corresponding to a solvent content of 55.4% by volume. The results of data collection and processing are given in Table 1. The structure of MPD-inhibited SPS-40 was isomorphous to the native structure of SPS-40 (Srivastava *et al.*, 2007). Therefore, the coordinates of the native structure of SPS-40 (PDB code 2dpe) were used as the starting model for refinement. The crystallographic refinement procedure was carried out using *REFMAC* (Murshudov *et al.*, 1997). Model building was performed using the program *O* (Jones *et al.*, 1991). The difference $|F_o - F_c|$ Fourier map calculated when the value of R_{cryst} was 0.286 revealed excellent electron densities at a 3σ cutoff for two molecules of MPD in the proximity of Trp78. These positions correspond to subsites −2 and −1 as defined in previous reports (Srivastava *et al.*, 2007). The positions of 590 water molecules, 60 atoms of the carbohydrate residues from four glycan chains and nine atoms of ethanol were determined. The refinement converged to

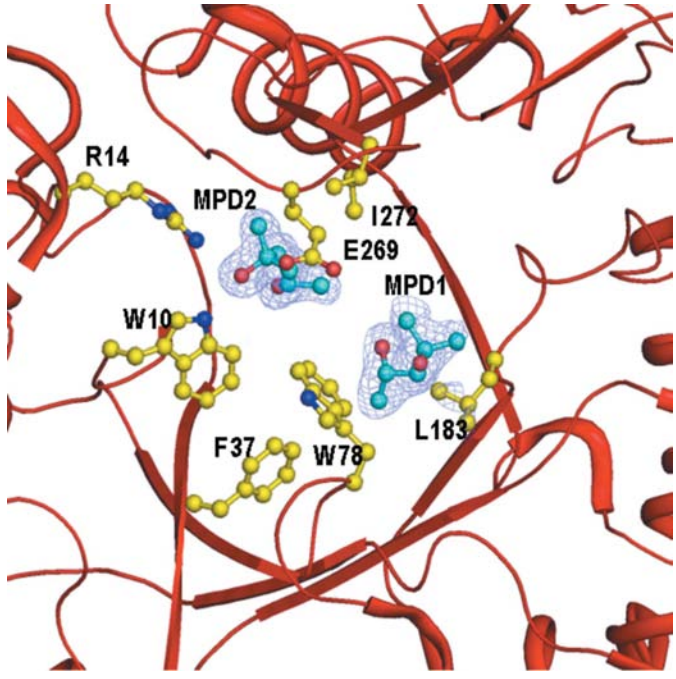

Figure 1
The $|F_o - F_c|$ electron-density map (contour level 3.5σ) computed without the atomic coordinates of MPD1 and MPD2. MPD1 is observed at subsite −1 in the proximity of residues Trp78 and Leu183 and MPD2 is present at subsite −2 near the residues Trp10, Glu269 and Ile272.

values of 0.167 and 0.217 for the R_{cryst} and R_{free} factors, respectively. The refinement statistics of the structure are summarized in Table 1.

3. Results and discussion

3.1. Overall structure of MPD-inhibited SPS-40

The $|F_o - F_c|$ map contoured at 3.5σ for the two ligand molecules is shown in Fig. 1. The Ramachandran plot (Ramachandran & Sasisekaran, 1968) calculated using *PROCHECK* (Laskowski *et al.*, 1993) showed 91.8% of residues to be in the most favoured regions, while the remaining 8.2% were found in additionally allowed regions. The polypeptide chain of SPS-40 folds into two globular domains: a large $(\beta/\alpha)_8$ triose phosphate isomerase (TIM) barrel (Banner *et al.*, 1975) and a small $(\alpha+\beta)$ domain. In order to understand the binding properties of the sugar-binding groove of SPS-40, the groove has been divided into several subsites on the lines of chitinase enzymes (Aronson *et al.*, 2003) and as reported previously for SPX-40 proteins (Kumar *et al.*, 2006; Srivastava *et al.*, 2007). The sugar-binding groove in SPS-40 is partially blocked by the protruding side chain of Trp78 and also by interactions between Tyr120 and Asp186 across the groove. The backbone structure of MPD-inhibited SPS-40 is essentially similar to the structure of native SPS-40 (Srivastava *et al.*, 2007), with an r.m.s. shift of 0.5 Å for the C^α trace. However, the conformations of the side chains of Trp78, Glu269 and Ile272 are remarkably different.

3.2. Binding of MPD to SPS-40

Bound MPD molecules (MPD1 and MPD2) were observed at the centre of the TIM barrel (Fig. 1). MPD is a solvent and is also used as cryoprotectant, but in this case MPD was used as a chemical entity based on its chemical structure and experimental observations which indicated that it inhibited the binding of sugars to SPS-40. As a consequence of the fact that it binds to SPS-40 at the sugar-binding site with considerable affinity, an examination of the binding properties of MPD to SPS-40 as a ligand may provide important clues for ligand design. MPD1 occupies subsite −1 and MPD2 was located at subsite −2. MPD1 forms one direct hydrogen bond to Tyr185 OH and interacts with Arg242 NH2 through water molecule OW569. It also interacts with Tyr120 through another water molecule OW167. MPD2 forms three direct hydrogen bonds to Trp10 $N^{\varepsilon1}$, Arg14 NH2 and Trp331 $N^{\varepsilon1}$. It also forms three hydrogen bonds to Thr8 $O^{\gamma1}$, Arg14 NH2 and Asp334 $O^{\delta1}$ through solvent water molecules as well as two hydrogen bonds to solvent water molecules. In addition to these interactions, both MPD1 and MPD2 are held in place by van der Waals forces involving residues Trp78, Leu119, Leu183, Tyr185, Asp186 and Trp331, and residues Trp10, Trp78, Thr267, Glu269 and Ile272, respectively. The extensive binding network between the protein and MPD molecules at the two important subsites in the sugar-binding groove induces a remarkable conformational change in several residues of the sugar-binding groove. The most important residue, Trp78, is part of the distorted type III β-turn conformation with torsion angles of $\varphi_{(i+1)}, \psi_{(i+1)} = -80.1^\circ, -29.4^\circ$ and $\varphi_{(i+2)}, \psi_{(i+2)} = -63.1^\circ, -46.2^\circ$ for the corner residues Trp78 and Asn79 at positions $(i + 1)$ and $(i + 2)$, respectively. However, this tight β-turn lacks the characteristic 4→1 hydrogen bond, indicating the lower stability of this conformation. The loss of this hydrogen bond is the consequence of backbone conformational perturbations of residues Gly77 and Phe80; the distance between Phe80 N and Gly77 O is 3.84 Å. It may be mentioned here that the φ, ψ values of Gly77 and Phe80 in the present structure are 88.7°, −179.1° and −64.5°, 119.8°, respectively, while the corresponding values in the structure of the native protein are 98.7°, 179.1° and −107.9°, 116.9°, respectively. It is noteworthy that the segment Gly77-Trp78-Asn79-Phe80 in the native structure adopts a type I β-turn with φ, ψ torsion angles $\varphi_1, \psi_1 = -71.2^\circ, -29.4^\circ$ and $\varphi_2, \psi_2 = -80.1^\circ, 11.2^\circ$ and the characteristic 4→1 hydrogen bond is formed. The conformation of the side chain of Trp78 in the structure of the native protein is defined by torsion angles $\chi^1 = -65.5^\circ$, $\chi^{2,1} = -78.8^\circ$, $\chi^{2,2} = 97.5^\circ$. The corresponding values in the structure with MPD are $\chi^1 = 55.9^\circ$, $\chi^{2,1} = 90.2^\circ$, $\chi^{2,2} = -88.9^\circ$, whereas in the complex with $GlcNAc_6$ the angles are −170.0°, −114.3° and 61.6°, respectively (Fig. 2). A comparison of three positions of the side chain of Trp78 in the structures revealed that the pinched conformation is stabilized mainly by van der Waals interactions involving the side chains of Trp10, Phe37 and Leu119, while in the stacked conformations the side chain of Trp78 forms a van der Waals interaction with Tyr120. In contrast, in the obstructive conformation Trp78 is held in place by several hydrogen bonds to Asn79 *via* water molecules as well as van der Waals interactions with Trp10 and Phe37.

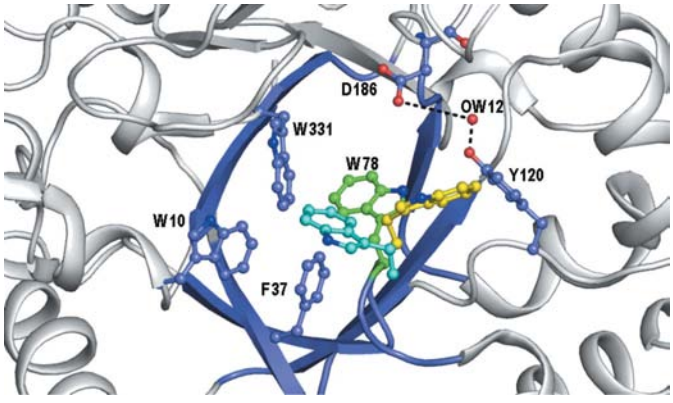

Figure 2
The three observed and superimposed conformations of Trp78 in SPS-40: Trp78 with pinched conformation in the resting state where Trp78 occupies the most favourable conformation in the least constrained state (green), the stacked conformation of Trp78 on hexasaccharide binding in which the side chain of Trp78 is stacked against a sugar residue on one side and Tyr120 on the other side (yellow) and the obstructive conformation observed when MPD binds at subsites −2 and −1 (light blue). The hydrogen bonds involving Tyr120 OH, OW12 and Glu186 are indicated as dashed lines.

4. Summary

The native SPS-40 protein consists of a sugar-binding groove which is partially blocked at the core of the protein by Trp78. However, upon binding to $GlcNAc_6$ (Kumar *et al.*, 2006; Srivastava *et al.*, 2007), Trp78 is displaced away from the groove to the stacking position, which coincides with the conformations observed for the corresponding Trp residue in chitinases and chitinase-like proteins. In a highly significant contrast, in the complex with MPD the specific binding of two MPD molecules in the TIM-barrel core results in considerable conformational perturbations in various residues (Gly77, Trp78, Phe80, Glu269 and Ile272), the most dramatic being Trp78, the side chain of which adopts a rarely observed conformation that blocks the binding of carbohydrates to the protein.

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