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FOR THE RECORD

Identification of a molecular switch that selects between two crystal forms of bovine pancreatic trypsin inhibitor

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Abstract: Two crystal forms of bovine pancreatic trypsin inhibitor are produced between pH 8.39 and 10.13 when crystals are grown at room temperature from solutions of 1.5 M potassium phosphate. Lower pH values favor the form II crystals, whereas higher pH values favor the form III. The transition from one crystal form to the other occurs at pH 9.35. We examined the crystal lattice contacts in both crystal forms and identified an unusual interaction we believe explains these observations. Spanning the crystallographic 2-fold axis in form III crystals, the Lys 41 side-chain amino nitrogens from 2 symmetry-related molecules are only 2.72 Å apart, implying they are hydrogen bonded to one another. In form II crystals, the Lys 41 side-chain amino group is protonated and forms a salt bridge with a solvent-derived phosphate group. For the Lys 41 side-chain amino groups to hydrogen bond in form III crystals, at least 1 member of the pair must be deprotonated. The transition that occurs at pH 9.35 marks the pK_a for deprotonation. In solution, the pK_a for the Lys 41 side chain is around 10.8. The pK_a for one of the interacting Lys 41 side chains in form III crystals is therefore shifted downward by about 1.5 pH units. The energy for lowering the pK_a value comes from the many additional intermolecular hydrogen bonds that are present in form III crystals: 19 compared to only 8 in form II crystals.

Keywords: amine–amine hydrogen bonds; BPTI; crystal lattice contacts; molecular switch; multiple crystal forms

Many proteins produce multiple crystal forms. This usually occurs as a consequence of differences in pH, ionic strength, the presence or absence of bound ligands, or chemical modifications to a protein. Comparisons of multiple crystal forms have examined how crystal lattice contacts influence the structure and dynamics of proteins (Artymiuk et al., 1979; Sheriff et al., 1985; Phillips, 1990; Gallagher et al., 1992; Sobek et al., 1992; Tong et al., 1993) and ligand binding to proteins (Gros et al., 1989; Heinz et al., 1991). Comparisons of multiple crystal forms can also be used to assess the magnitude of errors remaining in a crystal structure after the refinement process (Wlodawer et al., 1987a; Madden et al., 1989).

Some proteins produce multiple crystal forms under nearly identical conditions. The form II and form III crystals of bovine pancreatic trypsin inhibitor (BPTI) are an example (Wlodawer et al., 1987b). Such systems provide an opportunity to examine the environmental factors and protein interactions that select for different crystal forms. We have mapped out a pH-dependent phase diagram for form II and form III crystals of BPTI crystals in the pH range 8.39–10.13. By examining the lattice contacts in the crystal structures for both forms, we have identified an interaction we believe accounts for the pH-dependent selection of form II versus form III crystals of BPTI.

As part of an earlier study (Gallagher et al., 1992) we made numerous bulk preparations of form II and form III crystals of BPTI. The details of the procedures used to grow and identify these crystal forms are given by Wlodawer et al. (1987b). The crystals were grown at room temperature from a 1.5 M potassium phosphate solution at various pH values. Twelve crystal preparations were made at pH values between 8.39 and 10.13. At pH values less than 9.35, only form II crystals were observed, whereas at pH values greater than 9.35, only form III crystals formed. The crystal forms for 5 of the preparations were confirmed by X-ray diffraction. The identity of the crystal forms in the remaining preparations was based on crystal morphology. Five of the crystal preparations had pH values between 9.3 and 9.4, so we were able to observe that the pH range where the transition occurs is very narrow. This implies the selection of a particular crystal form is a cooperative process. We also observed that, when the pH of a suspension of form III crystals is dropped to pH 7.7, the form III crystals fracture and begin to fall apart within hours. Within 1 day, the suspension contains nearly all form II crystals.

A preliminary description of form II crystals of BPTI was reported by Walter and Huber (1983). The crystal structure was

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subsequently determined by joint refinement of neutron and X-ray diffraction data (Wlodawer et al., 1984). Form II crystals (5PTI) have the orthorhombic space group $P2_12_12_1$. The unit cell contains 4 protein molecules, along with 1 bound phosphate ion per protein molecule (Kinemage 1). The structure of form III crystals (6PTI) reported by Wlodawer et al. (1987b) was determined by X-ray diffraction alone. Like form II crystals, form III crystals are also orthorhombic, but the space group is $P2_12_12$, with one of the crystallographic 2-fold screw axes found in form II crystals replaced by a pure 2-fold axis. Each unit cell also contains 4 protein molecules, along with 1 bound phosphate per protein molecule (Kinemage 2).

The most extensive lattice contact for both crystal forms involves a loop extending from residues 35 to 44 (see contact 1 in Kinemages 1 and 2, and Kinemage 3). It is particularly extensive in form III crystals, where it spans the 2-fold crystallographic axis of symmetry. This contact contains intermolecular hydrogen bonds involving the main chains and side chains of Arg 39, Lys 41, Arg 42, and Asn 44 (see Table 1 of Wlodawer et al. [1987a] and Table 3 of Wlodawer et al. [1987b]). This lattice contact also involves the bound phosphate, which is hydrogen bonded to the side chains of Arg 20 and Tyr 35 from a symmetry-related molecule. Closer examination of this contact in form III crystals reveals an unusual interaction. Spanning the 2-fold crystallographic axis of symmetry, the N_ζ nitrogen of Lys 41 comes within 2.72 Å of the N_ζ nitrogen of Lys 41 on the symmetry-related molecule (Kinemage 3). Because this distance is less than the sum of the van der Waals radii for 2 nitrogens, it suggests that the 2 are hydrogen bonded to one another.

This raises some questions. Although primary amines can serve as both hydrogen bond donors and acceptors in the absence of water, in aqueous solutions they are more basic than water and are usually found in their protonated form. The protonated form is positively charged and lacks a nonbonded pair of electrons to serve as a hydrogen bond acceptor. The close approach of 2 positively charged amines, which cannot hydrogen bond to one another, is very energetically unfavorable. We therefore propose that only one of the Lys 41 side-chain amino groups in the interaction is protonated, and that the 2 N_ζ atoms in this interaction share a hydrogen between them. The proposed interaction is shown in Figure 1 and Kinemage 4. The hydrogen atoms in these figures were placed by energy minimization using CHARMm v.21.3 (Brooks et al., 1983). This was done while constraining the coordinates of all heavy atoms to their form III crystallographic values.

The pH where the transition from form II to form III occurs, pH 9.35, represents the pK_a for the loss of the first hydrogen from the interacting pair of amines. The 2 N_ζ atoms are also hydrogen bonded to the Arg 39 main-chain carbonyl O of the symmetry-related molecule (Wlodawer et al., 1987b). The switch-like nature of the proposed interaction is supported by the observation that, when the pH of a solution containing form III crystals is dropped below pH 9.35, the form III crystals are replaced by form II crystals.

An experimental pK_a value of 10.8 for the side chain of Lys 41 of dissolved BPTI was determined by Brown et al. (1976). A calculated value of 10.3 has also been reported (Yang et al., 1993). The Lys 41 amino groups in the unit cell of form II crystals are known to be protonated because hydrogens and deuterium atoms can be located by neutron diffraction. Upon formation of the form III crystals, the pK_a value of 1 of the 2 interacting Lys 41 side-chain amino groups is, therefore, shifted downward by 1 to 1.5 pH units.

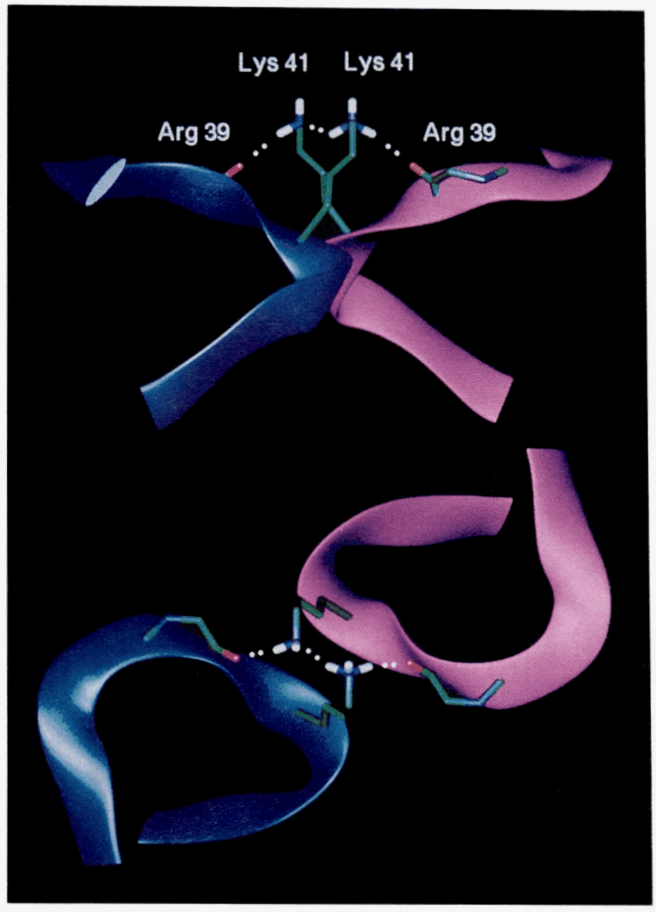

Fig. 1. The proposed molecular switch as it exists in form III crystals of BPTI. Top, looking at the interaction at right angles to the 2-fold crystallographic axis of symmetry. Bottom, looking at the interaction down the 2-fold crystallographic axis of symmetry. The blue and magenta ribbons trace the polypeptide backbones from residues 35 to 44 for 2 symmetry-related molecules. Placement of the hydrogens on the ϵ-amino groups of the Lys 41 side chains was done by energy minimization using CHARMm (Brooks et al., 1983). The models were produced using the Ribbons program (Carson, 1987).

The direction and magnitude of this shift in pK_a value is reasonable. Similar shifts of equal or greater magnitude are predicted when 2 groups having similar pK_a values interact strongly with one another (Yang et al., 1993). Other charged groups in the vicinity of the Lys 41 side-chain amine could also affect its apparent pK_a. In the absence of symmetry-related molecules, there are 3 charged groups within 10 Å of the Lys 41 side chain: the positively charged guanidinium side chain of Arg 39 at 6.60 Å, the negatively charged carboxylate side chain of Glu 7 at 6.79 Å, and the positively charged guanidinium side chain of Arg 42 at 9.86 Å (Kinemage 3, form III). The effects of the Arg 39 and Glu 7 side chains should nearly cancel one another, whereas the Arg 42 side chain, at nearly 10 Å away, should have a minimal effect at high salt concentrations. When form III crystals form, 2 additional positively charged groups are placed in the vicinity of the Lys 41 amino side chain (Kinemage 3, form

III): the Lys 41 amino group from a symmetry-related molecule at 2.72 Å and the Arg 39 guanidinium group from the same symmetry-related molecule at 4.70 Å. The resulting increase in the interaction energy due to charge–charge interactions will favor deprotonation of the Lys 41 amino side chain. According to the calculations of Yang et al. (1993), a shift of 1–1.5 pH units corresponds to an interaction energy of less than 2 kcal/mol. In form II crystals, the Lys 41 amino side chain is also involved in an intermolecular hydrogen bond. It forms a salt bridge with the negatively charged phosphate group that is bound to a symmetry-related molecule (Kinemage 3, form II).

The positive interaction energy needed to bring the 2 Lys 41 amino groups together in form III crystals implies other interactions must exist that favor the form III crystal over the form II crystals. When comparing all of the crystal lattice contacts in the 2 crystal forms (Kinemages 1, 2), there are 19 protein-to-protein intermolecular hydrogen bonds in form III crystals compared to only 8 in form II crystals. The greater number of intermolecular hydrogen bonds in form III crystals can also explain an observation we have made (Gallagher et al., 1992) that form III crystals have a higher thermal stability than form II crystals: when form II crystals are taken to 35 °C at pH 9.0, they crack and begin to break into smaller pieces within hours. On the other hand, when form III crystals are taken to 35 °C at pH 9.8, they remain stable for as long as 4 months.

In summary, we have identified a molecular switch that selects between form II and form III crystals of BPTI. The switch comprises the side-chain amino groups of 2 lysines from symmetry-related molecules, which hydrogen bond to one another in form III but not form II crystals. The interaction results in a downward shift of the pK_a for 1 of the 2 interacting amino groups by 1–1.5 pH units. At pH values above 9.35, where this interaction is observed, the form III crystal is favored over the form II crystal because of the many additional intermolecular hydrogen bonds that form as a consequence. When the pH drops below 9.35, both amino groups are protonated and form salt bridges to phosphate ions, and the formation of form II crystals becomes favored over form III crystals.

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