Peptidyl-prolyl Cis/trans Isomerase Activity In The Functioning Of Native Folded Proteins

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Abstract

The finding that cis/trans isomerization of proline peptide bonds can provide the basis for conformational changes in native folded proteins, ensuring transition between functionally different states, has led to marked progress in this field of research. Presently, there are reasons to assume that the proline switch can serve as a precise regulator of biological function in native folded proteins. This review aims to summarize the available information on proline switch mechanisms in different proteins, focusing the attention on features pointing to similarities or distinctions between these mechanisms.

Two groups of proteins are considered. The first one includes proteins which require the presence of an external factor accelerating the process of isomerization. These proteins are interleukin-2 tyrosine kinase SH2 domain and the Crk adaptor protein. Each of them is able to catalyze an intrinsic intramolecular switch afforded by prolyl cis/trans isomerization, but this process is very slow; it can, however, be significantly accelerated by cyclophilin A (CypA), a peptidyl-prolyl cis/trans isomerase. The second group, comprising 5HT3 receptor of neurotransmitter-gated ion channel, cytochrome P450 cam, and molecular chaperone Hsp70, can do without external catalysts; these proteins are able to accelerate cis/trans isomerization due to intrinsic structural features that facilitate this process. In each case, the likely mechanism of proline switch is discussed. Special attention is given to the possibility that a conformational switch may result from a shift of equilibrium between coexisting cis and trans conformers.

Introduction

Proteins exhibit considerable conformational dynamics, undergoing remarkable structural fluctuations and inner motions during the folding process and while performing their biological functions. Backbone flexibility in polypeptides results mainly from the rotational freedom of the single bonds, characterized by torsion angles $\phi$ and $\psi$. The peptide bond shows considerable double-bond character, the distance between the carbonyl carbon and the nitrogen being 0.15 Å shorter than expected for a C-N single bond. As a consequence, peptide bonds are planar. The properties of the rigid peptide bond ($\omega$ angle) substantially restrict the available conformational space, allowing molecules to populate only two ground state conformations, cis and trans, for rotation about the C-N bond.

In proteins, most peptide bonds are in the trans conformation, which is favored energetically over the cis in both folded and unfolded states. Yet an appreciable proportion of the peptide bonds preceding proline (prolyl bonds) are in the strained cis conformation. For peptide bonds preceding residues other than proline, the cis state is strongly disfavored. Because of this, non-prolyl cis peptide bonds are very rare in native, folded proteins. On the other hand, for Xaa-Pro peptide bonds, the cis and trans conformations are much more similar energetically because $\phi$ and $\psi$ of Xaa in each case are located close to a cis carbon atom, either the $C_\alpha$ or the $C_\delta$ of the proline. Since prolyl cis/trans isomerization involves rotation around a partial double bond, it is a very slow reaction with an activation barrier of about 20 kcal/mol and a half-time that ranges between 10 and 100 s [1]. The process takes so long because of the need to overcome the energy of resonance between C=O and N of the prolyl bond. The reaction can be accelerated when resonance is decreased, e.g. by protonating the carbonyl oxygen or in non-polar solvents. Because the trans and cis forms of X-Pro peptide groups are almost isoenergetic, (the trans conformation being only 2 kJ/mol more stable than the cis conformation [2]), isolated peptides exhibit a mixture of cis and trans forms [3]. Moreover, it has been shown that cis and trans isomers of some folded proteins may coexist in solution [4-7]. Owing to the fact that proline is the only naturally occurring amino acid for which the thermodynamic stability of the cis and trans isomers differs by a mere 1 kcal/mol, the proline-containing polypeptide backbone can populate discrete conformational and dynamic states separated by a modest interconversion activation energy [8]. So, a drastic structural rearrangement induced by peptidyl-prolyl isomerization can be expected to serve as an intrinsic molecular switch that controls protein function. This assumption was proved experimentally.
in several laboratories; the results obtained have demonstrated the validity of the hypothesis that cis and trans conformers represent functionally distinct conformational states of a protein. Understanding the mechanisms of molecular switching is crucial in order to appreciate the processes that control cellular signaling. In what follows, we shall consider the mechanisms and consequences of a “proline switch” in various systems.

Review

Proline isomerization as a molecular switch responsible for a protein’s transition from one functional state to another

Interleukin-2 tyrosine kinase SH2 domain: a molecular switch controlling ligand recognition

The nonreceptor tyrosine kinase, interleukin-2 tyrosine kinase (Itk) plays a role in the maturation of thymocytes, is required for intracellular signaling following T cell receptor crosslinking, and is involved in the generation of second messengers that mediate cytoskeletal reorganization. Itk is homologous to Src tyrosine kinases, being composed of SH2, SH3 and catalytic kinase domains. The SH2 and SH3 domains have important modular functions in targeting signaling molecules to specific locations and in regulating intramolecular interactions [9]. In contrast to Src tyrosine kinases, Itk lacks the C-terminal tail that contains the regulatory tyrosine. However, activation of Itk depends on SH2-mediated interactions with phosphorylated signaling partners [10], suggesting a regulatory role for the Itk SH2 domain despite the absence of a Src-like C-terminal regulatory tyrosine residue [11].

Structural analysis of the Itk SH2 domain, performed using NMR spectroscopy, has shown two well-defined, low energy conformations in slow exchange, populated in solution [12,11]. This conformational heterogeneity is entirely caused by cis/trans isomerization about the imide bond between Asn286 and Pro287, as the mutation of Pro287 to Gly leads to greatly simplified spectra, consistent with a single equilibrium system involving conformer-specific interactions [12,8]. In addition to the large displacement of the CD loop, statistically significant conformational differences were apparent between the structures of the cis and trans forms in the AB and BG loops and in other regions of the SH2 domain [12].

It is noteworthy that, by virtue of the structural and dynamic differences between the cis and trans isomers, they are able to bind different ligands. A phosphotyrosine-containing peptide preferentially interacts with the trans form of the SH2 domain. An addition of excess phosphopeptide to the Itk SH2 domain shifts the cis/trans equilibrium in favor of the trans conformer [11]. On the other hand, the cis form is responsible for the association between SH2 and the SH3 domains belonging to different Itk subunits. Indeed, an addition of excess Itk SH3 domain to the SH2 domain shifts the equilibrium to favor the cis SH2 conformer [11].

Taken together, these results are consistent with a model according to which interconversion between two conformationally and functionally different cis and trans SH2 domain isomers can directly regulate the SH2 domain substrate recognition, and, by consequence, influence the functioning of Itk. As demonstrated by Brazin et al.[11], this process can be regulated by peptidyl-prolyl cis/trans isomerase, cyclophilin A (CypA). Both in vitro and in vivo the data reveal a stable CypA-Itk complex in T cells and point to a role for CypA in repressing Itk kinase activity [11]. One possible mode of CypA action in vivo is shown schematically in Fig.1. It is proposed that CypA may facilitate rapid interconversion between trans and cis Itk conformers and thus between the phospholigand-bound and dimerized states, respectively. By lowering the kinetic barrier to interconversion, CypA can catalyze cis/trans isomerization of the conformationally heterogeneous prolyl imide bond within the SH2 domain. This way, CypA may play an integral role in a coupled equilibrium system involving conformer-specific recognition. Such a mechanism would allow Itk to switch binding partners more rapidly in response to exogenous signaling events.

Illustration 1
Proline switch in the Crk adaptor controls autoinhibition of this signaling protein

Crk family adaptors mediate the timely formation of protein complexes elicited by a variety of extracellular stimuli [13]. The regulatory mechanism in Crk includes interconversion between an open conformation, capable of associating with its biological partners, and an autoinhibited conformation. Cellular Crk consists of a single SH2 domain, an N-terminal SH3 (SH3N) domain, and a C-terminal SH3 domain (SH3C), see Illustration 2. The SH3N and SH3C domains are tethered by a 50-residue long linker containing a tyrosine residue that becomes phosphorylated by ?-Abl kinase [14]. Crk complex formation with a target protein is mediated by the SH2 and SH3N domains of Crk, which selectively bind to pY-x-x- P and P-x-L-P-x-K motifs, respectively [15, 16]. On the other hand, the SH3C domain does not bind to conventional polyproline II (PPII) -type sequences [17], and its biological function remained elusive until recently. Illustration 2

Using an integrated NMR, thermodynamic and biochemical approach, P. Sarkar et al. [18] have demonstrated the presence of a unique regulatory mechanism in Crk, controlled by an intrinsic molecular switch afforded by cis/trans isomerization of a proline residue located in the linker tethering the two SH3 domains. Isomerization of a single proline modulates the structural features of one of the domains of Crk in such a way that the two isomeric forms acquire different binding properties, rendering only one of them capable of inducing the autoinhibitory conformation. The principal findings of this study can be summarized as follows. First of all, the authors have found that part of the linker tethering the two SH3 domains interacts with SH3C, and that the interaction is modulated by a linker proline residue (Pro238) that exhibits cis/trans isomerization. Illustration 2 shows the construct that was used, which comprises the SH3C domain and the part of the linker containing all the conserved proline residues (hl-SH3C). Characteristic NOE correlations [19] between Gly237 and Pro238 indicated that the Gly237-Pro238 prolyl bond exists both in the cis and trans conformations. Mutant Pro238Ala adopted a unique conformation in contrast with the two conformations adopted by wild type hl-SH3C construct. NMR analysis demonstrated that all proline residues located within the boundaries of the SH3C domain adopt uniquely the trans conformation. It was concluded that the source of conformational heterogeneity in hl-SH3C is the presence of cis/trans isomerization about the Gly237-Pro238 prolyl bond, and that the two conformational states have similar stability. The consequences of the Gly237-Pro238 prolyl bond isomerization are pronounced. For some of the SH3C residues significant differences in the chemical environment of nuclei in the two conformations were observed. The residues mostly affected form a contiguous region that faces the linker. These data suggested that the presence of the linker affects the conformation of the SH3C domain, most likely through a direct linker-SH3C interaction. As a consequence of altered linker-SH3C interactions, the effect of cis/trans isomerization at Pro238 is not confined to SH3C, but extends to the linker as well, where large chemical shift differences between the two conformations were observed. The residues of the N-terminal half of the linker are not affected by the isomerization process; the overall results showed that only the residues at the C-terminal half of the linker participate in the interaction with the SH3C domain. Further study revealed that although the two SH3 domains are tethered with a long linker, they can interact with each other. Thus, chemical shift analysis of the SH3N domain in isolation and in the context of the SH3N-fl-SH3C construct showed that the SH3N-SH3C interaction is mediated by residues lining the conventional PPII- binding site of SH3N. This site, which specifically interacts with short sequences encompassing a P-x-L-P-x-K motif [20,16], is used by Crk to form a variety of protein complexes [21].

P. Sarkar et al. [18] suggested that such an intramolecular structural arrangement of Crk would be particularly important because the binding sites on SH3N for PPII ligands and SH3C would be overlapping. This hypothesis was proved experimentally, confirming that the binding sites on SH3N for PPII ligands and SH3C are mutually exclusive. It was therefore suggested that the residues forming a contiguous surface on SH3C may serve as the binding region for the SH3N domain. Combined, the NMR results raised the possibility that the SH3N/SH3C intramolecular arrangement in Crk may inhibit the binding affinity of SH3N for PPII ligands, thereby forming the basis of an autoinhibitory mechanism. Using isothermal titration calorimetry, the authors showed that PPII peptide binds to isolated SH3N with a KD ≈ 2 µM, whereas the affinity of PPII peptide binding to SH3N in the context of SH3N- fl-SH3C construct is lowered by a factor of 10. These findings corroborated the NMR data that SH3N is intramolecurarily inhibited by SH3C, structurally occluding the PII-binding site. An important observation from the NMR data was that only one set of peaks for the linker-SH3C region was present in the spectrum of the SH3N-fl-SH3C construct. This was in contrast to the conformational
heterogeneity seen for isolated SH3C. Chemical shift and NOE data demonstrated that in a SH3N-fl-SH3C structure the Gly237-Pro238 prolyl bond only exists in the cis conformation. Once the SH3C is displaced from SH3N by PPII peptide binding, the Gly237-Pro238 prolyl bond again adopts both the cis and trans conformations. These results are in line with the conclusion that the prolyl cis conformer stabilizes the autoinhibitory Srk conformation, whereas the trans conformer destabilizes it.

One of the most significant results was obtained upon subsequent detailed analysis of the SH3N-fl-SH3C spectra, which revealed the presence of a minor conformation, corresponding to ≈ 10% of the population of the major, autoinhibited conformation. The chemical shifts of the minor conformation resonances correspond to the isolated SH3N and SH3C domains. Interestingly, only the trans conformer of the Gly237-Pro238 prolyl bond was present for the peaks of the SH3C region in the minor conformation. It also has been established that the autoinhibitory conformation, which is stabilized only in the cis form of Pro238, exists in equilibrium with a low-populated uninhibited conformation favored by the trans form. Because the chemical shift in the resonances of the minor conformation corresponds to the characteristic chemical shifts of the unoccupied SH3N domain, these data suggest that the minor conformer may adopt an open, uninhibited state. In that case the PPII peptide is expected to preferentially bind to the uninhibited conformation as opposed to the autoinhibited one. The results presented by P. Sarkar et al. provide direct evidence thereof.

Illustration 3 schematically shows the conformational states of the Srk SH3N-fl-SH3C polypeptide and presents a model of its activation and autoinhibition. In a non-inhibited state, a single prolyl bond in the linker (Gly237-Pro238) undergoes cis/trans isomerization, giving rise to two different conformers which are equally populated. Because the topology of the SH3C binding surface is different in the cis and trans conformers, two alternative surfaces are presented for binding to SH3N. Only the trans conformer favors an open structure wherein the PPII-binding site is accessible to ligands. In the absence of PPII ligands, the free PPII binding area on the SH3N domain is occupied by a surface region on SH3C domain, which becomes accessible only in the cis conformer. The two domains associate, forming a closed, self-inhibitory complex.

To activate Srk in response to the appearance of new portions of PPII peptides, the trans conformer is required. According to the elegant mechanism of Srk activation discovered and studied in detail by P. Sarkar et al. [18], a low-populated (10%) uninhibited conformation of the Srk molecules with an open structure where the PPII-binding sites on SH3N domains are completely unoccupied, exists in equilibrium with the autoinhibitory conformation. Ligands will bind preferentially to the 10% of the Srk molecules with accessible SH3N-binding sites, shifting the equilibrium toward the uninhibited conformation. Taken together, the results of this study offer a striking example of how structural differences between the cis and trans conformers of a protein, arising from the isomerization of a single prolyl bond, can be a decisive factor in a regulatory mechanism.

Another property of the Crk activation process is the fact that the kinetics of interconversion between the cis and trans conformers can be regulated. A determination of the rate constant of Gly237/Pro238 isomerization in Crk has revealed that the kinetics of the switch is very slow (≈ 0.01 s^-1 at 22οC). To find out whether this process can be accelerated by a peptidyl-prolyl cis/trans isomerase (Cyp A), the fl-SH3C polypeptide was studied in the presence of catalytic amounts of CypA using 1H-15N NMR exchange spectroscopy [22]. The appearance of exchange peaks provided direct evidence that CypA catalyzes the Gly237-Pro238 cis/trans isomerization process. The exchange rate was found to be ≈ 80 s^-1 at 22οC. This suggests that the Gly237-Pro238-Phe239 motif in Crk contains a suitable binding site for peptidyl-prolyl-cis/trans isomerases, in accordance with the data by Piotukh et al. [23]. This indicates that if Pro238 is accessible to peptidyl-prolyl-cis/trans isomerase, the faster, CypA-mediated kinetics of the proline switch will contribute significantly to the rapid activation that Srk should exhibit in response to ligand binding in certain signaling pathways [18]. At the same time, the effectiveness of CypA as a catalyst can be regulated as well by modulating its accessibility to Pro238.

Cis-trans isomerization at a proline controls the opening and closing of the pore of a neurotransmitter-gated ion channel 5-Hydroxytryptamine type 3 (5-HT3) receptors are members of the Cys-loop receptor superfamily [24]. They are thus structurally and functionally distinct from the other classes of 5-HT receptors, which are all coupled to G-proteins and mediate slow-modulator responses via second-messenger signaling pathways. Ligand-gated ion channels are responsible for the rapid chemical transmission of nerve impulses at synapses, where the binding of a neurotransmitter to its receptor results in the prompt opening of an integral ion-selective pore. The speed of transduction, an
The 5-HT3 receptor is a large (487 amino acids) glycoprotein assembled from a ring of five homologous subunits, each containing one extracellular (predominantly β-sheet) and four (M1-M4) transmembrane α-helices [26-28]. Illustration 4 A shows schematically the topology of a subunit with an extracellular N-terminal domain followed by four consecutive membrane passes, a large intracellular loop between the M3 and M4 helices, and an extracellular C-terminus. The neurotransmitter-binding site is located in the extracellular domain, about 60° from the channel pore; its critical ligand-binding residue, Trp183 [26] is designated W. Communication between the neurotransmitter binding site and the structural elements involved in channel gating (the M2-M3 loop) is achieved through interaction of this loop with loops 2 and 7 of the extracellular domain. The apex of the M2-M3 loop contains a conserved proline (Pro308, designated P) that is ideally placed to provide a hinge for the movement of the channel-lining M2 helix. The key role of this helix in the structure of the pore region is firmly established, and it is believed that the side chains of Leu287 (located in the M2 helix), by virtue of their side-to-side interactions with neighboring M2 segments, constitute the gate of the pore.

Illustration 4

On the other hand, evidence has been obtained that Pro308 is essential for receptor function in the 5-HT3 receptor. According to Lummis et al. [29], a replacement of Pro308 with Gly, Ala, Cys, Val, Lys or Asn results in receptors that are properly trafficked to the membrane and display wild-type binding properties towards a radio-labeled antagonist, but all those receptors are non-functional. This indicates that mutations at Pro308 affect receptor function by altering gating, but not ligand binding, folding or structural elements involved in channel gating. Thus, several workers have noted the proximity of loops 2 and 7 to the M2-M3 loop [30?34]. The overall gating mechanism in the 5-HT3 receptor can be described as follows. In the closed state of the channel, Pro308 is in the trans conformation. This conformational isomerization at residue 308 and the gating of the 5-HT3 receptor. To obtain further support for the notion that a proline in the M2-M3 loop can serve as a structural switch, Lummis et al. [29] used NMR spectroscopy to examine the structure of a 20-amino acid peptide spanning the M2-M3 loop (SDLPATAIGTPLIGVYFVV; Pro308 is shown in italic). They observed with interest the clear presence of two conformers in the peptide structure, with the major form about five times more prevalent than the minor one. In the major conformer, Pro308 was assigned the trans conformation, based on observed Overhauser effect connections.

The overall gating mechanism in the 5-HT3 receptor is largely determined by the incorporation of the ion channel into the same protein that contains the neurotransmitter-binding site. This same property makes it possible to analyze the opening of single channels, and therefore the activation of this class of proteins has been studied exceptionally well [25].

The arrival of a neurotransmitter causes a movement of Trp183. This tryptophan residue is directly linked to loop 7 by a six-amino acid stretch of sheet structure, termed β7. Therefore, the binding site Trp183 may be the actuator that, perhaps via β7, releases the clamp on M2-M3. Pro308 can then undergo a spontaneous (or protein-aided) cis/ trans isomerization, gating the channel (Illustration 4). It is noteworthy that the study described above has made a significant contribution to the understanding of the role of cis/trans isomerism in the function of many other membrane proteins. In fact,
The use of multidimensional NMR methods, combined with extensive sequence-specific backbone resonance assignments, has allowed Pochapsky et al. to obtain a more complete characterization of the complex formed by CYP101 and Pdx [40-44]. Detailed information was received on the conformational changes in this enzyme induced by the effector. A conformational shift was detected in the active site of CYP101 upon Pdx binding, which results in a reorientation of the bound camphor in a manner appropriate for hydroxylation.

The most significant outcome of this study was the finding that the conformational change takes place with a rate constant between 150 and 200 s\(^{-1}\) at half-saturation at 25°C. This led the authors to suggest that the process might involve a cis/trans isomerization of an X-proline imide bond, a reaction which is implicated in a number of biological switches. To investigate the possibility that the conformational change induced by Pdx binding to CYP101 might have to do with an X-Pro isomerization, Pochapsky et al. [43] chose a proline residue (Pro89) which seemed a likely candidate for isomerization. It is one of the three prolines identified in all crystal structures of CYP101 as being in a cis conformation around the peptide bond with the previous residue Ile88, and the only one of those three not involved in a regular type VI turn.

Illustration 5

As shown in Illustration 5, which presents a portion of secondary structural elements in CYP101 most uniformly perturbed by the binding of Pdx, Pro89 is located at the N-terminal end of the B1 helix. The carbonyl oxygen of Pro89 provides the N-terminal hydrogen bond acceptor stabilizing the B1 helix. Tyr95, the last residue in the B1 helix, provides a hydrogen bond to the carbonyl of the bound camphor, and the B1 helix has also been implicated in gating access to the CYP101 active site [44]. Hence the conformational change of the Ile88-Pro89 imide bond is important in determining the shape and the accessibility of the active site of CYP101 [43].

Spectroscopic and site-directed mutagenesis experiments have supported the notion that the binding of Pdx to CYP101 drives the isomerization of the Ile88-Pro89 imide bond from a trans, or a distorted trans, conformation in the absence of Pdx to one that becomes cis in the Pdx-bound form. The authors propose that the structural perturbations detected by NMR in the active site and other distal locations of CYP101 upon Pdx binding are due primarily to this isomerization [43]. In other words, these results suggest that the cis conformer of the Ile88-Pro89 bond is the one favored upon binding of Pdx, is prompted, inter alia, by

Illustration 5

Further studies into this mechanism hold out considerable promise. They might, for example, provide new evidence to support the notion postulated in 1986 by Brandl and Deber that long-range interactions that stabilize the tertiary structure of a protein within a membrane can influence the rate of prolyl cis/trans isomerization of proteins isomerases?

As proposed by the authors of the study [29], the structural properties of the receptor itself could serve to accelerate the isomerization rate significantly. The mechanism could be similar to that used by prolyl

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the results obtained with a Tyr29Phe mutant. The phenolic OH group of Tyr29 stabilizes the cis conformer of the Ile88-Pro89 imide by hydrogen bonding with the carbonyl oxygen of Ile88. It has been found that the above mutation increases the KD for Pdx binding to CYP101 by an order of magnitude relative to the wild type enzyme. This change in KD corresponds to a destabilization of the Pdx-bound form of the Tyr29Phe mutant by about 6 kJ/mol, which is appropriate for the loss of a weak to medium hydrogen bond [43].

Assuming that cis-trans isomerization of the Ile88-Pro89 imide bond is indeed the structural switch responsible for the changes observed in CYP101 upon binding of Pdx, Pochapsky et al. reasoned that a comparison between the structures of the cis and trans conformers should show perturbations corresponding to those observed spectroscopically. To make this comparison, they performed a series of molecular dynamics simulations on CYP101 with the Ile88-Pro89 imide bond in either the cis or the trans conformation [45]. Based on this comparison, the authors predicted the structural consequences of the isomerization and suggested a mechanism for the effector activity of Pdx. They concluded that in the Pdx-free form of CYP101 the Ile88-Pro89 imide bond at the N-terminal end of the B1 helix is in a distorted trans conformation. In the trans conformer, the substrate access channel is open, and camphor is more solvent-exposed. Binding of Pdx on the proximal face of CYP101 forces the isomerization of the Ile88-Pro89 bond from trans to cis via a mechanical linkage provided by the C helix, the B1-C loop and the B1 helix. The isomerization moves the bound substrate into the correct orientation for subsequent hydroxylation, and closes the active site to substrate and/or solvent access [45].

The value of the rate constant for the conformational exchange occurring upon Pdx binding to wild type CYP101 (between 150 s−1 and 200 s−1) [41] indicates an activation free energy of ≈ 60 kJ/mol, which is below the value required for an uncatalyzed X-Pro isomerization (≈ 80 kJ/mol). This suggests that the process of isomerization needs a catalyst. Since the activation free energy barrier for reactions catalyzed by peptidyl-prolyl cis/trans isomerasers, e.g. cyclophylin A, is ≈ 50 kJ/mol, the authors (OuYang et al. [43]) proposed that the structure of the CYP101 molecule near the Ile88-Pro89 dipeptide may have some features in common with the active sites of known peptidyl-prolyl cis/trans isomerasers. It is generally accepted that these enzymes catalyze X-Pro cis/trans isomerization by creating a hydrophobic environment for the imide carbonyl (thereby destabilizing the charge-separated O- - C=N+ resonance structure that imparts a double-bond character to the C-N bond) and by providing a localized hydrogen bond donor to stabilize the lone pair on the imide hydrogen due to developing sp3 character in the transition state. It is interesting that the structure of CYP101 in the vicinity of the Ile88-Pro89 imide bond meets both of these requirements. The closest side chains to the Ile88-Pro89 imide (Met28, Tyr29, Phe87, Ile88, Pro89 and Ile395) are hydrophobic, and there are no water molecules within hydrogen-bonding distance of the imide in the crystal structure. The side chain hydroxyl of Tyr29, the only hydrogen bond donor to the Ile88-Pro89 peptide, is positioned so as to stabilize the cis conformation of this imide, but not the trans conformation. This hydroxyl group is also in a position to stabilize a developing electron lone pair on the Pro89 imide nitrogen by hydrogen bonding in the transition state of the isomerization [43].

Proline switch in allosteric regulation of molecular chaperone Hsp70

Molecular chaperone Hsp70 (DnaK in E.coli), whose main function is to assist the folding of newly synthesized polypeptides, is a monomer consisting of two major structural elements: an N-terminal 44-kDa nucleotide-binding domain connected by a hydrophobic 10- to 12- amino acid linker to a 26-kDa substrate binding domain [46-49]. The interaction of the substrate-binding domain with hydrophobic peptide stretches within substrate polypeptides is regulated by the nucleotide status of the nucleotide-binding domain. Thus, the binding of ATP induces conformational changes leading to the opening of the substrate binding pocket, and the affinity for substrates is lowered [50-52]. Conversely, substrate binding to its binding pocket in the substrate-binding domain, in synergy with the action of the J-domain cochaperone (DnaJ in E.coli), triggers ATP hydrolysis in the ATPase domain and the subsequent reversion of the conformational changes in the substrate binding domain to the high affinity state [53-56]. In the ADP state of the nucleotide binding domain, substrate protein is bound stably. The release of the tightly bound ADP is accelerated by nucleotide exchange factors [57-59]. Rebinding of ATP and the concomitant dissociation of the nucleotide exchange factor complete the reaction cycle.

The mechanism of ATP-dependent intramolecular allosteric regulation between the nucleotide binding and substrate binding domains of Hsp70 has been the subject of intense studies. Although the structures of the individual domains have been available [47], structure determination of a full-length Hsp70 protein...
was performed only recently. Chang et al. [60] reported the crystal structures of DnaK from Geobacillus kaustophilus HTA426 and the 70-kDa heat shock cognate protein from Rattus norvegicus, both complexed with ADP. The results of this study made it clear that in the ADP-bound conformation, the nucleotide-binding and substrate-binding domains of Hsp70 are markedly separated from each other.

In order to explore the full allosteric cycle in Hsp70 proteins, Swain et al. [61] employed a combination of NMR and other biophysical methods to study both the isolated domains and the two-domain DnaK protein from E. coli. A global model for Hsp70 allosteric regulation proposed by the authors suggests that in the ADP-bound state, the two domains are independent and connected by a flexible linker. ATP binding causes a docking of the substrate-binding domain onto the ATPase domain with concomitant major structural changes in both domains. The binding of substrate leads to uncoupling of the two domains and to enhancing the ATP hydrolysis rate of the ATPase domain.

However, the above studies did not elucidate the allosteric control mechanism effecting the ATP-regulated conformational changes in separate domains of Hsp70. A great stride forward was made by Vogel et al. [62], who discovered a role of a proline switch that assumes alternate conformations in response to ATP binding and hydrolysis. One problem to be solved was explaining the mechanism used to overcome the high energy barrier between the two states of the chaperone: the ATP-bound state with high association and dissociation rates for substrates, and the ADP-bound state with two- and three orders of magnitude lower association and dissociation rates. Indeed, the spontaneous transition from the ATP-bound state to the ADP-bound state, coinciding with spontaneous ATP hydrolysis, occurs with a time constant of up to 30 minutes. This points to a high energy barrier between the two states [62]. Numerous studies, including NMR spectroscopy [63-64], have failed to clarify the nature of the energy barrier and the residues involved.

Vogel et al. reasoned that any allosteric control mechanism effecting ATP-regulated conformational changes in separate domains needs four elements to work: an ATP sensor, a transducer, a switch, and a lever. The ATP sensor must be part of the ATP-coordinating residues, and the lever part of the target domain, i.e. the substrate-binding domain in the case of Hsp70. Transducing the ATP binding event could involve many residues in separate subdomains, or a transduction path of interconnected residues [62]. To identify the hydrogen bond network involved in these effects, published crystal structures of bovine Hsc70 ATPase domains in different nucleotide states were analyzed. This resulted in the discovery of a hydrogen bond network converging onto the surface-exposed, universally conserved Arg155 (Illustration 6). Between Arg155 and the Mg2+ and ATP γ-phosphate-coordinating residue Glu175 was identified a proline residue (Pro147), located in a small loop (residues Val146-Pro147-Ala148-Tyr149) and connected to several key residues of this network. Illustration 6

The carbonyl oxygen of Pro147 is about 3.5Å apart from one of the terminal nitrogens of Arg155, and the imido group of the Pro147 is located at a distance of 3.3 Å from the Glu175 side chain carboxyl group. In addition, the side chain of Lys71, which is believed to act as catalytic residue by positioning a water molecule for the nucleophilic attack on the γ-phosphate [65], is in close proximity to the side chain of Pro147 [65]. The authors [62] hypothesized that Pro147 might constitute the molecular switch that, in response to ATP binding, assumes an alternate conformation, inducing and stabilizing the open conformation of the substrate binding domain via Arg155. The reversion of the conformational change, which occurs either spontaneously at a very low rate or is triggered by synergistic action of a substrate and a J domain protein, elicits γ-phosphate cleavage by positioning the side chain of Lys71 and, coordinated by its amino group, a water molecule at the perfect distance for nucleophilic attack on the γ-phosphate of ATP (Illustration 7).

Illustration 7

This hypothesis was tested by several experimental approaches. First, Vogel et al. exchanged the corresponding residues in E. coli Hsc homologue DnaK: Pro143 (corresponding to Pro147 in Hsc70) for Ala (P143Ala) and Gly (P143G), and Arg151 (Arg155 in Hsc70) for Ala (R151A) and Lys (R151K). In addition, Glu171 (Glu175 in Hsc70) was replaced with Asp (E171D) and Gln (E171Q). An examination of the effects of these amino acid replacements on ATPase activity indicated that Pro143 and Glu171 are important for catalysis. Thus, replacements of these residues reduced the basal ATP hydrolysis rate of DnaK by one to two orders of magnitude, and the γ-phosphate cleavage rate of the mutant proteins was not stimulated by DnaJ. Notably, while the overall decrease of the ATPase rate following a replacement of Glu171 is easily explained by its functioning in coordination with the catalytically important Mg2+, consistent with earlier findings for Hsc70 [66], the effects of Pro143 replacement cannot be explained by a direct role in catalysis, yet they are consistent with
the hypothesis that Pro143 is important for the positioning of the catalytic residues by virtue of its contacts with Lys70 and Glu171.

In contrast to the DnaK variants discussed above, replacement of Arg151 resulted in an increase of the basal rate of ATP hydrolysis. The ATPase rate of such mutant proteins could not be further stimulated by DnaJ or substrate, indicating that this residue is essential for the allosteric regulation of ATPase activity by the substrate binding domain. These data strongly suggested that Arg151 is part of the mechanics that trigger the conformational changes in the substrate-binding domain upon alteration of the nucleotide status. In an attempt to identify other residues that continue the signal transduction pathway from the surface of the ATPase domain into the substrate-binding domain, Vogel et al. found two positively charged, surface-exposed residues in the ATPase domain (Lys155 and Arg167), and a negatively charged residue in the linker connecting both domains (Arg393) that are important for interdomain communication. This led the authors to suggest that this linker most likely serves as the lever that is widened by the substrate-binding domain and the cochaperone (DnaJ) onto the ATPase domain to induce a conformation favorable for ATP hydrolysis [67].

Considering that the flexible linker exists in two alternative conformations, and the point in the crystal structure of the substrate-binding domain at which the linker bends into these two conformations is precisely Asp393, the authors proposed that ATP binding to the ATPase domain causes a bending of the linker at position 393 with the consequence of inserting it between the ATPase domain and the substrate-binding domain. This movement gives rise to the low affinity state of the substrate-binding domain. Substrate and DnaJ binding lead to a repositioning of the linker, which in turn sets off a conformational change in the ATPase domain, most likely involving Lys155, Arg167 and Arg151, which finally causes the proline switch to transit into the conformation optimal for ATP hydrolysis [67].

Taken together, the results of this study show that the peptide backbone in position 143 in the ATPase domain of DnaK constitutes a switch in mutual allosteric control between the ATPase domain and the substrate-binding domain [62]. The prime role of the proline residue in the realization of this effect is supported by findings about the energy barrier controlling the switch position: Pro143 increases the enthalpy of activation for ATP hydrolysis by 40% and by 100% as compared to the R143A and R143G mutants, respectively. The Pro143 position is relayed through the universally conserved surface-exposed Arg151 to the substrate-binding domain. Replacement of this residue by alanine obliterates the mutual allosteric control of the ATPase domain and the substrate-binding domain.

A question arises as to the nature of the alternate conformations of Pro143. To address it, Vogel et al. focused on the mechanism of cis/trans isomerization of a proline-preceding peptide bond as a process which results in the interconversion of two conformers which differ little in stability (the trans conformation is only 2 kJ /mol more stable than the cis conformation [2]). Because of a high energy barrier between these two states, their intrinsic interconversion rates are low. However, the process can be accelerated under conditions affecting the local environment of the proline residue. Such is probably the case of transitions between the two alternate states of DnaK, although a direct demonstration of proline isomerization is not possible since the protein molecule is too big for this kind of NMR studies. The energy barrier that controls the two states in DnaK (103.8 kJ/mol) has a value consistent with cis/trans isomerization of a proline-preceding peptide bond. It remains unknown which form (cis or trans) emerges as a result of the switch, although a cis-peptidyl-prolyl bond in position 143 can be fitted into the ATPase domain structure.

In summary, the results of this study suggest that the mechanism of allosteric regulation of Hsp70 relies on the existence of two different conformers of this chaperone which are interconverted as a result of cis/trans isomerization of a proline peptide bond. Such conformers are shown schematically in Illustration 8. Illustration 8

Proline switch in phage infection performs a dual function, operating as a molecular timer. The gene-3-protein (G3P) of filamentous phages is essential for the infection of E. coli. G3P consists of three domains. The C-terminal domain is partly embedded in the phage coat and serves to anchor G3P to the phage tip, whereas the N-terminal N1 and N2 domains protrude from the phage surface and are used for sequential interaction with the target cell during infection. N2 can be further decomposed into the globular subdomain, which resembles N1 is size and chain topology, and the hinge subdomain, which provides most of the contacts with N1 [68,69]. In the latent state of the phage, N1 and N2 are tightly associated, forming a bilobal structure in which the larger N2 domain wraps around the smaller N1 domain, ensuring high long-term stability of G3P but rendering the phage incompetent for infection. In a step-wise process, this resting form of the phage first
binds with a surface-exposed region of the N2 domain to the tip of the bacterial F pilus. This activates G3P by exposing the binding site for TolA, which is the ultimate phage receptor at the bacterial cell surface [70]. The TolA binding site is located on the N1 domain at the N1-N2 interface and thus is protected in the closed form of G3P. In the course of phage infection the open, binding-competent form is deployed (Illustration 9 A). It must persist until the N1 domain reaches the C-terminal domain of TolA [71,72]. Therefore, a local unfolding reaction is required to activate the G3P protein for the infection.

Illustration 9
The subsequent step, i.e. the refolding of N2 and association of the domains, appears to be very slow, showing a time (τ) of 6,200 s at 250C. As discovered by Martin and Schmid [73], this process is controlled by cis/trans isomerization of the Gln212-Pro213 bond in the hinge subdomain of N2, near the interface between the two domains. As this takes place, the conformational energy accumulated in the course of refolding becomes available to shift the prolyl cis/trans equilibrium [74]. The G3P gets locked in a stable but non-infectious form. The slow switch provided by the isomerization would ensure that domain reassembly is suppressed long enough so that N1 can interact with TolA after the pilus has been retracted. To clarify the structural basis of proline-limited domain docking in G3P, the role of the hinge subdomain of N2 was studied in detail.

The central part of the hinge is formed by two non-contiguous segments of the polypeptide chain that run in opposite directions (see Illustration 9). The first segment (residues 88-123) connects the glycine-rich linker between N1 and N2 with the globular subdomain of N2. The second segment (residues 203-217) is located after the globular subdomain of N2 and leads into the other linker that connects N2 with the C-terminal domain. The chain region 208-216, which includes Pro213, forms numerous hydrogen bonds with the N1 domain. Notably, the side-chain amide of Gln212, which precedes the cis peptide bond, forms two H bonds with the side-chain amide group of Gln52 in N1. It is also hydrogen bonded with the CO of Pro211 and thus fixes the backbone adjacent to the cis Gln212-Pro213 bond. In view of these facts, the authors suggested that the network of interdomain interactions around the Gln212-Pro213 bond is required for a firm docking between N1 and N2, and that it will be lost when this prolyl bond isomerizes from the native cis to the incorrect trans conformation [73]. They also proposed that the isomerization at Pro213 triggers a movement of the entire N2 domain relative to N1, beyond a hinge located at residues Gly99 and Ser208 [73]. It therefore seems likely that the "open" and "closed" states of G3P can be described as different (trans and cis) conformers of this protein.

To investigate whether the unusually slow, proline-limited domain movement in G3P is also important for the function of the protein during phage infection, F. Schmid and colleagues performed a series of in vivo experiments [75]. It was shown that phage infectivity correlates inversely with the rate of trans-to-cis isomerization of the peptide bond between residues 212 and 213, and thus with the lifetime of the open form of G3P. Mutations at either side of Pro213 modify the isomerization rate. In the purified N1-N2 protein, the Q212A and P214A mutations decrease the time constant of isomerization from 6,200 s to 3,800 s and 1,900 s, respectively, whereas the P213G mutation accelerates isomerization 26-fold.

In their entirety, these results were summarized as follows. For phage infection, the prolyl isomerization in G3P is used as both a timer and a switch. With a cis-Pro213 the timer is switched off, and in this state the phage is stable and robust but not infectious. The timer is turned on when the N2 domain of G3P binds to the F pilus. This binding weakens the N1-N2 domain interactions, presumably by local unfolding, and thus relieves the conformational tension that keeps Pro213 in the cis state. In a spring-like action, Pro213 reverts to the more stable trans state, and thus the timer is set [75].

Conclusion(s)
Several examples of proline switches considered in this review demonstrate the existence of two groups of proteins employing different mechanisms. The first group requires the presence of an external factor accelerating the process of isomerization. It includes proteins such as interleukin-2 tyrosine kinase SH2 domain and the Crk adaptor protein. Although each of them is able to catalyze an intrinsic intramolecular switch afforded by prolyl cis/trans isomerization, this process is very slow; it can, however, be significantly accelerated by CypA, a peptidyl-prolyl cis/trans isomerase. Since the participation of an enzyme catalyst in the functioning of the above proteins has
been firmly established, it is reasonable to conclude that peptidyl-prolyl cis/trans isomerases can play an important role in the regulation of processes associated with a proline switch. The second group of proteins catalyzing a proline switch can do so without external assistance; they are able to accelerate cis/trans isomerization by virtue of their own structural features that facilitate this process. Such is the case of the proline switch that controls the opening and closing of the pore of a neurotransmitter-gated ion channel [29]. A good testimony to the ability of the 5-HT3 receptor to accelerate the isomerization of Pro308 for himself is the fact that the opening rate of the 5-HT3 receptor pore is several orders of magnitude higher than the intrinsic prolyl cis/trans isomerization rates. Taking into account that even a simple hydrogen bond to the proline imide nitrogen can accelerate isomerization ≈ 260-fold [76], the authors suggest that specific features of the receptor provide the means to hasten the isomerization rate significantly.

Another example illustrating the ability of a protein to catalyze cis/trans isomerization of prolyl residues included in the structure of that same protein is cytochrome P450cam. Considering that the activation free energy barrier for the conformational exchange observed upon effector binding to this protein (≈ 60 kJ/mol) is close to the activation barriers for reactions catalyzed by peptidyl-prolyl cis/trans isomerases (≈ 50 kJ/mol), the authors [43] proposed that the structure of the cytochrome P450cam molecule near the Ile88-Pro89 bond which undergoes isomerization, has features in common with the active site of known peptidyl-prolyl cis/trans isomerases. It is generally accepted that these enzymes catalyze X-Pro isomerization by providing a hydrophobic environment for the imide carbonyl and by procuring a localized hydrogen bond donor to stabilize the lone pair on the imide hydrogen. In line with that assumption, the structure of cytochrome P450cam in the vicinity of the Ile88-Pro89 imide bond meets both of these requirements.

In the case of the proline switch in molecular chaperone Hsp70 [62], transition from one conformer to another is triggered by changes in the hydrogen bond network which connects the nucleotide binding site with the peptide backbone including Pro143 and its environment. Given the location of Pro143 in close proximity to catalytic residues Glu175 and Lys71, as well as about 3.57 from one of the terminal nitrogens of conservative Arg175, it can be suggested that moderate alterations in these interactions would trigger isomerization. One might expect other events demonstrating the ability of native folded proteins to catalyze cis/trans isomerization of prolines integrated in their own structure to be uncovered in the near future.

In spite of the fact that the mechanisms of proline switch considered above differ from one another, one may point out the general principles observed in all cases. In each and every one of them the proline switch brings about an interconversion between cis and trans conformers, which differ both structurally and functionally. The effector triggering the switch may be the substrate of an enzyme (as in the case of interleukin-2 tyrosine kinase SH2 domain [8]), or an effector protein (as in the case of cytochrome P450cam [43]), a neurotransmitter (as in the case of the 5HT3 receptor of neurotransmitter-gated ion channel [29]), a specific ligand (as in the case of the Crk adaptor [18]), or an allosteric effector (as in the case of molecular chaperone Hsp70 [62, 67]). Interaction with an effector results in an accumulation of conformational energy which becomes available to drive prolyl isomerization [74]. Because the trans and cis forms of X-Pro peptide groups are almost isoenergetic (the trans conformation being just 2 kJ/mol more stable than the cis conformation [2]), the proline-containing polypeptide has the ability to populate two discrete conformations, which coexist with each other. In a number of studies reviewed above, a coexistence of cis and trans prolyl isomers was detected by NMR spectroscopy (the cases of interleukin-2 tyrosine kinase SH2 domain, the 5HT3 receptor of a neurotransmitter-gated ion channel, and the Srk SH3N-fl-SH3C polypeptide). In the latter case, the coexistence of cis and trans conformers of this polypeptide was shown to play a pivotal role in the mechanisms of its autoinhibition and activation (see Illustration 3). As considered above, the autoinhibitory conformation has been shown to exist in equilibrium with a low-populated (10%) uninhibited conformation of the Srk molecules representing the trans conformer. When new portions of substrate appear, they bind preferentially to the ≈ 10% of the Crk molecules with an accessible SH3N-binding site, which shifts the equilibrium toward the uninhibited conformation. Therefore, to activate Srk the substrate does not need to induce any specific conformational changes in the SH3N/SH3C-binding interface, but may instead drive the activation by shifting the equilibrium between cis and trans conformers [18]. These results are noteworthy since they offer impressive evidence that the function of an effector may be restricted to a selection between two preexistent conformational states. One is tempted to suggest that similar mechanisms may be at work also in other cases of proline cis/trans isomerization, which...
await further study. Another point of interest lies in a new area of research concerned with the functioning of cis/trans isomerization as a molecular timer to help control the amplitude and duration of cellular processes. As considered in the final part of the review relating to the mechanism of prolyl isomerization in phage infection, this exciting field of investigation is progressing successfully.

References

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Illustrations

Illustration 1

Illustration 1. Equilibrium model encompassing the CypA-catalyzed Itk SH2 cis/trans interconversion, cis-mediated SH3 binding (Itk dimerization), and trans-mediated phospholigand (pY) binding. Adapted from Brazin et al. [11].

Illustration 2

Illustration 2. Schematic diagram of domain organization in Crk. The various Crk fragments used in the study are indicated: hl-SH3C denotes the part of the linker connected with the SH3C domain (a.a. residues 210-297); fl-SH3C denotes the full linker bound to the SH3C domain (a.a. acid residues 190-297), and SH3N-fl-SH3C designates both SH3 domains bound to the linker (a.a. residues 135-297). The position of Pro238 is indicated. Adapted from Sarkar et al. [18].
Illustration 3

Illustration 3. Model of the equilibrium of conformational states of Crk SH3N-fl-SH3C polypeptide, its activation and autoinhibition. In the noninhibited state SH3N and SH3C domains do not interact owing to the presence of substrate ligands (shaded ellipses) forming complexes with SH3N domains. In the absence of substrates, the cis conformer of SH3C associates with the SH3N domain, stabilizing the inactive state. Activation occurs by substrate binding to a low population of uninhibited states wherein the SH3N-ligand binding site is accessible, thereby shifting the equilibrium toward the SH3N-substrate bound state. Adapted from P. Sarkar et al. [18].

Illustration 4

Illustration 4 A. Schematic representation of two neighboring subunits of the 5-HT3 receptor. In the closed state of the channel (left), Pro308 (designated P) is in the trans conformation, which is stabilized by interaction between the loop connecting helices M2 and M3, and loops 2 and 7 of the extracellular domain (this interaction is shown as a series of horizontal lines). Binding of the neurotransmitter (5HT) initiates a structural change in the extracellular domain, which is transmitted to the M2-M3 linker, releasing the clamp on M2-M3 and allowing Pro308 to isomerize to the cis form. This change at a crucial pivot point reorients the M2 transmembrane helix, opening up the channel (right). The M2 helix contains the gate of the channel, Leu287 (designated L). B. Schematic structure of the entire receptor, viewed from the synaptic cleft. M2 is shown lining the pore (dark spheres). Adapted from Lummis et al. [29].
Illustration 5

Illustration 5. Elements of CYP101 structure markedly perturbed by binding of putidaredoxin (Pdx). cam a?" camphor. Adapted from OuYang et al. [43].

![Illustration 5](image)

Illustration 6

Illustration 6. Model of the ATP binding site within the Hsp70 nucleotide-binding domain. Shown in stick representation are the nucleotide and the amino acid residues which are supposed to participate in a hydrogen bond network connecting the ATPase site with the structural elements of the substrate-binding domain. Putative hydrogen bonds are indicated as dashed lines. Dotted lines indicate the close proximity of Glu147OE to Pro147N and the preceding carbonyl of Val146, both at a distance of 3.3Å. In italics are the residue numbers of the corresponding residues in E. coli Dnak. Adapted from Vogel et al. [62].

![Illustration 6](image)
Illustration 7

Illustration 7. Proposed mutual allosteric mechanism controlling conformational changes in the substrate binding domain and ATP hydrolysis in Hsp70. A. ATP binding is sensed by Lys70 and Glu171 through water-bridged hydrogen bonds to the γ-phosphate and the Mg2+. B. This interaction triggers the conformational change of Pro147 and moves Arg151 toward the substrate-binding domain, leading to the opening of this domain. C. Substrate and DnaJ interaction through Arg151 trigger the reversion of the conformational change in Pro147, moving Glu171 and Lys70 into a perfect position for catalyzing ATP hydrolysis (hydrolysis transition state). Dotted lines indicate hydrogen bonds; arrows indicate movement. Adapted from Vogel et al. [62].

Illustration 8

Illustration 8. Different states of the Hsp70 molecule. (1) Apo-form. Nucleotide-binding (left) and substrate-binding (right) domains do not interact. (2) ATP binds to an active center which is not yet optimally formed for catalysis. A net of intramolecular interactions which affect the microenvironment of Pro143 side chain is altered, triggering peptide bond isomerization; the effect is transmitted to the substrate-binding domain. The two domains come close together, and a conformer with low affinity for substrate is stabilized. (3) Binding of substrate (S) and cochaperone (J), together with the effect of linker (L), induce conformational changes resulting in the stabilization of another conformer and triggering one more peptide bond isomerization. This second conformer has an active center optimally suited for ATP hydrolysis and exhibits a high affinity for the substrate. (4) ADP is bound; the substrate becomes encapsulated in its domain; the nucleotide-binding and substrate-binding domains are separated.
fig. 8
Illustration 9

Illustration 9. Schematic representation of the trans (A) and cis (B) conformers of G3P. In the infectious state (A), N1 and N2 domains are separated. The network of interdomain interactions around Gln212-Pro213 bond is lost. In the stable non-infectious form of G3P (B), N1 and N2 are tightly associated; numerous hydrogen bonds exist between the hinge and the N1 domain, which assures the firm docking between N1 and N2. F- pilus binding site and TolA- binding site are indicated, as well as the position of Pro213 in the second segment of the hinge subdomain of N2. See text for details. Adapted from Eckert et al. [75].
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