Minireview

The mode of action of peptidyl prolyl *cis/trans* isomerases in vivo: binding vs. catalysis

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Abstract Polypeptides often display proline-mediated conformational substates that are prone to isomer-specific recognition and function. Both possibilities can be of biological significance. Distinct families of peptidyl prolyl *cis/trans* isomerases (PPIases) evolved proved to be highly specific for proline moieties arranged in a special context of subsites. Structural and chemical features of molecules specifically bound to the active site of PPIases served to improve catalysis of prolyl isomerization rather than ground state binding. For example, results inferred from receptor Ser/Thr or Tyr phosphorylation in the presence of site-directed FKBP12 mutant proteins provided evidence for the crucial role of the enzymatic activity in down-regulating function of FKBP12.

1. Introduction

Proteins, in having binding capacity for other molecules, control cellular reactions differently. In this way, enzyme proteins have to convert biomolecules chemically whereas binding proteins release biological effects from complexes by realizing togetherness. In both cases capacities for ligand binding are required at a single site of the protein. Differentiating between the alternative functions is crucial in understanding cellular effects but not always an easy task for protein–ligand interactions. Commonly, difficulties in studying reversible reactions are encountered in cases of minor differences of chemical properties between the reactant and product state. Typically, the assignment to a cellular function of peptidyl prolyl *cis/trans* isomerases (PPIases), which catalyzes equilibration of conformers, suffers from the lack of pronounced chemical differences between the interconverting molecules. Unlike other enzymes utilizing polypeptide substrates (proteases, protein kinases, protein phosphatases) the catalytic mechanism of PPIases promotes a permanent population of two ground state Michaelis complexes upon joining a proline-containing peptide chain and a PPIase. Originally discovered as helper enzymes for accelerating restructuring of the polypeptide backbone [1], these proteins have a proline-directed binding capability proposed to be of exclusive biological importance [2,3]. Moreover, catalysis of prolyl bond[1] isomerization was discussed as a side effect attributable to the hydrophobic nature of the substrate binding site of the PPIases.

To date, the existence of three families of PPIases has been established represented by numerous proteins ubiquitously occurring from primates to Archaea [4,5]. The families were named cyclophilins (Cyp), FK506 binding proteins (FKBPs) and parvulins [6]. The competitive inhibitors rapamycin, FK506 and cyclosporin A display both tight binding to and high specificity for differentation of the PPIase families. However, dissecting PPIase functions in vivo will not succeed with these inhibitors because active site competition simultaneously affects both complex formation and catalysis. Furthermore, significance to cell signalling of enzyme activity cannot be inferred from inhibitory effects because of the additional bioactivity associated with the PPIase-inhibitor complexes themselves [2].

However, there is a major difference between the alternative modes of action of PPIases that may serve to make a decision. Evolved to perform catalysis, an enzyme promotes transition state binding. Ground state binding to ligands has been the driving force for a binding protein to evolve. Obviously, natural substrates are expected with most pronounced effects in differentiating both situations but, when still unknown, structure-function relationships within series of peptides may serve to approximate the cellular state. Similarly, site-directed mutagenesis of PPIases may provide a valuable tool to distinguish between those functional alternatives thought to be differently affected in binding and catalysis of ligands by the amino acid substitution.

2. Conformational diversity is intrinsic to prolyl bonds

The traditional electron resonance model describes most of the biologically important properties of secondary amide and imide peptide bonds, including hindered bond rotation, to be involved. It confers splitting of prolyl bond rotational isomers between two energetically preferred states, *cis* and *trans*, leaving aside regions of dihedral angles ω different from the values of $0 \pm 15°$ and $180 \pm 15°$, respectively. Crossing of the barrier between the conformations is rather costly in terms of energy. Both states have to become populated in linear proline peptides unless severe constraints are imposed by tertiary interactions, crystal packing effects and internal strain. In addition, immediately after ribosomal synthesis *trans* prolyl bonds are expected throughout. Adjacent amino acids exert local sequence effects on the *cis/trans* ratio but cannot liberate more than about 8 kJ/mol free energy differences between the gene-

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[1] The term prolyl isomerisation is used throughout the paper for the *cis/trans* isomerisation of the peptide bond preceding proline in an amino acid sequence. Similarly, the term prolyl bond is synonymous with the peptide bond preceding proline.

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coded residues [7,8]. This energetic contribution does not suffice to shift the *cis*/*trans* equilibrium completely to a certain side.

The coexistence of the isomers remains constant in time in the absence of energy-coupled vectorial processes involving the conformers differently, like membrane transport, bond formation, bond cleavage and folding.

The vectorial events correlate with the re-equilibration of isomers to occur. The equilibration rate is controlled by a first-order rate constant k_{obs} equivalent to the sum of the *cis* → *trans* and *trans* → *cis* isomerization rate constants as an upper rate limit when the coupled process is of much slower rate than *cis*/*trans* isomerization. At high velocity of the coupled reaction two limiting rates become alternatively possible, depending on which of the isomers, the minor or the major one, is preferentially sequestered into the coupled event. For prolyl isomerizations half times may cover the range of seconds to many minutes at ambient temperature.

Excluding N-terminal proline the number of *cis*/*trans* isomers theoretically formed is given by 2^n with n accounting for the number of prolines within the sequence. The peptide chain must have a sequence-derived predisposition to distribute to all *cis*/*trans* states in aqueous solution. Conformational homogeneity roughly parallels increasing chain-length under native conditions as was comprehensively indicated by the exclusive isomeric state of most prolyl bonds, either *cis* or *trans*, in biologically active proteins. The energy costs associated with the preferential population of a certain isomer have to be covered by favorable tertiary interactions.

Under denaturing conditions in the presence of high concentrations of urea or GdmHCl the peptide chain becomes uniformly solvated. Three out of four prolines of RNase A exhibit isomerization rates and *cis*/*trans* ratios typical of those found in short oligopeptides [9].

More native conditions existed when NMR spectra revealed the 16 possible prolyl isomers in a prolactin receptor-derived octapeptide consisting of a four-proline array supplemented with non-polar amino acids [10]. The dominance of local effects is still evident by having present all isomers at a fraction $> 1\%$. However, the first indication of the relevance of long-range interactions comes from the enhanced *cis* fraction (70%) of the Val-Pro-Gly moiety that does not fit by about 8kJ/mol into the structure–*cis*/*trans* ratio relationship derived from short oligopeptides [7,8].

Dramatic alterations in the *cis*/*trans* ratio were found for three out of four prolyl bonds in a 25-mer oligopeptide corresponding to residues 81–125 of the HIV-1 p24 capsid protein [11,12]. Energetically, the Gly-Pro^{90}-Ala segment behaves normally in that the ratio of conformers did not reflect any influence of remote parts of the molecule. The segments His-Pro^{85}-Val, Ala-Pro^{93}-Gly and Glu-Pro^{99}-Arg, however, completely lack the conformational multiplicity expected stabilized in the *trans* state throughout. Curiously enough, using different structural probes evidence for the existence of any of the common secondary structure elements was lacking for the 25-mer. This observation raises the question of the origin of energy stabilizing the pure conformational state of these prolyl bonds. The increasing frequency of reports about prolyl isomerization in native proteins confirms that the coexistence of conformationally homogeneous and heterogeneous prolyl bonds in a single, folded peptide chain is a general phenomenon. [13–16].

3. The choice of the prolyl isomer for mediating bioactivity

There is no need to discuss enzymes that catalyze the interconversion between biologically indistinguishable molecules. However, *cis*/*trans* isomers do not belong to this type of compound. They cannot sample the same conformational space around proline [7,17], thus presenting a distinct topography to interacting groups. In this way, a precondition for resulting isomer-specific reactions is the inability of macromolecules to provide sufficient binding energy to preorganize ligands into reactive conformations. Usually, proteins are not able to adopt ligands containing wrong prolyl isomers giving rise to isomer-specific recognition. The question about which molecule is biologically relevant among a diversity of prolyl isomers was already answered in a few simple cases, but needs to be addressed as a central concern in others. Regarding isomer specificity we adopt the theory that either the ligand or the acceptor protein undergoes isomerization. In the first case analyses were easy to perform by measuring enzyme kinetics under specific conditions as was shown for proline-containing substrates underlying proteolysis. Dependent on the proline position relative to the scissile bond, a moderate to absolute specificity in discriminating *cis* prolyl bonds was found for many proteases [18]. The presence of PPIases completely abolished realization of isomer specificity of substrate degradation in vitro [1,5] and in vivo [19,20], provided that the proline peptide could also be used as a substrate of the respective PPIase.

Of the *cis*/*trans* conformers of Cyp18 substrates in solution, the PPIase sequesters more the *cis* form, leading to a slightly altered *cis*/*trans* ratio of the Michaelis complexes near unity [21]. According to Burbaum et al. [22] this ratio may postulate that cytoplasmic Cyp18 is likely to operate in vivo nearby the *cis*/*trans* equilibrium rather than far away from it. Because the latter situation is realized for nascent polypeptide chains cyclophilins might be targeted to other folding states of proteins.

Turning to another protein function, the intestinal H^+/peptide symporter was shown to transport only *trans* prolyl dipeptides from the uptake medium into Caco-2 cells, leaving the fraction of the *cis* isomers unbound and thus outside the cell [23]. In this case, kinetic control of uptake by isomer specificity cannot be dissolved in cells enzymatically because PPIases capable of catalyzing dipeptides have not yet been detected. In other cases the bioactivity of peptide ligands was identified to relate to either *cis* [15,24,25] or *trans* isomers [26] using analogues that constrain critical peptide bonds.

What is more important, several examples show that the function of native proteins can be separated by prolyl bond isomerization [27–29]. For example, the Gly-Pro^{17} moiety has been addressed to be the likely candidate of an ATP-dependent induced-fit movement in rabbit muscle. Indeed, Cyp18 caused a two-fold increase in rate for this domain movement [30]. On the contrary, Cyp18 proved to be unable to catalyze the native-state isomerization of Gly-Pro^{43} in calbindin D_9K [16], eventually indicating lack of productive binding by sterical crowding.

4. Subsite interactions correlate with catalysis

Extended chain segments represent the neighborhood of proline attached to the active cleft of complexes of Cyp18 with proline-rich polypeptides totalling nine amino acids in

length [3,31]. This mode of binding is similar to that of ligand peptides described for the Hsp70 family. These proteins are representatives of another class of folding helper proteins, the chaperones [32]. However, so far no evidence for a chaperone-like activity of a prototypic PPIase was obtained. Thus, Cyp18 failed to increase the refolding yield of carbonic anhydrase protein that suffers from aggregation-prone folding intermediates [33]. In addition, the prototypic PPIases exhibit low affinity for unfolded polypeptide chains, since relevant V/[S] characteristics did not reveal substrate saturation up to the upper μM range [34]. Thus, the K_m values of multidentate polypeptides approach those of short peptides in the millimolar range. Similarly, the native state interaction of Cyp18 with HIV-1 capsid protein was found to exhibit a relatively high affinity >10 μM despite the use of multiple binding sites [35]. For comparison, dissociation constants have been measured for SH3 domains which evolved to mediate proline-directed protein–protein contacts. Octapeptides excised from SH3 domain ligands bind in the range of 10 μM [36] already, and HIV-1 Nef exhibits avid binding below the μM range. Systematic extension of peptide chain lengths led to quite different effects regarding ground state and transition state binding in PPIases. According to the catalytic model of Cyp18 [21] the K_m values approximate ground state binding K_s by $K_s \geq K_m$ whereas k_{cat}/K_m represents complementarity to the transition state. In going from a *cis* dipeptide to a *cis* tetrapeptide ground state affinity increases 70-fold [37]. However, k_{cat}/K_m increases $>10^6$-fold, indicating that binding energy of additional subsites is mainly used to increase k_{cat}. Also, introduction of side chain phosphate on threonine in a -Ser-Pro-Arg-moiety causes 1300-fold increase in k_{cat}/K_m for the PPIase activity of the parvulin-like human Pin1 [38] indicating marked transition state preference of phosphate group binding capability. In contrast to conventional PPIases like FKBP12 and Cyp18, Pin1 (and the yeast analogue ESS1) evolved to utilize binding energy of just a single polar subsite to increase k_{cat} enormously.

Beside the prototypic members of the PPIase families larger proteins exist that possess a PPIase core supplemented with domains of different functions. Only a few investigations exist about the coexistence of the extra modules with the catalytic core. However, it has been shown that the 48-kDa trigger factor, which is a FKBP-like PPIase bound to the 50S subunit of the *E. coli* ribosome, is constructed from a central PPIase core flanked by two autonomous folding modules. The considerable affinity to unfolded polypeptide chains of the flanking modules did not serve to increase catalysis, but is solely used for ground state binding [34]. Again, the excised catalytic module alone lacks enhanced ground state affinity. Consequently, proline recognition is not involved in the chaperone-like affinity of the flanking domains [39]. These domains can thus enable a PPIase core to approach selectively cell sites prone to protein unfolding. A similar function can be hypothesized for FKBP52 because active site blocking does not deplete its aggregation suppressing properties [40]. Regarding domain function the large bovine RanBP2 and fly NinaA [41,42] might represent cyclophilin counterparts of the FKBP-like trigger factor. Indeed, contingent on the presence of the flanking Ran-binding domain 4 of RanbP2, R/G opsin binds stably to the cyclophilin-like PPIase. NinaA does not have a Ran-binding domain 4 but a hydrophobic C-terminal extension of the catalytic core which is well suited for binding of a subset of opsins. Both stable binding to and PPIase activity of the cyclophilin domain were necessary for production of visual pigments in COS-1 cells [41].

5. Dissecting functions by site-directed mutagenesis

Once site-directed mutations are made to explore catalytic functions of PPIases it is necessary to establish structural integrity, residual activity toward a broad range of substrates and conservation of the binding function of the active site cleft. Obviously, prerequisites have been evaluated only in part for most mutant PPIases hitherto reported. For example, FKBP12 dependent Ca^{2+} channel properties of the muscle ryanodine receptor could be restored with the apparently inactive Phe^{99}-Tyr FKBP12 variant, suggesting that enzyme activity was dispensable for biological function [43]. This FKBP12 variant maintained binding to the receptor, but in a reduced manner. However, this FKBP12 variant was not completely devoid of activity in other PPIase assays. Even more, the ratio of activity of wt FKBP12 to mutant enzyme depended on the amino acid sequence of the substrate [44,45]. Considering these results functional complementation by the Phe^{99}-Tyr FKBP12 variant does not yet rule out the necessity of PPIase activity for ryanodine receptor function. Other results with mutant PPIases also need re-interpretation in the light of activities monitored by improved PPIase assays [46].

Receptors provide exquisitely sensitive probes for evaluating the effects of mutant PPIases because of signal amplification. Fortunately, it was possible to make use of the transforming growth factor-β (TGFβ) signal transduction to dissect FKBP12 functions. FKBP12 inhibits TGFβ type I receptor (TβR-I) signalling function by preventing TβR-II-coupled phosphorylation of the Gly/Ser/Thr-rich motif of TβR-I [47]. The Leu-Pro^{194} moiety succeeding the phosphorylation site in TβR-I proved to be critical to the FKBP12-TβR-I interaction, since the Leu-Gly^{194} but not the Leu-Phe^{194} mutation eliminates FKBP12 effects. With oligopeptides, kinetic data demonstrated that the Leu → Gly substitution in the FKBP12 substrate exclusively deteriorates k_{cat}/K_m, leaving binding avidity unaltered [48]. Similarly, wt FKBP12-mediated decrease in TβR-1 phosphorylation could not be mimicked by the Phe^{36}-Tyr FKBP12 variant in cotransfected R1B cells [47]. Remarkably, the same FKBP12 mutant protein leads to contrasting results with the ryanodine receptor discussed above: restoration of biological activity with the ryanodine receptor vs. inability to replace wild-type enzyme with the TβR probe. Such results have to be expected from the substrate specificity of enzymes.

In another example, the effect on signalling of the partial disruption of the catalytic machinery of the Phe^{99}-Tyr FKBP12 variant became also apparent, but even in a simple cell-free assay. The sequential signalling events induced by binding of EGF to the EGF receptor (EGFR) include receptor dimerization, stimulation of its intrinsic tyrosine kinase activity and finally autophosphorylation of defined tyrosine residues of the receptor protein. Negative regulation of autophosphorylation after EGF stimulation was obtained by externally added human recombinant FKBP12 in plasma membrane fractions of A431 fibroblasts [49]. At high concentrations of FKBP12 even the basal level of EGFR autophosphorylation is almost invisible. Replacement with low-activity FKBP variants, like Phe^{99}-Tyr FKBP12 and Asp^{37}-Leu

FKBP12, of wt FKBP12 can restore its inhibitory potential to a small extent only. The remaining inhibitory potential was thought to indicate residual PPIase activity of the mutant enzymes as measured in the oligopeptide assay. According to this model, ground state binding affinity to oligopeptides of the low-activity Phe^{99}-Tyr FKBP12 variant was only slightly reduced, ruling out binding as a source of the inhibitory effect of wt FKBP12 on EGFR autophosphorylation (T. Zarnt, unpublished). It is worth noting that PPIase specificity plays a crucial role in the inhibitory effect. Despite high enzyme activity toward standard substrates, neither Cyp18 nor the FKBP-like catalytic domains of the *E. coli* trigger factor were able to mimic the effect of FKBP12.

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