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Structure-Function Correlation for ras p21 and the Molecular Origin of Cancer

Final Report

In the past five years we followed different routes in correlating the structure and function of $p21^{ras}$ on an atomic level. Our main project focused on understanding the GTPase mechanism catalyzed by $p21^{ras}$ and other GTP-binding proteins. The progress on this front is summarized below.

Our starting point was the crystal structure of $p21^{ras}$ that was solved by the Kim group[1] and the Wittinghofer group[2] and paved the way for any attempt of understanding the hydrolysis mechanism in this protein. The crystallographic analysis has identified a water molecule (Wat175) in a position that makes it likely to be able to act as the nucleophile in the hydrolysis reaction. This water is directly located between the γ–phosphate and the side chain of Gln61 in one of its possible orientations. This arrangement and the fact that mutations of Gln61 decrease the GTPase reaction rate[3] led to the suggestion that this residue plays an important role in catalysis by acting as the general base for the nucleophilic water molecule and that it is assisted by Glu63.[2]

In examining this proposal we noted that at least in aqueous solution the amide group side chain of Gln is a very weak base with a pK_a of about -0.5. Yet, it was still possible that the protein makes Gln61 a reasonable base. In order to resolve this crucial problem we applied our *Empirical Valence Bond* (EVB) and the Protein Dipoles Langevin Dipole (PDLD) approaches. These approaches allows one to convert the issue of the energetics of proton transfer steps in proteins to an almost purely electrostatic question, giving the free energy of such steps as the sum of the experimentally known energy in solution (this energy is given by the pK_a difference between the protein donor and acceptor) and the change in electrostatic energy of moving the reacting groups from water to the protein active site. Our study indicated quite accurately that the $GlnH^+$ OH^- ion pairs in the protein are even less stable than in water. Thus, we concluded that a proton transfer from Wat175 to Gln61 in the active site of $p21^{ras}$ is less favorable than in the corresponding process in aqueous solution (where the activation barrier is already too high to account for the observed rate in $p21^{ras}$).The error range in our electrostatic calculations was smaller than the difference between the predicted activation barrier for the Gln61 as a base mechanism and the observed activation barrier.[4] Our result also suggested that Glu63 will have little or no effect for the GTPase reaction. In fact later we could demonstrate with site-directed mutation that Glu63 indeed is not important for catalysis[5]. In another study Schultz and coworkers[6] replaced Gln61 in $p21^{ras}$ by an isoelectronic nitro-analogue that has a pK_a some ten units lower than the amide group of Gln61, and yet has the same catalytic activity. On the basis of these results one can conclude that Gln61 is not likely to be the general base for the GTPase reaction of $p21^{ras}$.

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Using our computer simulation approaches, we analyzed several alternative mechanisms.[7] The only tested mechanism that was in agreement with the energetics of this reaction is a mechanism where the substrate GTP itself is the general base of the reaction. Further, as a result of our study we proposed that the pK_a of the γ-phosphate of GTP bound to $p21^{ras}$ must be around three if this group indeed would qualify as general base for the reaction mechanism. On the basis of these studies it was proposed that GTP hydrolysis in $p21^{ras}$ is initiated by the abstraction of a proton from the catalytic water molecule by the γ-phosphate of protein-bound GTP. The generated nucleophilic hydroxide ion subsequently attacks the protonated γ-phosphate in an S_N2-like mode and thus creates a trigonal bipyramidal transition or intermediate state, respectively.

To test this *GTP as a base* proposal we determined the corresponding pK_a of bound GTP in collaboration with the Wittinghofer group from the Max-Planck-Institute. With ^{31}P-NMR and via the pH-profile of the intrinsic GTPase reaction we could indeed demonstrate that the relevant pK_a is 2.9 for wildtype $p21^{ras}$[5]. One of our major goals was to understand why the exchange of a single residue in some positions in $p21^{ras}$ could decrease the GTPase rate and thus could stimulate cell growth and induce cancer. To address this question and to also test the proposed *GTP as a base mechanism* we determined the pK_a of GTP bound to many different Ras-mutants. To our surprise we found a linear relationship between the logarithm of the intrinsic GTPase rate at neutral pH and the relevant pK_a of bound GTP[5]. This relationship provides a very strong indication that the γ–phosphate of the GTP itself is the general base of the reaction.

We use this *Linear Free Energy Relationships* (LFER) to examine the mechanism of the intrinsic as well as the GAP-stimulated reaction of Ras- and Ras-related proteins. When LFER are observed it implies that the mechanism of the reaction is unchanged despite small changes in the reaction conditions. That is, one can use LFER not only to obtain new insights in a reaction mechanism for a particular protein but also to examine whether certain mutants or even different proteins follow the same mechanistic reaction route. In order to test if different Ras-mutants have functional similarities we extended the original LFER found for $p21^{ras}$ to a wide range of oncogenic as well as non-transforming $p21^{ras}$-mutants.[8] In this work we could demonstrate that 14 Ras and 2 Rap1A proteins follow the observed LFER. From this result we can conclude that these proteins exhibit the same *GTP as a base* reaction mechanism that was proposed before for Ras-wild type. The difference in GTPase rate seems to be a consequence of a pK_a change of the terminal GTP phosphate due to the corresponding mutation: The higher the pK_a of the γ-phosphate and the stronger its proton abstraction potential the faster is the reaction rate at neutral pH. In fact from this relationship alone, one can conclude that the γ-phosphate of the GTP itself is the *general base* of the reaction. Interestingly, most of the common known oncogenic mutants of $p21^{ras}$ follow this relationship. This suggests that they follow the same reaction mechanism as Ras-wild type does. A pK_a deviation of less than half a unit seems to be enough to slow down the intrinsic GTPase reaction rate in oncogenic mutants. This effect might be indirectly responsible for the oncogenic potential that these mutants display. In another study we could show that the increase in GTPase rate due to an exchange of the Ras-bound Mg^{2+} by Mn^{2+} is also a result of an increase in pK_a by almost half a unit.[8] Furthermore, it was found that Rap1A proteins follow the same type of LFER as $p21^{ras}$ does. Thus we conclude that Rap1A proteins probably also follow the same proposed *GTP as a base* mechanism. This supports the notion that many if not all members of the superfamily of *guanine nucleotide binding proteins* are not only structurally closely related to each other but also share a common reaction mechanism. In fact, structural studies with transducin[9] and the muscle motor ATPase myosin[10] reveal that

the active site of these proteins resemble that of $p21^{ras}$ and it was suggested that the *GTP (ATP) as a base mechanism* is also operative in these proteins.

Our studies have provided strong indications that the mechanism of GTP hydrolysis by GAP-activated $p21^{ras}$ is probably closely related to that in $p21^{ras}$ alone. A LFER similar to the one applied to the intrinsic GTPase of $p21^{ras}$ reveals that the rate of the GAP-stimulated GTPase of $p21^{ras}$ correlates with the basicity of bound GTP in Ras alone. This indicates that the GAP-stimulated reaction might also follow the *GTP as a base* mechanism.

While in case of the intrinsic GTPase reaction most of the Ras mutants display a Brønsted slope of $\beta = 2.1$ (a small set of oncogenic mutants exhibit a $\beta = \infty$) the corresponding Brønsted slope for the GTPase reaction of $p21^{ras}$ in presence of GAP is about $\beta = 4.9$. In a recent theoretical analysis we established the basis for such LFER in general and apply these concepts to $p21^{ras}$ and related systems.[11] It was demonstrated that the optimal way to analyze LFER is using *Marcus' type parabolas* that represent the reactant, intermediate and product state of the reaction in a relevant energy diagram. The observed LFER was used[11] to analyze the different feasible mechanisms and reaction paths for the intrinsic GTPase reaction in $p21^{ras}$.

In another study we examined the electrostatic control of GTP and GDP binding $p21^{ras}$.[12] In this work the crystal structures of $p21^{ras}$ were correlated with the binding affinities of GTP and GDP by calculating the relevant electrostatic energies. It was demonstrated that such calculations can provide a road map to the location of "hot" residues whose mutations are likely to change functional properties of the protein. Furthermore, calculations of the effect of specific mutations on GTP and GDP binding were found to be consistent with those observed. This helps to locate functionally important parts of the protein and to shed light on its interaction with exchange factors.

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