Signaling mechanistics: **Aluminum fluoride for molecule of the year**

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Recent three-dimensional structures of phosphoryl transfer enzymes in their aluminum fluoride bound state and corresponding biochemical data have shown how diverse biological problems can be investigated using this small inorganic molecule.

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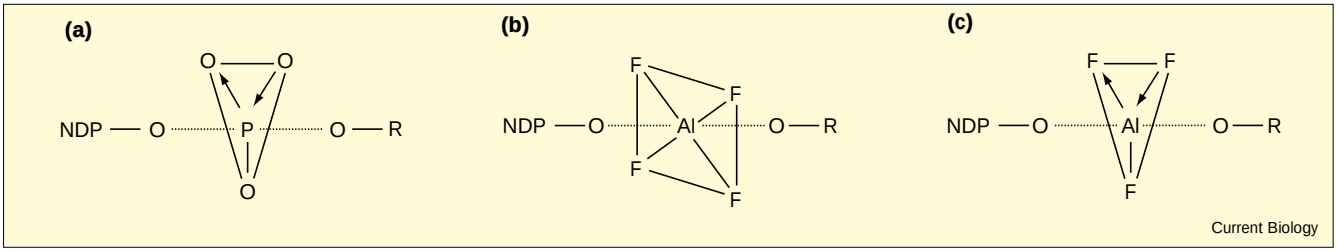
I do not know who decides what should be the biological molecule of the year. For 1997, I vote for aluminum fluoride. The choice of this small inorganic molecule may seem strange, but this year a number of reports on its use have appeared, with far-reaching consequences for our understanding of some very fundamental processes in biological systems.

Why aluminum fluoride? The answer is that aluminum fluoride complexes with proteins can be used to study the mechanistic aspects of phosphoryl transfer reactions in biology. These are of fundamental importance, as phosphoric acid anhydrides such as ATP and GTP control most of the reactions involved in metabolism, growth and differentiation. Aluminum fluoride entered the scene with the accidental finding by Sutherland and co-workers in 1958 that adenylate cyclase is activated by fluorides [1]. At the time, this made no sense in molecular terms and it took Gilman and co-workers [2] another 20 years or so to solve the puzzle.

They found that the target of the activation was in fact a heterotrimeric G protein and that the active stimulatory agent was aluminum fluoride (AlF_4^-), which is present as a leached-out impurity in millimolar solutions of fluoride in glass (*in vitro*, literally) [2]. When complexed with the protein, the aluminum fluoride was believed to mimic an oxygen-bound phosphate, since aluminum and fluoride have properties similar to phosphorus and oxygen, respectively, and the P–O bond length is similar to that of Al–F. As G proteins are activated when they go from the GDP-bound to the GTP-bound state, it was assumed that the aluminum fluoride occupies the γ-phosphate-binding site on the protein and together with bound GDP makes the G protein act as if it has bound GTP [3]. Later it was found that aluminum fluoride is a good analogue for a γ-phosphate for a number of other ATP- and GTP-converting enzymes.

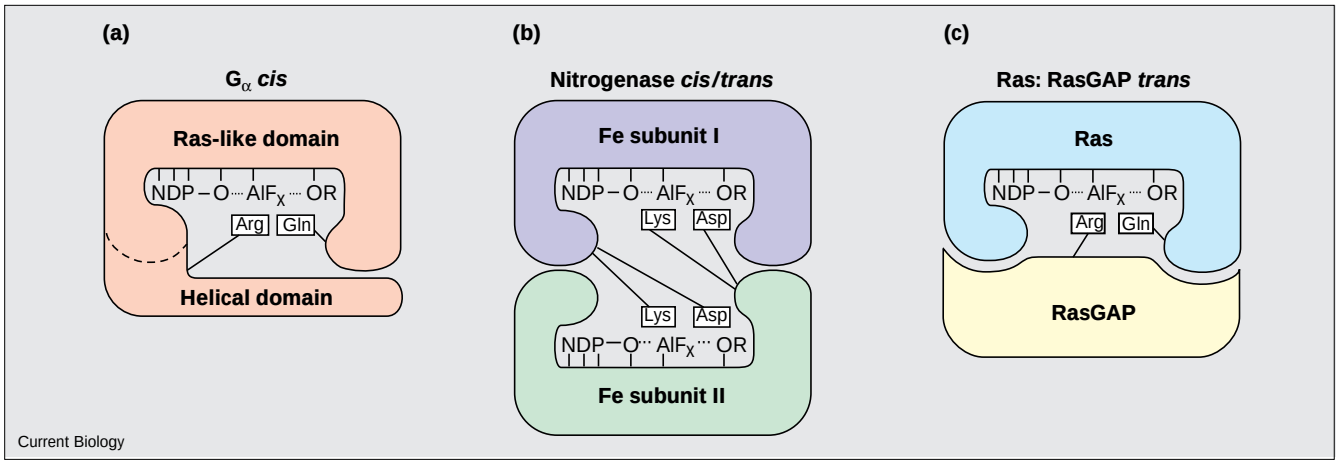
The next milestone was the determination in 1994 of the three-dimensional structures of heterotrimeric G proteins bound to GDP and aluminum fluoride [4,5]. As expected, aluminum fluoride is located in the γ-phosphate-binding site of these proteins. Unexpectedly, however, it mimics the transition state of the phosphoryl transfer reaction rather than the ground state. This is because aluminum is bound to four fluoride ligands in a square-planar coordination with two oxygen ligands at the apical position of the resulting octahedron (Figure 1a,b). One oxygen ligand is a β-phosphate oxygen, the leaving group in the transfer reaction, whereas the other is the oxygen from water believed to represent the attacking nucleophile of the hydrolysis reaction. Invariant arginine and glutamine residues, which are required for GTP hydrolysis but not for its binding, stabilize the aluminum fluoride binding to Gα·GDP (Figure 2a). The structure determination both supports the conclusion that aluminum fluoride binding mimics the transition state of

Figure 1



Schematic drawing of (a) a phosphoryl transfer reaction transition state, which is mimicked by (b) aluminum tetrafluoride and (c) aluminum trifluoride. Dotted lines indicate that the degree of bond making and bond breaking determines whether the transition is more dissociative, with a metaphosphate-like intermediate, or associative, with a pentavalent intermediate. Charges have been omitted for clarity. NDP, nucleoside diphosphate; R, nucleophile.

Figure 2



Schematic drawing showing the close similarity of the transition state mimic in **(a)** G_α proteins, **(b)** nitrogenase and **(c)** the Ras:RasGAP complex. In each case a crucial positive charge is supplied into the active site either in *cis* from the same molecule (a), in *cis/trans* from another subunit of the homodimer (b) or in *trans* from a different molecule (c). In each case the active site is complemented by a crucial and structurally homologous Gln (a,c) or Asp (b), which in the case of nitrogenase comes from the other subunit.

the reaction and identifies the role of the invariant arginine and glutamine in GTP hydrolysis.

Work published more recently on the three-dimensional structures of complete nitrogenase [6] and of the Ras:RasGAP complex [7] highlight the usefulness of aluminum fluoride to study even more complex biological problems. Nitrogenase is the enzyme that reduces elemental nitrogen (N_2) to ammonium (NH_4^-) in biological nitrogen fixation [8]. It couples the transfer of electrons for the reduction of nitrogen to the hydrolysis of ATP; two molecules of ATP are hydrolyzed per electron transferred. Nitrogenase is composed of an Fe protein, which contains an Fe-S cluster and a P-loop motif found in many ATP- and GTP-binding proteins [9], and an Fe-Mo protein, an $\alpha_2\beta_2$-heterotetramer with two metal ion clusters. The individual structures of these proteins have been solved by X-ray crystallography, and indicated how nitrogen might bind to the Fe-Mo protein. In the three-dimensional structure of the Fe protein dimer, however, only one ADP per dimer was bound in a mode that does not resemble the binding of other P-loop proteins [9]. With the structures of the enzyme subunits available, the main question remaining was how ATP hydrolysis is coupled to the electron transfer reaction.

As a major step towards this goal, the structure of the complex between the Fe protein and the Mo-Fe protein has recently been solved in the presence of ADP and aluminum fluoride [6]. Starting from the observation that the two proteins bind to each other reasonably well only in the presence of ADP and aluminum fluoride [10,11], Schindelin *et al.* [6] prepared such a complex with a relative molecular mass of 360K and solved its three-dimensional structure. It shows major rearrangements in the complexed Fe protein compared with the uncomplexed protein. The major conformational change involves movement of the two Fe subunits towards each other, which modifies the interface between the two subunits around the nucleotide-binding sites and at the same time shifts the 4Fe-S cluster away from its solvent-accessible location observed in the uncomplexed Fe protein.

In contrast to the individual Fe protein structure determined earlier, this structure shows two nucleotides bound per dimeric Fe-S protein, and bound in the same way as expected for a P-loop protein. Most remarkably, for each subunit, three residues from the other subunit contribute to the binding of the ADP·AlF_4 moiety. The most important of these residues seem to be a lysine, which contacts a phosphate oxygen of ADP, and an aspartic acid, which is in a suitable location to serve as a potential general base for activation of water (Figure 2b). The structure of the transition state mimic rearranges the interface between the Mo-Fe protein and the Fe protein such that the 4Fe-S cluster of the latter is now close to the interface of the former and is located such that an electron transfer path between one of the ion clusters of the Mo-Fe protein can now be traced to the Fe-S cluster. This path would not exist without the structural rearrangement.

An important element of this conformational change is a DxxGD motif, the glycine of which is involved in a main-chain interaction to a fluoride. Assuming a similar interaction to the γ-phosphate oxygen, this interaction is also found in the same motif in GTP-binding proteins, where

the glycine in DxxGQ is part of the universal conformational switch mechanism of these proteins [12]. The second aspartic acid is homologous to the invariant glutamine in GTP-binding proteins. As GTP hydrolysis drives the conformational change and is responsible for interrupting the interaction with the effector, the analogy with nitrogenase suggests a way in which ATP hydrolysis and a subsequent conformational change would break the interaction between the ATPase and the electron transfer system.

Another variation on the molecular switch system has been observed in the Ras:RasGAP complex [7]. The proto-oncogene product Ras is a small GTP-binding protein [12] that is a component of intracellular signalling pathways involved in cell growth and division. It has a very low intrinsic GTPase reaction rate that is stimulated 10^5-fold by GTPase-activating proteins (RasGAPs) that downregulate the accumulation of Ras·GTP. Ras binds aluminum fluoride only in the presence of RasGAP, and an efficient GTPase site is only created by the addition of stoichiometric amounts of RasGAP. One particular arginine residue is required for the catalytic activity of RasGAP and its ability to induce formation of the transition state [13]. These findings are complemented and highlighted by the determination of the structure of a complex between RasGAP and Ras·GDP in the presence of aluminum fluoride [7]. It shows that aluminum fluoride, in this case aluminum trifluoride, forms a pentagonal bipyramid, with the fluorides forming the trigonal base with two apical oxygen ligands (Figure 1c). From this structure, it was concluded that RasGAP supplies an arginine finger into the active site to contact one of the fluorides. Extrapolating from this to the real transition state the authors conclude that RasGAP stabilizes the transition state by neutralizing developing charges on the γ-phosphate during phosphoryl transfer. This agrees very well with the structure of the transition state in G_α proteins [4,5], the difference being that in G_α the arginine is supplied in *cis* from the same molecule, and in *trans* in the Ras:RasGAP complex (Figure 2c).

GAP proteins are specific for their respective Ras-related protein and the structure of RhoGAP in complex with Cdc42 has been solved by X-ray crystallography [14]. Functional RhoGAPs also contain an invariant arginine, which is essential for GAP activity. In the complex between Cdc42 and RhoGAP, this arginine is not in the active site and does not contact the γ-phosphate. This suggests that the arginine is not required to form the ground state, but is probably required for the transition state of the GTPase reaction. This is in line with biochemical experiments on G_{ai} [5] and Ras [13]. Also, from phosphorus NMR experiments with Ras·GppNHp, no change is observed on addition of RasGAP, making it unlikely that an arginine of RasGAP contacts a phosphate oxygen in the ground state [15].

GAPs for heterotrimeric G proteins, called RGS (for regulator-of-G protein signalling), have recently been identified. Biochemical evidence showed that RGS proteins bind with higher affinity to G_α·GDP·AlF_4 than to the triphosphate state of G_α, indicating that RGS stabilizes the transition state of the G_α GTPase. This was confirmed by the three-dimensional structure of the RGS·$G_{\alpha i1}$·GDP·AlF_4 complex determined by x-ray crystallography, in which RGS contacts the regions of G_α involved in GTP hydrolysis [16].

In the case of G_α proteins, in myosin [17] and in nitrogenase, AlF_4^- is the active site species, whereas in Ras:RasGAP complex, it is AlF_3. It is not clear whether these differences reflect some fundamental difference between these proteins or, more likely, are just due to technicalities. The structures of the two metabolic enzymes nucleoside diphosphate kinase [18] and uridylate monophosphate kinase [19] also showed AlF_3 is the transition state mimic. The reason for the differences are not clear at present; however, a pentavalent aluminum more closely resembles the real transition state of the phosphoryl transfer reaction (Figure 1).

Another important issue addressed by the recent structures of aluminum fluoride complexes with phosphoryl transfer enzymes is whether the transition state is mostly dissociative, with a metaphosphate-like intermediate, or associative, with a pentavalent phosphorus. The structures of the transition state mimics show distances between aluminum and the leaving group and nucleophilic oxygen that are intermediate between van der Waals and covalent bonds [4,5,18,19], suggesting a mostly associative character. More important, the fact that in most cases positively charged groups contact the phosphoryl group to be transferred is an even stronger argument for an associative mechanism, since positive charges contacting the phosphoryl group would be anticatalytic in the case of a dissociative mechanism [20]. In the case of the Ras:RasGAP interaction, the positively charged arginine supplied in *trans* is a major factor in converting the intrinsically slow GTPase into a fast GTPase switch, an important aspect for regulation of cell growth through Ras.

In summary, the use of the small inorganic molecule aluminum fluoride has led to a quantum jump in our understanding of the biophysical mechanisms of enzyme catalysis underlying such diverse biological processes as metabolism, signal transduction and tumor formation, the common denominator of which is the transfer of a phosphoryl group. Considering that these reactions are fundamental for nearly all biological systems, I hope that everybody ticks the right box when it comes to electing the molecule of the year.

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