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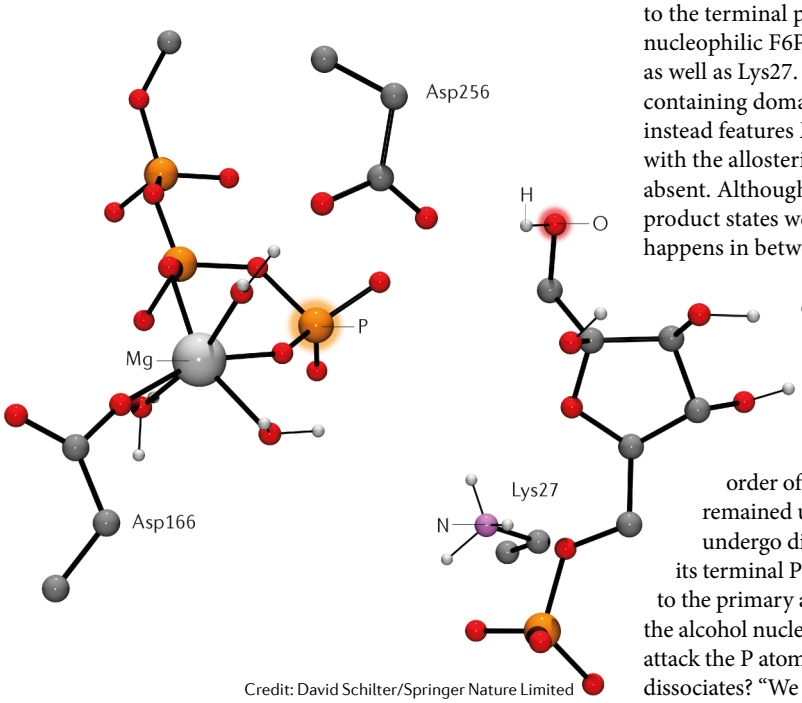
ENZYME MECHANISM

# A dissociative path to phosphorylation

> We localized the minimum free energy path from reactants to products by using the string method, a powerful approach for exploring complex processes

Phosphorylation reactions, in which a phosphoryl group is added to an organic compound, abound in biology. For example, an important step in glycolysis — the energy-releasing metabolic pathway in cells — involves transfer of a phosphoryl group from an ATP to the primary alcohol group of fructose-6-phosphate (F6P) and is catalysed by phosphofructokinases. Despite phosphofructokinases being highly conserved across many species, the mechanisms by which they operate are not well understood. Writing in *Chemical Science*, Julio Caballero, Iñaki Tuñón, Ricardo Cabrera and colleagues determine the X-ray structure of *Escherichia coli* phosphofructokinase 2 (Pfk-2) bound to both substrates (F6P and $MgATP^{2-}$) and products (fructose-1,6-bisphosphate (FBP) and $MgADP^{-}$). With coordinates of the latter in hand, the team used quantum mechanics/molecular mechanics (QM/MM) simulations to investigate the Pfk-2 phosphorylation mechanism for which they propose a dissociative path.

"One of the main difficulties with exploring this mechanistic question is to obtain a crystal structure of the enzyme in the presence of substrates and products," says Cabrera, and the team did just that. The domain featuring the substrates includes two $MgATP^{2-}$ complexes, one of which is bonded to the Asp166 residue and the other is located at an inhibitory allosteric site. Proximal to the terminal phosphate is the nucleophilic F6P primary alcohol as well as Lys27. The product-containing domain is similar, but instead features $MgADP^{-}$ and FBP, with the allosteric $MgATP^{2-}$ being absent. Although both substrate and product states were known, what happens in between?




Credit: David Schilter/Springer Nature Limited

The conversion of F6P into FBP involves phosphoryl transfer as well as deprotonation of the primary alcohol, but the order of these two steps remained unclear: does ATP undergo dissociation before its terminal P atom bonds to the primary alcohol, or does the alcohol nucleophilically attack the P atom before the ATP dissociates? "We localized the minimum free energy path from reactants to products by using the string method, a powerful approach for exploring complex processes," says Tuñón. The team calculated the free energy barriers of two possible mechanisms and considered the roles of the Asp and Lys residues by performing QM/MM simulations. In the dissociative pathway, the free Asp256 residue deprotonates the F6P primary alcohol to afford an alkoxide, which attacks the terminal P atom of $MgATP^{2-}$. In the associative pathway, the base is instead one of the terminal O atoms of the terminal phosphate. According to the respective free energy barriers, the team found the dissociative mechanism to be more favourable. The calculated free energy barrier for the dissociative path (21.0 kcal $mol^{-1}$) is lower than for the associative path (55.2 kcal $mol^{-1}$). Thus, the former appears more likely, and its barrier is comparable to that determined experimentally (15.2 kcal $mol^{-1}$). As part of the dissociative mechanism, Lys27 forms a hydrogen bond to the reactive phosphate group, thereby stabilizing the transition state for dissociation.

The work of Tuñón and colleagues exemplifies the value of combined experimental and theoretical studies in unravelling the mechanisms of enzymes. But despite these findings, certain aspects of catalysis by Pfk-2 remain unclear. "The inhibition mechanism by its own substrate, ATP, involves interactions that could directly affect phosphoryl transfer," says Caballero. "We think this effect could be further explored." The team also hopes that these findings could pave the way for further study of similar kinases.

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**ORIGINAL ARTICLE** Murillo-López, J. et al. Studying the phosphoryl transfer mechanism of the *E. coli* phosphofructokinase-2: from X-ray structure to quantum mechanics/molecular mechanics simulations. *Chem. Sci.* https://doi.org/10.1039/c9sc00094a (2019)