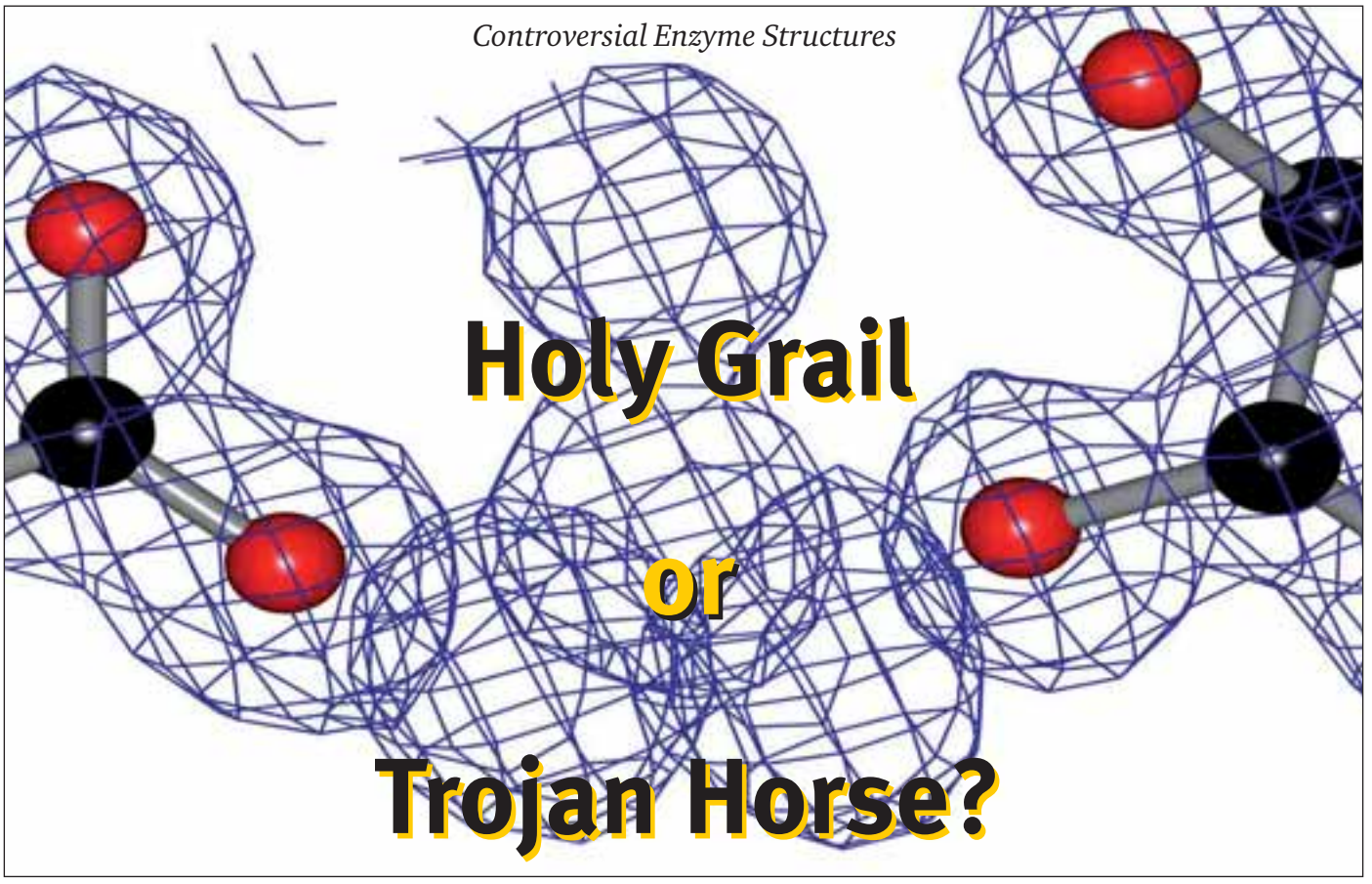
Controversial Enzyme Structures



Holy Grail or Trojan Horse?

For years, biochemists had aspired to visualise a high energy transition from substrate to product. In 2003, a paper claimed to have achieved exactly that. But it was just the start of a harsh dispute that is still ongoing.

"Take nobody's word for it". This, or rather the original Latin version, *nullius in verba*, remains the motto of the British Royal Society (RS). What is a rather commonplace concept today, was revolutionary stuff when the RS was founded in the seventeenth century. Until then, knowledge was jealously guarded by those in authority, such as priests and university teachers, and debates were based on authoritative books using pure logic. Eventually, however, the RS founders and others decided to challenge the authorities by taking observation and experimentation as their sources of the truth. To this day, its empirical methods are a major strength of that new philosophy called science.

An extraordinary claim

Michael Blackburn, emeritus professor of biochemistry at the University of Sheffield, must have felt like one of those early scientists when he decided not to believe the claims he had just read in *Science* (Lahiri *et al.,* 2003, 299:2067). The authors reported the crystallisation of an enzyme and concluded to have succeeded in "the visualization of an intermediate species of high intrinsic energy". The problem is that such a high energy reaction is usually extremely short-lived. What Blackburn did not know at the time was that his doubts would become the start of a seven-year refutation process.

The group that made this claim, led by Karen Allen, professor of biochemistry at Boston University, had presented a high resolution X-ray diffraction structure of a beta-phosphoglucomutase containing an "amazing" ligand. In the cell, this enzyme catalyses the phosphate transfer from position one to position six of a glucose molecule. The most likely reason the Allen *et al.* structure made it into *Science* is that the enzyme was apparently caught in the act of transferring the phospho group from the glucose phosphate ligand to the catalytic aspartate residue. The intermediate thus contained a phosphorous atom bound to the unusual number of five oxygens at the same time. The ligand fitted so well that the authors were left with no other interpretation than having obtained the "unequivocal, direct visual evidence of enzyme catalysis by way of intermediate stabilization." A first!

Pauling's theory

Theory states that a chemical reaction rate is determined by the highest energy level on the reaction path: the transition state. Due to this high energy level any species going through the transition state must be highly unstable and will instantaneously drop into the stable energy minimum of the substrate, product or a stable intermediate. It was Linus Pauling who proposed that enzymes speed up reactions by specifically stabilising the transition state and, therefore, lowering the activation energy needed for the reaction. This results in spectacularly speeding up the corresponding reactions by factors of 106 up to 1,021.

The theory is so powerful that biochemists, of course, have long-yearned for a glimpse of a transition state which, according to the theory itself, is close to impossible. No wonder that one of them, the late Jeremy Knowles, professor of biochemistry and former dean of the Faculty of Arts and Sciences at Harvard University, was so fascinated by Allen's paper that he abandoned his long-held reservations about solving chemical mechanisms by crystallography. In the same issue of *Science* (Knowles, 2003, 299:2002) he asked, "Could this actually be the transition state [...]?"

An alternative interpretation

Blackburn's answer, however, was a definite "No!" He and a couple of other colleagues had a clear idea of what might have been wrong with Allen's structure and two weeks later wrote in a comment to *Science* (Blackburn *et al.,* 2003, 301:1184), "[...] MgF3- is a good mimic of the transition state of phosphoryl transfer reactions and

can be formed under conditions such as those employed [...].” MgF3- rather than PO3- could well explain the illusion of the transition state. Wham! But the Allen group criticised the precedent offered by Blackburn and colleagues in the same issue (Allen *et al.,* 2003, 301:1184): “[The precedent] offers no proof of the existence of magnesium trifluoride in solution [...].” Therefore, MgF3- is nothing but pure speculation and does not fit the data. Pow!

Blackburn was convinced,

(1) that the bond lengths between the phosphorus, the oxygens linking it to the sugar and the catalytic residue were too long to be stable, and

(2) that there were some inconsistencies with the published electron density maps.

Allen, however, insisted,

(1) that the experimentally determined ratio between protein and phosphate in the crystals was just right,

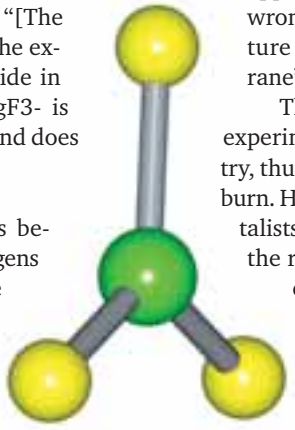
(2) that the observed lengths between phosphorus and the oxygens perpendicular to the direction of transfer were too short for magnesium-fluorine bonds,

(3) that the anomalous X-ray scattering matched phosphorus,

(4) that magnesium fluoride was not inhibiting the enzyme reaction as should be expected, and

(5) that pentacoordinated phosphorus, in contrast to magnesium fluoride, has a proven chemical model.

There they were: one group’s word against another.



What has the beta-phosphoglucomutase enzyme actually transferred? Trifluoromangensate or...

Simulation vs. experimentation

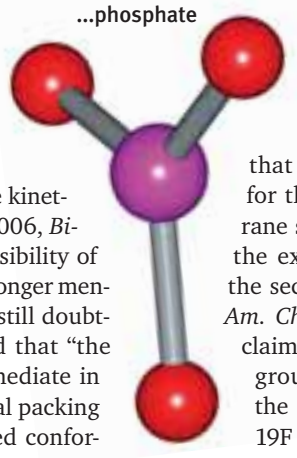
Usually, such deadlocks are most effectively resolved by a different group employing yet another method. And indeed, help came from chemical simulations. As many as three groups subsequently published results on the question. The first (Webster, 2003, *J. Am. Chem. Soc.,* 126: 6840), only one year later, made the solomonic judgement that pentacoordinated phosphorous really was the transition state of the native reaction but that “the structure is consistent with the previous proposal of a transition state analogue containing MgF3- in the active site”. The second group (Berente *et al.,* 2007, *Theor. Chem. Acc.* 118:129), three years later, also came to the conclusion that “the best agreement with experiment is observed for [...] a model where the phosphate group is replaced by a MgF3- anion”. Another three years later, the third simulation group (Marcos *et al.,* 2010, *Proteins*, 78:2405) went as far as to state in their abstract that their simulation “strongly supports that Allen and co-workers wrongly assigned the X-ray structure to a pentavalent phosphorane”.

The simulations, based on prior experimental knowledge of chemistry, thus apparently supported Blackburn. However, hard-core experimentalists often consider themselves as the real heirs of the empirical scientists and generally distrust computational methods.

Corroborating evidence

Two years after their original publication, the Allen group published new data (Tremblay *et al.,* 2005, *J. Am. Chem. Soc.*, 127:5298). In the introduction they wrote, “The Blackburn hypothesis was sufficiently credible to create a controversy” and “because of the significance of the proposed phosphorane intermediate, it is imperative that this controversy be resolved.” Their new data consisted of quantifications of protein and phosphate in crystals confirming their original interpretation. In addition, they presented a new X-ray crystal structure of beta-phosphoglucomutase with galactose-phosphate (instead of glucose-phosphate) that contained no MgF3- as they expected. Finally, they also cited another similar publication containing a high energy intermediate structure.

For the Allen group this seemed to have settled the dispute. All the more so as they added another article, in which they closely analysed the kinetics of the reaction (Dai *et al.*, 2006, *Biochemistry*, 45: 7818). The possibility of MgF3- in the active site was no longer mentioned. Instead, for those who still doubted their results they presumed that “the stability of this reaction intermediate in the crystal is the result of crystal packing forces stabilizing the cap-closed conformation”.



...phosphate

NMR reveals Trojan horse

Blackburn, however, an experimentalist himself, came up with some results which he published one year later under the title, A Trojan Horse Transition State Analogue Generated by MgF3- Formation in an Enzyme Active Site (Baxter *et al.*, 2006, *PNAS*, 103:14732). His group had, meanwhile, introduced an experimental method that was complementary to the crystallography: a new 19F NMR method, with which they showed the existence of three fluorines in three different chemical environments that specifically interacted with residues in the active site of beta-phosphoglucomutase. The Blackburn group therefore claimed that their NMR method “provides a sensitive probe of subtle changes within the enzyme that would be invisible to other structural biology methods”. Furthermore, they showed that fluorine was indeed an inhibitor.

Was this a convincing refutation? Not for everybody. A Chinese group, for example, soon joined the dispute by citing both groups and noted that “the structure of this intermediate is still a matter of argument” (Wang *et al.*, 2006, *JBC*, 281: 39642).

Neither was the Allen group impressed. In two articles, two and three years after publication of the NMR data, they analysed the geometry of the transition state (Lu *et al.*, 2008, *PNAS*, 105:5687) and other structural specificities (Dai *et al.*, 2009, *Biochemistry*, 48:1984). However, they discussed their structure, whilst mentioning neither Blackburn’s opposing interpretation nor the NMR data.

Build-up of refutation

The Blackburn group was now properly cooking. They published three articles in two years on the subject. The first (Goličnik *et al.*, 2009, *J. Am. Chem. Soc.*, 131:1575), although without Blackburn himself as co-author, re-analysed the kinetics of the reaction. The authors concluded that “the components required for the formation of a phosphorane species do not persist under the experimental conditions.” In the second (Baxter *et al.,* 2009, *J. Am. Chem. Soc.,* 131:16334) they claimed that, although the Allen group didn’t observe MgF3- in the crystals with galactose, the 19F NMR actually showed its existence in solution, which “refutes the major remaining piece of evidence used to support the putative existence of a pentavalent phosphorane in complex with beta-phosphoglucomutase”.

The third article from the Blackburn group (Baxter *et al.*, 2010, *PNAS*, 107:4555)

was intended to be the final blow to the pentavalent phosphorane theory. They reproduced the Allen crystal structure under similar conditions to the NMR experiments. Their own crystal structure matched MgF3-well, whereas anomalous X-ray diffraction did not show phosphorous at the presumed site of magnesium. NMR spectra of the crystallisation solution also lacked the claimed phosphorous. The authors also reprocessed the original crystallographic data from the Allen group, which revealed a surplus of electron density at the phosphorous and a lack of density just beyond the oxygens surrounding the phosphorus. Blackburn and co. therefore concluded that "these results leave no doubt that the transition-state-like complex for beta-phosphoglucomutase has MgF3- coordinated" and that "there is no evidence that supports the presence of a pentacoordinate phosphorane under any conditions".

Good for science

This was bad news for biochemists, as they dealt with the fact that the transition state had still not been visualised. The Blackburn group tried to brighten up the picture a bit by stating that "the metal fluoride complexes offer opportunities to measure properties of near-transition state complexes that are currently unmeasureable for phosphorus oxide species". Thanks to intense scientific debate, the Blackburn group has developed a powerful new technique. That was the good news.

Photo: Universities of Sheffield and Boston, modified: K. Gransalke

It's not a laughing matter for Karen Allen and Michael Blackburn anymore.

However, would the Allen group agree? What do other structural biologists and enzymologists think of it? Unfortunately, this question cannot be answered here as nobody contacted by *Lab Times* wanted to comment on the case. It seems that talking about scientific disagreements in public is taboo.

Extraordinary evidence

Whatever the final conclusion on the pentavalent phosphate intermediate or MgF3- transition state analogue, questions about the original publication remain. Although crystallography is surely an amazing technique, publishing a paper with an extraordinary claim based on one single technique is not enough. Martin Rees, astronomer and president of the Royal Society, in a lecture about the future of science once stressed, "Extraordinary claims demand extraordinary evidence." In peer-reviewed science it is the task of the reviewers to demand the corroborating evidence.

So the Allen-Blackburn controversy rumbles on. But controversy has always been a crucial part of science. Or, as Rees puts it, "What's crucial in sifting error and validating scientific claims is open discussion."

Florian Fisch