Inferring the chemical mechanism from structures of enzymes

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Chemistry has once again embraced the study of enzyme mechanism as a core discipline. Chemists are uniquely able to contribute to the analysis of enzymes through their understanding of the reactivity of atoms. In this *tutorial review* for the Corday-Morgan medal, I will concentrate on the work from my lab over the past six years. I discuss enzymes which transform carbohydrates and incorporate halogens. The *tutorial review* will emphasise the strengths and limitations of structural biology as a means to deducing the chemical mechanism.

Introduction

It is important to try to distinguish between enzyme mechanism and the chemical mechanism of an enzyme transformation, although the terms are frequently used interchangeably. The most clear definition I have is from a talk by Perry Frey, who pointed out that enzyme mechanism is a model of when, how and at what rate each atom and electron of substrate and protein moves during the reaction. It can never be proven but each movement can and should be experimentally characterised. To many this seems a council of perfection but there are many examples of this being achieved. For a particularly beautiful example, I would recommend reading the story of lysine 2,3 (and 5.6)-aminomutase. This work led by Frey spans nearly 20 years and almost 40 publications, most recently the crystal structure of the enzyme.[1] It is worth noting that the crystal structure is almost at the end of the story, the careful biochemistry which mapped out a mechanism preceded the structure. This is almost the exact opposite of many contemporary studies, mainly because of the growth in structural information. Structure provides a three dimensional template to think about mechanisms before the first kinetic experiment. At its best, structural data can be used to infer the likely movements of atoms during a transformation and is often useful for eliminating possible mechanisms. In contrast to enzyme mechanism, the chemical mechanism is the description of the transformation of a substrate by the enzyme in a series of bond-breaking and bond-making steps, crucially it lacks the rates and often the timing of various steps (concerted *vs.* stepwise) seen in enzyme mechanisms. The chemical mechanism is not an end in itself but the starting point for detailed biochemical analysis. For many years, the chemical community in the UK has seen biochemistry as a discipline outside chemistry, this has not been true in other countries, most notably the US. The proliferation of structural studies of enzymes has, in recent years, been one of the factors that led to re-integration of biochemistry into the central identity of chemistry. This is because, in essence, enzymes are chemical puzzles in a biological context.

My own lab has been particularly interested in carbohydrate transformations and halogenation reactions. Carbohydrates are central to biology; they are involved in molecular recognition and metabolism as well as in forming important biological materials such as cellulose. Sugars are unusual molecules; in comparison to amino acids, they are much more densely functionalised. Glucose in its pyranose form is capable of donating 5 hydrogen bonds and accepting 12 hydrogen bonds. In comparison, lysine can accept one and donate four hydrogen bonds. The presence of multiple stereogenic centres in carbohydrates means that even for a simple 6 carbon carbohydrate there are 16 distinct diastereomers, each with a different presentation of hydrogen bond donors and acceptors. This combination of diversity and functionality makes them particularly interesting targets for study. Rather than generate all possible conformers, biology transforms one into another. In addition biology can alter the reactivity of the sugar by chemical modification, for example dehydration, oxidation, methylation and amidation. Biology faces two particular issues in manipulating sugars. Firstly the stereocontrol of reactivity must be exquisite; in organic chemistry the protection and deprotection of carbohydrates is a significant challenge to

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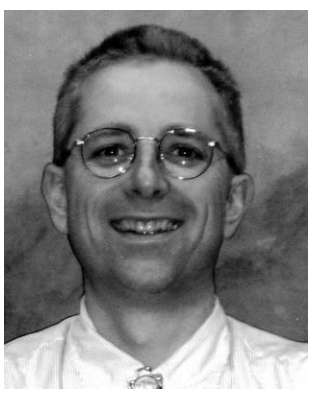

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methodology. Enzymes overcome this by careful positioning of the substrate relative to the active components of the protein. The second problem, which is often ignored, is that the recognition of simple monosaccharides in water is non-trivial. Monosaccharides are highly solvated and often binding gains in hydrogen bonds to the protein are offset by losses of water–carbohydrate hydrogen bonds. The lack of hydrophobic surface on the sugar makes it difficult to gain affinity by van der Waals interactions. There are many enzymes which can bind simple monosaccharides and process them, but for more complex transformations such as epimerisation or oxidation of secondary alcohols it is much more common to utilise sugars linked to nucleotides. The nucleotide serves as an affinity handle allowing the enzyme to hold its substrate. I will discuss some insights into the chemistry carried out by a class of enzymes, the short chain dehydrogenases, which modify sugars attached to nucleotides.

In contrast to carbohydrate chemistry, which is often a minor component of the undergraduate course, the incorporation of halogens into molecules is central to the teaching of organic chemistry. There are several thousand halogenated natural products, the overwhelming majority of which contain chlorine, followed by bromine, with a handful containing iodine and fluorine. The dominance of chlorine is not surprising, it is abundantly available in sea water as the solvated ion. Equally, the almost complete absence of fluorine is not surprising, given the low abundance of the solvated ion in the ocean and unreactive nature of the solvated ion. Fluorine and chlorine have very different chemistries and I will compare the quite different chemical approaches taken by biology to achieve their incorporation.

Sugar nucleotide enzymes from the short chain dehydrogenase family

Short chain dehydrogenase/reductase (SDR) enzymes encompass a wide variety of enzyme activities. At their heart is the catalysis of transfer of hydride between a nicotinamide cofactor and a substrate (Fig. 1). The simplest example of this type of chemistry is seen for RmlD,[2] part of the rhamnose biosynthetic pathway[3] (Fig. 2). RmlD simply catalyses the transfer of hydride from NADH (and unusually NADPH) to dTDP-4-keto-L-rhamnose to yield $NAD(P)^+$ and dTDP-L-rhamnose.[2] The basis of the SDR catalysis has been known for many years, the enzymes require the so called catalytic triad of LysxxTyr (x is any amino acid) and Ser (or Thr).[4,5] For many years our understanding of the role of the three residues came from the seminal work of UDP-galactose/glucose epimerase (GALE).[6–10] This enzyme oxidises UDP-glucose at the C4 position, the keto sugar rotates at the active site and is reduced

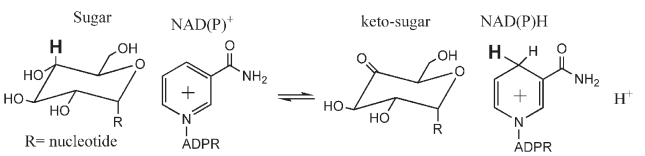

Fig. 1 The transfer of pro-*S* hydride to and from $NAD(P)^+$ seen in SDR enzymes. ADPR is adenosine diphosphate ribose.

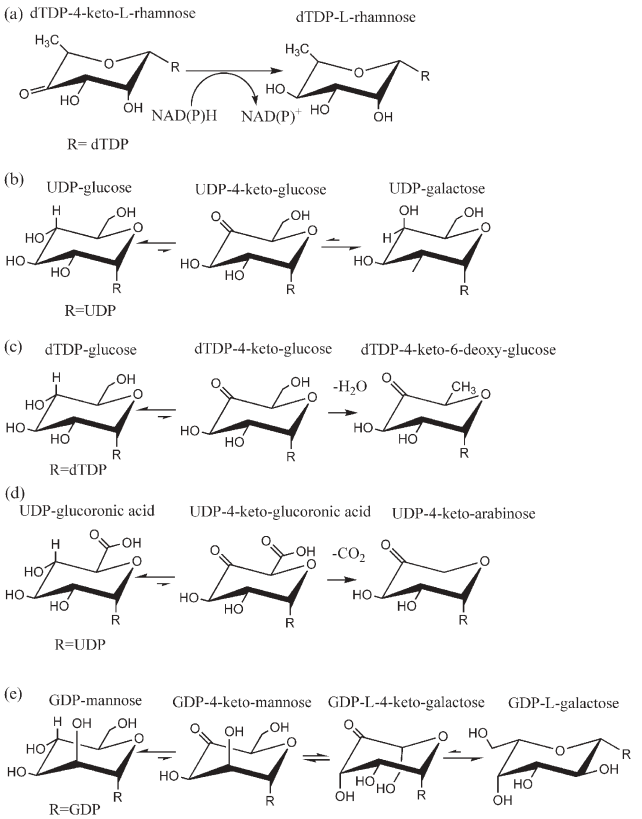

Fig. 2 Despite acting on a wide range of substrates and with seemingly different reactions, at their heart the following SDR enzymes are based on hydride transfer between NAD(P) and the C4 position of a carbohydrate. (a) RmlD (b) GALE (c) RmlB (d) ArnA (e) GME.

at the opposite face, to give UDP-galactose (Fig. 2). These studies established that Tyr, found as tyrosinate, functioned as the terminal base; it abstracts a proton from the hydroxyl at C4 while hydride moves from the sugar to NAD. The change in pK_a to allow this results from the positive charges of NAD^+ and the Lys. However, in the structural studies the negatively charged tyrosinate oxygen was too far from the hydroxyl group to act directly. This led to the proton shuttle hypothesis, in which the third residue of the triad (Ser or Thr) acted as intermediary in allowing the proton to move from sugar to Ser and the proton from the Ser to the tyrosinate.[9] The proton shuttle mechanism is a question of detail and was based on several structures, however, several other studies suggest that it is not a component of the mechanism but results from a crystallographic artifact. In more recent crystallographic studies,[11–14] evidence emerged that the tyrosinate can function directly as a base. In particular, the validity of this hypothesis was strengthened by considering the trajectory of hydride transfer. One expects the nicotinamide ring and sugar ring to be approximately coplanar with the close approach of the carbon atoms, which exchange hydride. In a series of structures of RmlB with different substrates, including some at high resolution, this was shown to be the case[12,15] (Fig. 3). One of those studies described the puckering of the nicotinamide ring that occurs on its reduction to NADH, the first such structural description of NADH in an SDR enzyme.[15]

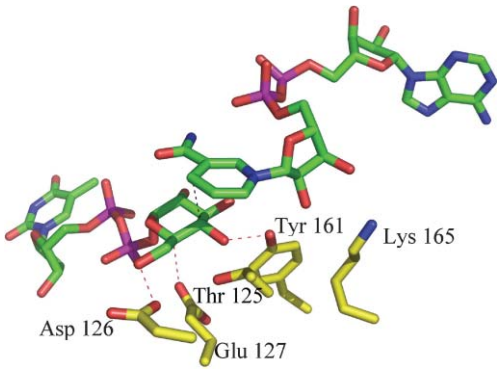

Fig. 3 RmlB with bound dTDP-glucose. The alignment of the sugar and nicotinamide ring is optimal for hydride transfer, a black dotted line indicates its route. Red dotted lines show the acid and base hydrogen bonds to the sugar that are required for the mechanism.[12] Side chains of the protein are coloured: carbon, yellow; nitrogen, blue; oxygen, red. The same colours are used for dTDP-glucose and NAD, except carbon is coloured green.

Although these studies apparently tie down the role of the LysxxTyr, they leave a gap in our understanding of the role of Ser, as no shuttle is necessary. The Ser is not essential for the reaction, its mutation in GALE significantly decreases but does not obviate activity.[9] In considering the transition state during proton transfer between Tyr and a sugar, an alternate proposal was made. In this model, the transition state has the proton between the sugar and the tyrosine. This could be a low barrier hydrogen bond,[16] which requires that the pK_a of the two groups is matched. Given that the pK_a of the tyrosine is about 6.7, at first glance it would seem unlikely as the pK_a of the sugar hydroxyl group is around 14 and that of the sugar keto group is about −2; neither is a good match. However, it is the transition state which matters and the pK_a of the C4 oxygen atom in the transition state may in fact be somewhere in the middle of the two extremes, around 7–8. The Ser residue hydrogen bonds to the substrate and Tyr, and its role may in fact be to fine tune the pK_a of the groups at the transition stage to promote the reaction.[2]

In sugar nucleotide chemistry hydride transfer is often coupled to a second activity. ArnA is an interesting case in point; the bifunctional enzyme has decarboxylase and formyl transferase domains which act on UDP-glucuronic acid[17] (Fig. 2). The decarboxylase domain (Fig. 4) belongs to the SDR superfamily and its first step is the oxidation of the C4 of the glucuronic acid and is thus essentially identical to RmlB and GALE. An interesting question is whether the decarboxylation is spontaneous? The 4-keto intermediate would be predicted to be unstable (spontaneous decarboxylation) in water and has not been observed experimentally. As the enzyme oxidises C4, it converts NAD^+ into NADH which can be monitored. Mutations away from the catalytic triad significantly reduce the rate of NAD^+ reduction, including a change from Glu to Gln which apparently kills the enzyme.[18] This is an interesting result which is difficult to explain. The K_M for the Glu to Gln mutant cannot be measured, but in a more radical Glu to Ala mutant which retains some activity, K_M is not dramatically changed.[18] If the Glu has a direct effect on the rate of hydride transfer this would be a very puzzling

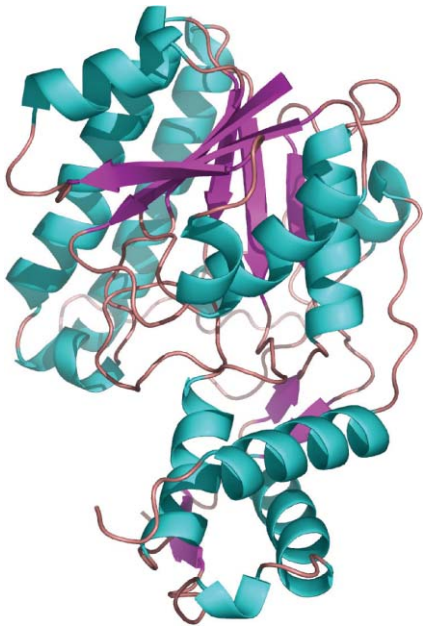

Fig. 4 The structure of ArnA shown in ribbon representation. ArnA has the classical extended SDR fold. The SDR fold comprises the 6 stranded β-sheet flanked by α-helices and this binds the NAD(P) nucleotide. The "extended" domain is mainly helical and, at the bottom third of the figure, this binds the nucleotide of the sugar nucleotide substrate.

result; the Glu in question is not conserved in SDR enzymes and is remote from the catalytic triad. The alternative explanation is that, in the enzyme, Glu is required for decarboxylation. The identity and role of residues involved in the decarboxylation is disputed but it does appear that, in the enzyme, decarboxylation is not spontaneous[18–20] as mutations which should not affect hydride transfer lead to a decrease in NADH production. As a direct corollary of this, the equilibrium between the keto sugar and NADH on one side and the sugar and NAD^+ on the other very much favours the substrate (Fig. 2). The Ala mutant may allow chemical rescue by permitting water access to the active site to mediate proton transfer. If not, the net formation of keto sugar would still cause conversion of NAD^+ to NADH. In the absence of a rapid irreversible step (decarboxylation) the keto sugar simply reverts back to the substrate and no consumption of NAD^+ is observed. This poising of the equilibrium of hydride transfer may in fact be a subtle form of control. Many of the substrates transformed by SDR enzymes are very similar and the potential for cross reactivity is high, with potentially damaging consequences. It is not clear from the many structures of SDR enzymes whether the enzyme can or cannot bind and oxidise alternate substrates. It is possible however, that net flux through the enzyme requires an appropriate match between the potential chemical reactivity (decarboxylation *vs*. dehydration) of the sugar substrate and the chemistry and spatial location of the enzyme amino acids around the sugar.

The spatial coupling of residues is also seen in the enzyme GDP-mannose epimerase (GME).[21,22] This enzyme epimerises the 3- and 5-positions of GDP-mannose to yield GDP-L-galactose; it has also been shown to produce GDP-L-gulose,[22]

by epimerisation at the 5-position only (Fig. 2). However, there is no base in biology capable of extracting the C3 or C5 proton from GDP-mannose. This conundrum is overcome by first oxidizing the sugar to the 4-keto form. This then activates both the C3 and C5 protons by lowering their pK_a. After epimerisation, GME reduces the 4-keto sugar back to the alcohol function; thus it combines three distinct chemical reactions, oxidation, proton transfer and reduction, in one active site. Unlike ArnA, there is no net transfer of electrons between NAD^+ and the substrate, therefore an HPLC assay was used. HPLC established that the enzyme establishes the equilibrium between GDP-mannose, GDP-L-gulose and GDP-L-galactose in the ratio 8.0:0.5:1.5. Structural studies of a mutant (catalytic Lys 178 was mutated to an Arg) of this protein trapped, for the first time, a keto sugar intermediate (GDP-4-L-keto-gulose) at an enzyme active site[23] (Fig. 5). This intermediate was very informative; it establishes that ring flip occurs with C5 epimerisation and that, in at least some cases, the enzyme operates on the C5 position first and the C3 position second. Structures with GDP-mannose and GDP-L-galactose gave a complete three dimensional description of each stage of the reaction.[23] These structures were, like RmlB and ArnA, consistent with the roles assigned to the catalytic triad discussed earlier. As with ArnA, we could not detect any free keto sugar in solution; the observation of it in the structure is most likely due to the particular conditions of the crystalline state. The collection of structures suggested that a single acid–base pair, Cys 145 and Lys 217, is responsible for both epimerisations. Both residues were mutated and in each case inactivated the protein; no accumulation of keto sugar was observed. Once again, it appears the equilibrium between the keto sugar/NADH and sugar/NAD^+ strongly favours sugar/NAD^+. The use of a single acid–base pair for both epimerisation requires that the protonation state is reset during turnover. Structural analysis does not reveal any obvious mechanism for this, highlighting its limitations. Although a

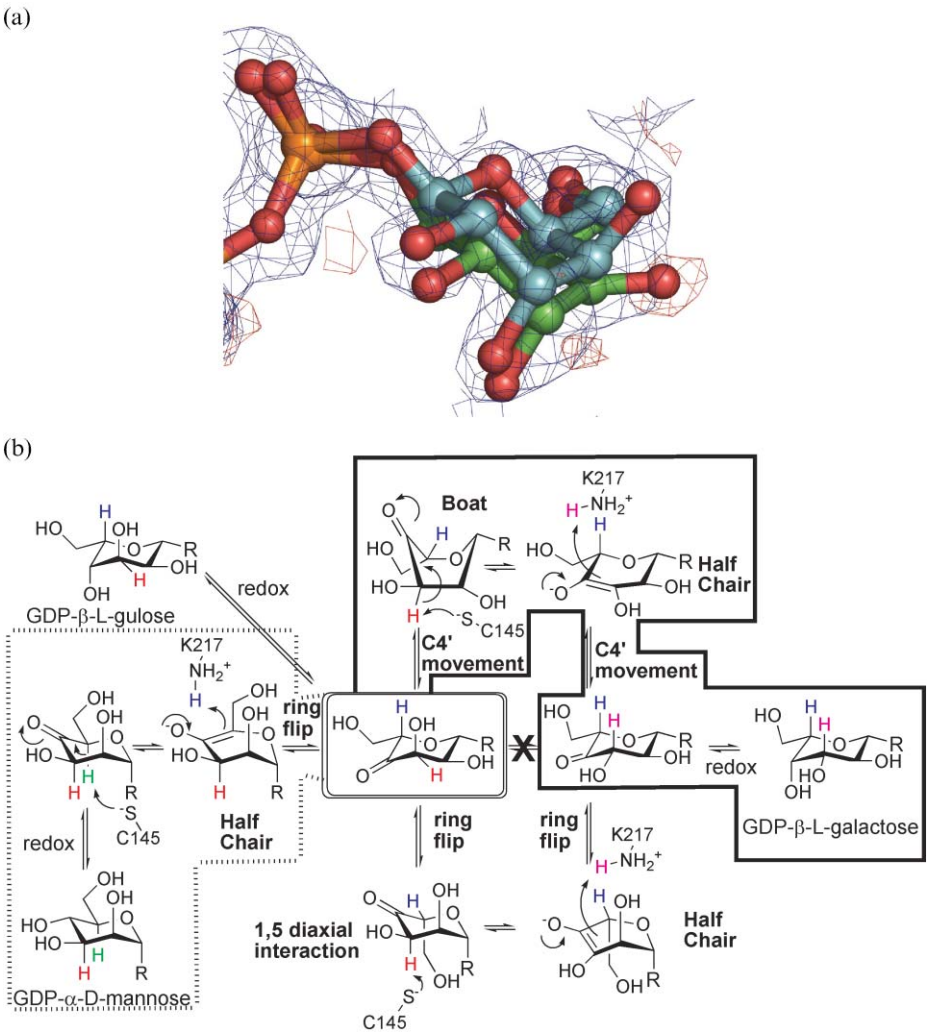

Fig. 5 (a) The final refined structure of GDP-L-gulose (carbon, blue; oxygen, red; phosphorus, orange)/GDP-L-4-keto-gulose (carbon, green; oxygen, red; phosphorus, orange) bound to GME. Shown in blue chicken wire is the $2F_o - F_c$ map contoured at 1σ, calculated with only GDP-L-gulose. In red chicken wire is the $F_o - F_c$ map (contoured at 3.5σ) showing the additional density for the keto sugar. (b) The mechanistic possibilities for GME. The first epimerisation is boxed in the broken line. The GDP-4-keto-gulose which is trapped in the crystal is boxed. The preferred route for the second epimerisation is shown highlighted in the bold box.

water network was observed which could function as a proton conduit. The structures are all high resolution and allow for the first time some insight into the conformation of the carbohydrate during turnover. In GDP-4-keto-mannose both protons are axial and thus *trans* to the keto function. Only in the *trans* position do the protons gain the orbital stabilisation from the antibonding orbital of the keto group. As GDP-4-keto-L-gulose must be a substrate for C3 epimerisation, it presents a problem with respect to the position of the C3 proton, which is now equatorial. Further, only in the axial position is the proton appropriately positioned for abstraction by Cys 145. The proton can only be extracted by ring flipping to a 1,5-diaxial compound (Fig. 5) or by going through a boat conformation. Examination of the active site suggests that an axial configuration for C5 (which has the CH_2OH group) would clash with the protein. Coupled to observations of twist-boat carbohydrates in several structures, this led us to favour a twist-boat intermediate rather than a 1,5-diaxial structure. This is a clear example of the limitations of structural data.

Significantly, the catalytic Cys 145 residue occupies the same position as the base (Glu 136) in RmlB. Dehydration does not occur because the acid required to protonate O6 is absent in GME. In GALE, there is no base at this position and therefore, although the keto sugar is also an intermediate, no epimerisation α to the keto group occurs. GME, like other extended SDR sugar nucleotide modifying enzymes, has quite an open carbohydrate site, allowing it to bind three quite different sugars (D-mannose, L-gulose and L-galactose). This accords with our suggestion that specificity comes from the chemistry and disposition of other residues around the substrate, rather than binding recognition *per se*.

Halogenation

Halogen reactivity is dominated by their electronegativity; the negatively charged ion is a powerful nucleophile. Fluorine is the most extreme, its chemical oxidation is effectively impossible and in the gas phase F^- is extremely nucleophilic. As one moves down the group, the ease of oxidation increases; most importantly for biological systems, molecular oxygen can oxidise Cl, Br and I (and their corresponding anions). This means, in addition to nucleophilic chemistry, biology has the option of electrophilic and radical chemistry for Cl, Br and I; these chemistries are not available for F in biology. The biological chemistry of F^- in water is almost non-existent because of the very high desolvation energies (400 kJ mol^{-1}). The key task for an enzyme is to catalyse F^- desolvation. The first report of enzymatic carbon–fluoride bond formation employed mutants of glycosyl hydrolase which create a very reactive electrophile and promote desolvation of the halogen anion.[24] Thermodynamically, the formation of a carbon–fluorine bond is quite favourable, the desolvation required to activate F^- is a perfect example of a kinetic barrier. The first natural enzyme to be isolated and characterised comes from the bacteria *Streptomyces cattleya*[25] and synthesises 5-fluorodeoxyadenosine from *S*-adenosyl methionine (SAM) and F^- (Fig. 6). The structure of the enzyme[26] reveals it to be an entirely novel fold; this is an exciting finding, quite in keeping with the highly unusual chemistry. The other side of this coin is that the standard tools of the structural biochemist are of little use in trying to think about mechanism. Traditionally one identifies conserved structural motifs which can guide the chemical interpretation, but for the fluorinase enzyme this was not possible. Structures with the substrate and product[26] did identify the active site and these showed an extensive set of interactions binding SAM to the structure (Fig. 6). The 5-fluorodeoxyadenosine complex was little changed from the substrate, suggesting the protein does not undergo a large conformational change during turnover. Assuming this to be correct, the organofluorine in the product is a guide to the location of the F^- anion. This places F^- in a hydrophobic pocket where it makes two hydrogen bonds to Ser 158, one to the backbone amide and the other to the side chain. The structure suggests a method by which desolvation would be achieved in stages as protein substitutes for water. The orientation of the F^- binding pocket relative to the C5 and S atoms of SAM, is consistent with S_N2 substitution of methionine by fluoride. The structural analysis is limited to a static picture. A very elegant computational study identified a more distant residue, Thr 80 as playing a key role in the desolvation.[27] This study is particularly interesting because structural analysis overlooked Thr 80. A further limitation of the structural analysis is that it gives no clue to the binding energies of the components; in understanding the mechanism of this process, this is particularly important. The strength of the structural work has been to be provide a template for further studies of this fascinating enzyme. A structure-based sequence alignment suggests that, although there are many

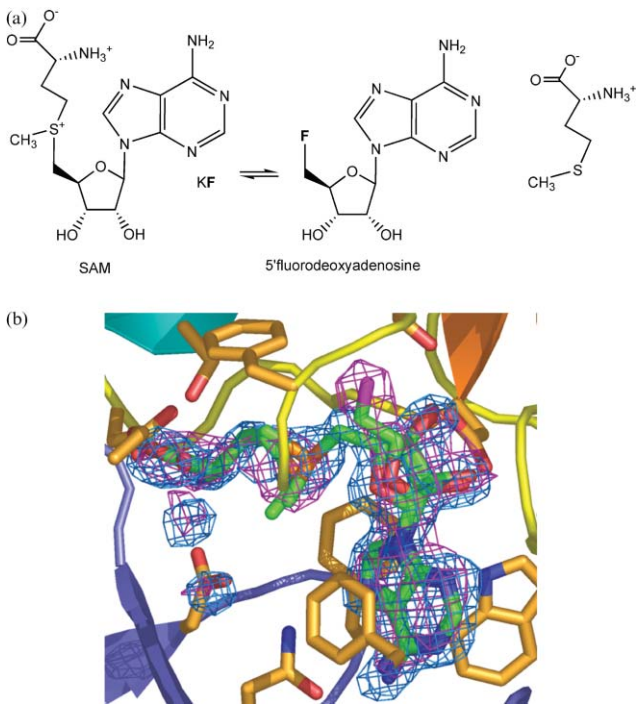

Fig. 6 (a) The formation of the carbon–fluorine bond. (b) The structure of the fluorinase with *S*-adenosyl methionine and 5-fluorodeoxyadenosine. The experimental $F_o - F_c$ electron density for each is shown contoured at 3σ. In the substrate and product, colouring is as follows: oxygen, red; nitrogen, blue; sulfur, yellow; carbon, green. In the protein, carbon is coloured in orange.

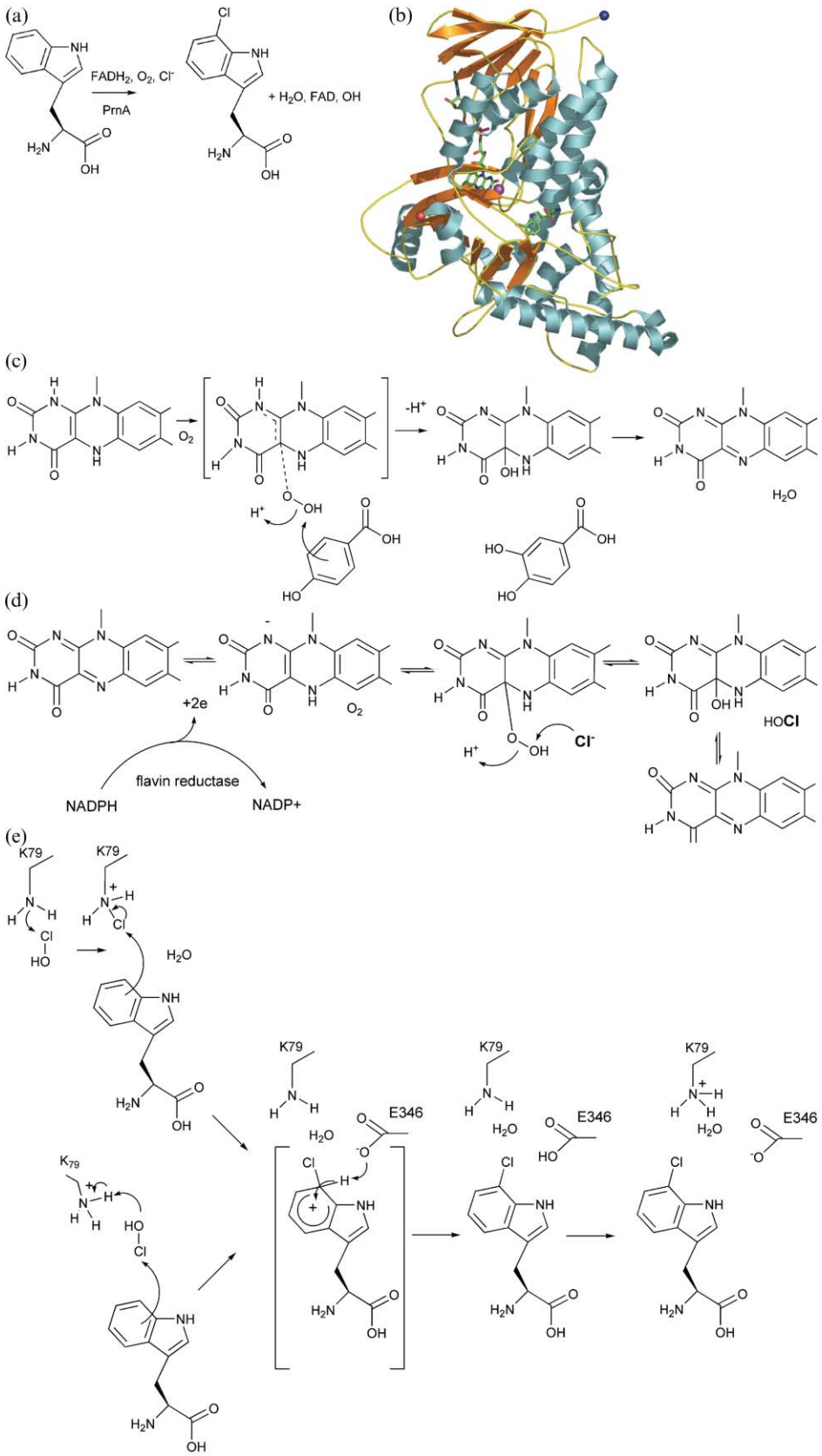

Fig. 7 (a) Formation of 7-chlorotryptophan. (b) The structure of *prnA* shows the substrate and FAD are far apart. *prnA* is drawn in ribbon representation, with a blue sphere at the N-terminus and a red one at the C-terminus. Cl is shown as a pink sphere, FAD and tryptophan are shown in stick form with carbon, green; nitrogen, blue; phosphorus, purple; oxygen, red. (c) The monooxygenase enzyme activates oxygen by forming an *endo*-peroxide at the C4 of the isoalloxazine ring.[50] (d) The mechanism for formation of HOCl. (e) The mechanism of chlorination. Both possible methods of activation of Cl by Lys 79 (chloroamine formation and proton addition).

other proteins with the same structure as fluorinase, only fluorinase contains a large loop which forms part of the binding site.[27] This predicts that fluorinase is a singleton and its homologues do not catalyse the same chemistry.

In contrast to the fluorinase enzyme, there are many different enzyme families which incorporate chlorine (and bromine), reflecting the thousands of chlorinated metabolites.[28] The first structural description of this chemistry came from studies of two classes of metal-dependent haloperoxidases: the iron heme-dependent enzymes,[29] and vanadium-dependent enzymes.[30] The chemistry in both systems is identical. Hydrogen peroxide binds to the metal and reacts with the halide ion, to generate metal-bound hypochlorite. The hypochlorite then diffuses off the metal and reacts with the substrate. This process has very little stereospecificity and was always a problem when thinking about how many natural products were synthesised in bacteria. Free hypochlorite is not reactive enough to chlorinate many of the aromatics seen in natural products. For those which HOCl is reactive enough to chlorinate, a mixture of chlorinated products or the "wrong" product will result. Dairi *et al*. identified the gene coding for a chlorinase in chlorotetracycline biosynthesis.[31] This protein showed no similarity to haloperoxidases and was a novel halogenase. Studies of the biosynthesis of the antifungal antibiotic pyrrolnitrin in *Pseudomonas fluorescens* identified two related genes (named *prnA* and *prnC*), each encoding one of these novel halogenases.[32] This novel class of halogenases binds flavin[31–35] and exhibits a distant relationship with monooxygenase enzymes,[36] which also bind flavin. *prnA* catalyses the regioselective chlorination of the 7-position of tryptophan[37] (Fig. 7) and requires $FADH_2$ (*in vivo* this is provided by flavin reductase) and O_2.[33] Flavin-dependent halogenases have been identified in the biosynthetic pathways of many biologically active halometabolites[38–43] including balhimycin,[44] vancomycin[45] and the antitumour agent rebeccamycin.[46] Despite its importance the mechanism remained unclear and the subject of speculation. Several structures of *prnA* were solved, giving an insight into tryptophan-, 7-chlorotryptophan-, $FADH_2$-, Cl^-- and FAD-binding by the enzyme.[47] The structures show the isoalloxazine ring is over 10 Å from the 7-position of tryptophan (Fig. 7). As had been suggested from sequence analysis, the flavin binding module of halogenases is related in structure to the monooxygenase class of enzyme, in particular to *para*-hydroxybenzoate hydroxylase. The substrate module is novel and is not conserved in the halogenase superfamily. Thus is appears that the enzyme is modular; a conserved monooxygenase module is linked to a variable substrate binding module. The conservation of the structure of the flavin module suggests a chemical relationship. Monooxygenases are known to bind molecular oxygen, forming the C4 endoperoxide at the isoalloxazine ring; this intermediate is then decomposed by "nucleophilic" attack by the electrons of *para*-hydroxybenzoic acid (Fig. 7). Using this as analogy, we suggested that in halogenases Cl^- attacks the *endo*-peroxide, liberating HOCl. We identified a tunnel at the protein active site, connecting flavin and the 7-position of tryptophan. We proposed that HOCl would be retained within the enzyme and directed to the 7-position, providing a rationale for the regioselectivity of *prnA* (Fig. 7). The lack of reactivity of HOCl with tryptophan could be overcome by HOCl activation by Lys 79. There are two chemically reasonable mechanisms for this activation; firstly proton transfer from Lys to HOCl.[47] An alternative mechanism (C. Bergt, personal communication) is the formation of a protonated chloroamine by nucleophilic displacement of water by Lys; such amine chemistry is well known. Both mechanisms increase the electrophilicity of Cl such that chlorination of tryptophan is now possible through the Wheland intermediate (Fig. 7). Although we have shown that the Lys is essential for chlorination, our structural and biochemical data have not yet clearly distinguished between these two possibilities.

Most recently a third new class of halogenating enzyme has been discovered, which is thought to work by a radical mechanism based on a non-heme iron centre.[48,49] Thus it seems biology has developed a range of chemistries to match different substrates, metal-dependent haloperoxidases work with the most reactive substrates, flavin halogenases with aromatic substrates and radical-based chlorinating enzymes with the least reactive (aliphatic) substrates.

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